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Volume 9

A Review of the Literature Published during 1976

Senior Reporter

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Organic formulae composed by Wright's Symbolset method

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Preface

This ninth Report reviews papers relevant to the chemistry of amino-acids, peptides, and proteins published during 1976. The overall arrangement is the same as in previous volumes, but some changes have been necessary within individual chapters.

Chapter 5 (Structure and Biological Activity) which was re-introduced into the series in Volume 5 has now become a major feature and occupied nearly one hundred pages in the last volume. The preparation of reviews of this length within the compressed time scale dictated by an annual series is proving too great a task for just one or two authors to tackle unaided. Accordingly, this chapter covering peptide hormones has now been sub-divided into five sections, each contributed by one or more specialists in individual aspects of peptide hormone chemistry. Inevitably this has resulted in selective coverage, and there are some regrettable omissions arising from lack of suitable authors. Chapter 2, Part III has been successfully arranged in a similar way since Volume 2, and this year Part I of the same chapter has also been divided into two discrete sections.

Once again it is pleasure to thank most warmly the many contributors to this volume who are listed opposite.

R. C. SHEPPARD

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Abbreviations

Abbreviations for amino-acids and their use in the formulations of derivatives follow, with some exceptions, the various Recommendations of the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature which have been reprinted in Volumes 4, 5, and 8 in this series.

Other abbreviations which have been used are listed here or are defined in the text and tables.

Ac	acetyl
Acm	acetamidomethyl
Ad	adamantyl
Adoc	adamantyloxycarbonyl
Amoc	9-anthrylmethyloxycarbonyl
Aoc	t-amyloxycarbonyl
Asu	α-aminosuberic acid
Asx	aspartic acid or asparagine (not yet determined)
ATP	adenosine 5′-triphosphate
Azoc	2-(4-phenylazophenyl)isopropyloxycarbonyl
Beoc	2-bromoethyloxycarbonyl
Boc	t-butoxycarbonyl
Bpoc	2-(4-biphenylyl)-isopropoxycarbonyl
BSA	bovine serum albumin
Btm	benzylthiomethyl
Bu^t	t-butyl
Bzh	benzhydryl (diphenylmethyl)
$Bzh(OMe)_2$	4,4′-dimethoxybenzhydryl
Bzl	benzyl
Bzl(4-Cl)	4-chlorobenzyl
$Bzl(2,6\text{-}Cl_2)$	2,6-dichlorobenzyl
Bzl(4-CN)	4-cyanobenzyl
Bzl(OMe)	4-methoxybenzyl
$Bzl(NO_2)$	4-nitrobenzyl
$Bzl(2\text{-}NO_2)$	2-nitrobenzyl
c.d.	circular dichroism
Cha	cyclohexylamine
Cm	carboxymethyl
Cmc	*S*-carboxymethylcysteine
CPh_2Py	diphenyl-4-pyridylmethyl
Dcha	dicyclohexylamine
Ddz	3,5-dimethoxy(αα-dimethyl)benzyloxycarbonyl

DMF	*NN*-dimethylformamide
DMSO	dimethyl sulphoxide
Dnp	2,4-dinitrophenyl
Dns	1-dimethylaminonaphthalene-5-sulphonyl (dansyl)
Dopa	3,4-dihydroxyphenylalanine
DP	degree of polymerization
Dpp	diphenylphosphinoyl
DPtd	4,6-diphenylthieno[3,4-*d*][1,3]dioxal-2-one 5,5-dioxide
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
Ec	ethylcarbamoyl
edta	ethylenediaminetetra-acetate
En	ethylene diamine
e.p.r.	electron paramagnetic resonance
e.s.r.	electron spin resonance
Et	ethyl
Gal	galactose
GC–MS	gas chromatograph–mass spectrometer combination
g.l.c.	gas–liquid chromatography
Glc	glucose
Glp	pyrrolid-2-one-5-carboxylic acid
Glx	glutamic acid or glutamine (not yet determined)
GTP	guanosine 5′-triphosphate
H.p.l.c.	high performance liquid chromatography
Iboc	isobornyloxycarbonyl
i.r.	infrared
Mal=	maleoyl
Man	mannose
Mbh	4,4-dimethoxybenzhydryl
Mbs	4-methoxybenzene sulphonyl
Me	methyl
Mea	mercaptoethylamine
Mhoc	1-methylcyclohexylcarbonyl
$MePh_2Peoc$	2-methyldiphenylphosphinioethyloxycarbonyl
Msc	2-(methylsulphonyl)ethoxycarbonyl
Mtc	2-methylthioethyloxycarbonyl
NAD	nicotinamide-adenine dinucleotide (NAD^+ oxidized: NADH, reduced)
NCA	*N*-carboxyanhydride
Nma	maleimido
N.m.r.	nuclear magnetic resonance
Nsu	Succinimido
Nps	*o*-nitrophenylsulphenyl
Np	4-nitrophenyl
ONp	*p*-nitrophenoxy
ONp(o)	*o*-nitrophenoxy
ONSu	succinimido-oxy
OPcp	pentachlorophenoxy

OPfp	pentafluorophenoxy
OPic	4-picolyloxy
o.r.d.	optical rotatory dispersion
OTcp	2,4,5,-trichlorophenoxy
Pac	phenacyl
Pcp	pentachlorophenyl
Peoc	2-triphenylphosphinioethyloxycarbonyl
Ph(SMe)	p-methylthiophenyl
Pic	4-picolyl
Picoc	4-picolyloxycarbonyl
Pipoc	piperidino-oxycarbonyl
Ppoc	phenylisopropoxycarbonyl
Ppt	diphenylphosphinothioyl
Pth-Gly	the phenylthiohydantoin derived from glycine, *etc.*
Pz	*p*-phenylazobenzyloxycarbonyl
SBu^t	t-butylthio
Scm	carboxymethylsulphenyl
SDS	sodium dodecyl sulphate
SPr^i	isopropylthio
Sub	5-dibenzosuberyl
Tac	toluene-*p*-sulphonylaminocarbonyl
Tcp	2,4,5-trichlorophenyl
Tfa	trifluoroacetyl
Thp	tetrahydropyranyl
T.l.c.	thin layer chromatography
Tmeda	*NNN'N'*-tetramethylethylene diamine
Tos	toluene-*p*-sulphonyl
Troc	2,2,2-trichloroethyloxycarbonyl
Trt	triphenylmethyl
Tse	2-(toluene-*p*-sulphonyl)ethyl
U.v.	ultraviolet
Z	benzyloxycarbonyl
Z(2-Br)	2-bromobenzyloxycarbonyl
Z(OMe)	*p*-methoxybenzyloxycarbonyl
Ztf	1-benzyloxycarbonylamino-2,2,2-trifluoroethyl

1
Amino-acids

BY G. C. BARRETT

1 Introduction

The format used in this chapter in previous volumes continues to provide a suitable framework for a review of current literature of amino-acids. There is an additional advantage in retaining a particular format in that readers may more easily trace specific topics back through the recent literature. Biological aspects and patent literature are excluded as in previous volumes of this series, but no other restraints have been imposed so that hopefully all chemistry-based work has been considered for inclusion.

Textbooks and Reviews.—Reference is made to relevant secondary and tertiary literature in the different sections of this chapter.

2 Naturally Occurring Amino-acids

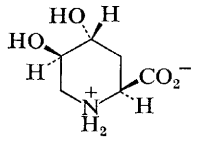
Occurrence of Known Amino-acids.—Reviews of the occurrence of non-protein amino-acids in plants [1–5] place emphasis on imino-acids and on *N*-heterocyclic amino-acids,[3] on D-amino-acids,[5] and on the roles played by free amino-acids,[1, 2, 4] which often exist in extraordinarily high concentrations in seeds [1] (*e.g.* 5-hydroxy-L-tryptophan comprises *ca.* 14% dry weight of seeds of *Griffonia simplicifolia* [6]).

Hydroxylated amino-acids existing in the free form, and their sources, as represented in the recent literature, are: (2*S*,3*R*,4*R*)-4-hydroxyisoleucine in steroidal sapogenin-yielding plants,[7] γ-hydroxyarginine from pea seedlings [8] and from lentil seeds [9] (which also contain γ-hydroxyornithine and homoarginine [9]), (2*S*,4*R*,5*R*)-2-carboxy-4,5-dihydroxypiperidine (1) from seeds of *Julbernardia paniculata*,[10] and its (2*S*,4*R*,5*S*)-isomer from *Derris elliptica*.[11] The (2*S*,4*S*,5*S*)-isomer of (1) is found in seeds of *Isoberlinia*, *Brachystegia*, *Cryptosepalum*,[10] and *Derris elliptica*,[11] but this is the first report [10] of the occurrence of the (2*S*,4*R*,5*R*)-isomer (1). 2,4,5-Trihydroxy-L-phenylalanine (*i.e.* 6-hydroxy-dopa) has been

[1] E. A. Bell, *F.E.B.S. Letters*, 1976, **64**, 29.
[2] A. Kjaer and P. O. Larsen, in 'Biosynthesis', ed. J. D. Bu'Lock (Specialist Periodical Reports), The Chemical Society, London, 1975, Vol. 4, p. 179.
[3] L. Fowden, *Heterocycles*, 1976, **4**, 117.
[4] L. Fowden, in 'Perspectives in Experimental Biology', Proceedings of the 50th Anniversary Meeting of The Society for Experimental Biology, ed. N. Sutherland, Vol. 2, Pergamon Press, Oxford, 1976, p. 263.
[5] T. Robinson, *Life Sci.*, 1976, **19**, 1097.
[6] E. A. Bell, L. E. Fellows, and M. Y. Qureshi, *Phytochemistry*, 1976, **15**, 823.
[7] R. Hardman and I. M. Abu-Al-Futuh, *Phytochemistry*, 1976, **15**, 325.
[8] T. A. Smith and G. R. Best, *Phytochemistry*, 1976, **15**, 1565.
[9] H. Sulser and F. Sager, *Experientia*, 1976, **32**, 422.
[10] P. R. Shewry and L. Fowden, *Phytochemistry*, 1976, **15**, 1981.
[11] M. Marlier, G. Dardenne, and J. Casimir, *Phytochemistry*, 1976, **15**, 183.

isolated from cultures of *Microspira tyrosinatica* grown aerobically in tyrosine-containing media.[12]

γ-Carboxyglutamic acid, a recently-identified addition to the protein amino-acids (Vol. 8, p. 5) has been found in human urine at low concentrations.[13]



(1)

The occurrence of γ-methyl-L-glutamic acid and its γ-ethyl-, -methylene, and -ethylidene analogues in seeds of *Julbernardia*, *Isoberlinia*, *Brachystegia*, and *Cryptosepalum* has been reported.[10]

Reports of studies of basic amino-acids form a large part of a *Proceedings* volume;[14] a report is included[14a] of the presence of basic and *N*-methylated basic amino-acids in frog tissue. Continuing studies of *Reseda odorata* (see Vol. 6, p. 3) reveal the presence of L-saccharopine and of 2*S*-amino-adipic acid.[15] One of the major cell-wall constituents of aerobic coryneform organisms from human skin is L,L-2,6-diaminopimelic acid;[16] however, the claim that this amino-acid exists in the free form in higher plants has been disproved,[17] providing something of a puzzle since the diaminopimelate pathway has been invoked for the biosynthesis of lysine in higher plants.

The first report of the existence of the D-enantiomer of α-amino-n-butyric acid in higher plants (legume seedling extracts) has appeared.[18] Other structurally simple amino-acids located in plants include glycine betaine in wheat,[19] and phenylalanine and tryptophan betaines in *Antiaris africana*.[20]

β-(Isoxazolin-5-on-2-yl)alanine occurs with β-(uracil-3-yl)alanine in root exudates of pea seedlings, and with small amounts of α-amino-γ-(isoxazolin-5-on-3-yl)butyric acid in sweet pea extracts[21] (see also Vol. 7, p. 4). The anthelmintic principle of *Quisqalis indica* is identical with quisqalic acid,[22] isolated from a related source (Vol. 8, p. 3).

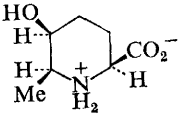
Fermentative production of amino-acids continues to generate a substantial

[12] D. O. Lunt and W. C. Evans, *Biochem. Soc. Trans.*, 1976, **4**, 491.
[13] P. Fernlund, *Clin. Chim. Acta*, 1976, **72**, 147.
[14] (*a*) J. Bolcs, in 'Proceedings of the 15th Hungarian Annual Meeting of Biochemistry', ed. B. Rosdy, Magy. Kem. Egyesulete, Budapest, 1975, p. 119 (*Chem. Abs.*, **85**, 189509); (*b*) E. Tyihak, I. Rusznak, and L. Trezl, *ibid.*, p. 19 (*Chem. Abs.*, **85**, 118182); (*c*) H. Kalasz, G. Kovacs, J. Nagy, J. Knoll, E. Tyihak, and A. Patthy, *ibid.*, p. 73 (*Chem. Abs.*, **85**, 119013); P. Miklos, J. Nagy, H. Kalasz, and J. Knoll, *ibid.*, p. 77 (*Chem. Abs.*, **85**, 119014); (*d*) A. Patthy, E. Tyihak, S. Ferenczi, S. Eckhardt, J. Kralovansky, H. Lapis, and B. Szende, *ibid.*, p. 57 (*Chem. Abs.*, **85**, 139313).
[15] H. Soerensen, *Phytochemistry*, 1976, **15**, 1527.
[16] D. G. Pitcher, *J. Gen. Microbiol.*, 1976, **94**, 225.
[17] P. O. Larsen and F. Norris, *Phytochemistry*, 1976, **15**, 1761.
[18] T. Ogawa, N. Bando, and K. Sasaoka, *Agric. Biol. Chem.*, 1976, **40**, 1661.
[19] R. B. Pearce, R. N. Strange, and H. Smith, *Phytochemistry*, 1976, **15**, 953.
[20] J. I. Okogun, A. I. Spiff, and D. E. U. Ekong, *Phytochemistry*, 1976, **15**, 826.
[21] Y. H. Kuo, F. Lambein, and R. Van Parijs, *Arch. Int. Physiol. Biochim.*, 1976, **84**, 169.
[22] P.-C. Pan, S.-D. Fang, and C.-C. Tsai, *Sci. Sin.*, 1976, **19**, 691.

literature, but can only be given passing mention here; representative papers [23, 24] describe the production of L-isoleucine [23] and the formation of L-norvaline and L-homoisoleucine as by-products in the conversion of threonine into L-isoleucine by *Serratia marcescens*.[24] For a more general review see reference 24*a*.

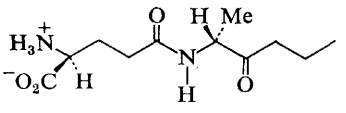
The area in which the non-occurrence of known amino-acids is perhaps a matter of as much importance as their occurrence, *i.e.* in extra-terrestrial samples, is represented by a report of analytical studies on lunar samples brought back on Apollo flights 11—17;[25] no amino-acids are present. The detection of amino-acids in meteorites and rock samples has been reviewed.[79]

New Natural Free Amino-acids.—Amino-acids reported in 1974 as 'unknown' constituents [26] of seeds of *Fagus silvatica* have now been characterized as (2*S*,5*S*,6*S*)-2-carboxy-5-hydroxy-6-methylpiperidine (2) and its 5*R*-epimer.[27]

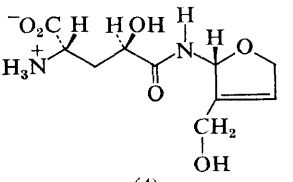


(2)

Further examples of new amino-acids reported in 1976 which also have relatively simple structures are L-2-amino-4-(2′-amino-ethoxy)butanoic acid (which accompanies the *trans*-3-butenoic acid analogue in a culture medium from an unidentified *Streptomycete*; see Vol. 7, p. 3);[28] α-methyl-L-arginine from a related source;[29] *N*-(L-γ-glutamyl)-2-aminohexan-3-one (3) from the mushroom *Russula*



(3)



(4)

ochroleuca;[30] and the L-allo-γ-hydroxyglutamine derivative (4) from *Hemerocallis fulva*.[31] The (*S*)-configuration can be proposed for the chiral centre adjacent to the ketone group in (3) on the basis of the octant rule (negative Cotton effect

[23] S. Ikeda, I. Fujita, and F. Yoshinaga, *Agric. Biol. Chem.*, 1976, **40**, 511; S. Ikeda, I. Fujita, and Y. Hirose, *ibid.*, 1976, **40**, 517; for a review see H. Matsushima, *Hakko Kogaku Zasshi*, 1976, **54**, 340.

[24] M. Kisumi, M. Sugiura, J. Kato, and I. Chibata, *J. Biochem.* (*Tokyo*), 1976, **79**, 1021.

[24a] D. R. Daoust, in 'Industrial Microbiology', ed. B. M. Miller and W. Litsky, McGraw-Hill, New York, 1976, p. 106.

[25] C. W. Gehrke, R. W. Zumwalt, K. Kuo, C. Ponnamperuma, and A. Shimoyama, *Origins Life*, 1975, **6**, 541.

[26] I. Kristensen, P. O. Larsen, and H. Soerensen, *Phytochemistry*, 1974, **13**, 2803.

[27] I. Kristensen, P. O. Larsen, and C. E. Olsen, *Tetrahedron*, 1976, **32**, 2799.

[28] J. P. Scannell, D. L. Pruess, H. A. Ax, A. Jacoby, M. Kellett, and A. Stempel, *J. Antibiotics*, 1976, **29**, 38.

[29] H. Maehr, L. Yarmchuk, D. L. Pruess, M. Kellett, N. J. Palleroni, B. La. T. Prosser, and T. C. Demny, *J. Antibiotics*, 1976, **29**, 213.

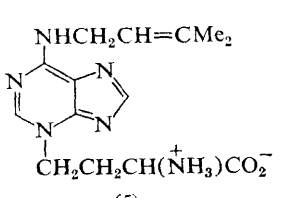
[30] A. Welter, J. Jadot, G. Dardenne, M. Marlier, and J. Casimir, *Phytochemistry*, 1976, **15**, 1984.

[31] G. J. Kruger, L. M. Du Plessis, and N. Grobbelaar, *J. South African Chem. Inst.*, 1976, **29**, 24.

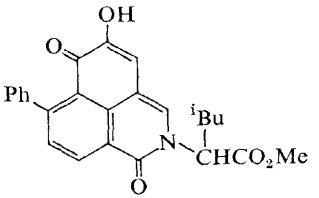
centred at 280 nm), but configurational assignments to acyclic ketones must be regarded as tentative when (as in this case) c.d. data for closely similar compounds of known absolute configuration are not available. The structure (4) was determined by *X*-ray crystal analysis,[31] establishing the configuration at the chiral centre in the dihydrofuran moiety to complete the structure assignment to oxypinnatanine, isolated from *Staphylea pinnata* and *Hemerocallis fulva* in 1973 (Vol. 6, p. 4), with which the amino-acid (4) studied by Kruger and co-workers is identical.[31]

The disulphiram-('antabuse')-like factor of the inky cap mushroom *Coprinus atramentarius* is N^5-(1-hydroxycyclopropyl)-L-glutamine (13), *alias* coprine.[36]

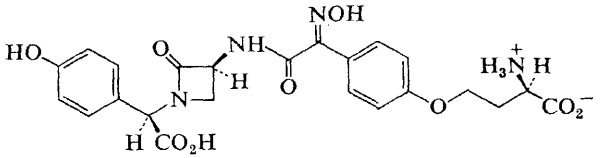
More complex structures are represented in discadenine (5), a spore germination inhibitor from the mould *Dictyostelium discoideum*,[32] the isoleucine derivative (6) (one of two new pigments from flowers of *Lachnanthus tinctoria*),[33] nocardicins



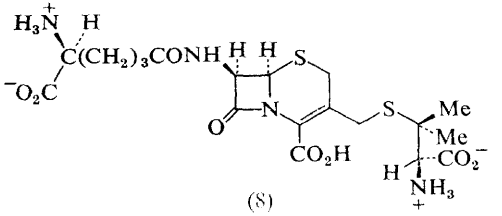
(5)



(6)



(7)



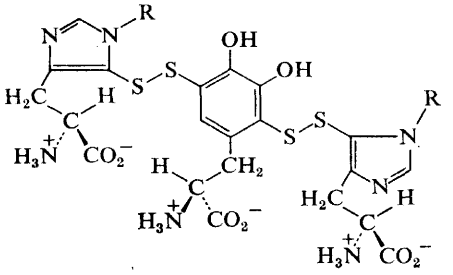
(8)

A and B from *Nocardia uniformis tsuyamaenis* [related to each other as geometrical isomers of the oxime (7)],[34] and the cephalosporin C analogue (8) produced by mutants of *Cephalosporium acremonium*.[35]

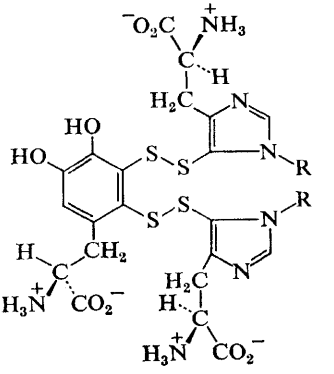
[32] H. Abe, M. Uchiyama, Y. Tanaka, and H. Saito, *Tetrahedron Letters*, 1976, 3807.
[33] A. C. Bazan and J. M. Edwards, *Phytochemistry*, 1976, **15**, 1413.
[34] M. Kurita, K. Jomon, T. Komori, N. Miyairi, H. Aoki, S. Kuge, T. Kamiya, and H. Imanaka, *J. Antibiotics*, 1976, **29**, 1243.
[35] T. Kanzaki, T. Fukita, K. Kitano, K. Katamoto, K. Nara, and Y. Nakao, *Hakko Kogaku Zasshi*, 1976, **54**, 720 (*Chem. Abs.*, **36**, 28440).

New Amino-acids from Hydrolysates.—Novel peptide antibiotics are often composed of an unusual variety of amino-acid residues. The antifungal antibiotic echinocandin B contains (2*S*,3*S*,4*S*)-3,4-dihydroxyhomotyrosine,[37, 38] (2*S*,4*R*,5*R*)-4,5-dihydroxyornithine,[38] and (2*S*,3*S*,4*S*)-4-methyl-3-hydroxyproline [38] among its complement of six amino-acids; the others are L-threonine (twice) and hydroxy-L-proline. A tripeptide from *Streptomyces plumbeus* contains D-2-amino-5-phosphono-3-pentenoic acid,[39] and provides a further example of an α-amino-acid with a phosphorus-containing functional group in the side-chain.

Hydrolysis of adenochrome, the iron(III)-containing pigment from the branchial heart of *Octopus vulgaris*, gives glycine and two novel iron-binding amino-acids, adenochromines A and B [(9) and (10) respectively], in the ratio 2 : 1.[40] Structure assignments were based upon reductive hydrolysis with HI and red phosphorus, which gives a new thiol-containing amino-acid, 5-mercapto-L-histidine, and L-dopa.[40] The adenochromines (9) and (10) are partially methylated (R = Me).[40]



(9; R = H, Me)

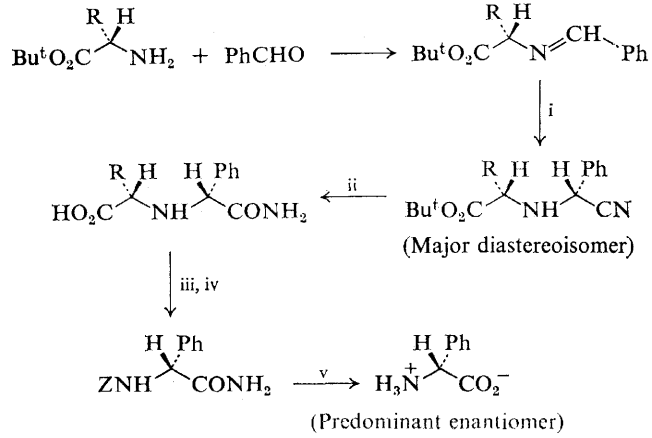


(10)

3 Chemical Synthesis and Resolution of Amino-acids

Asymmetric Synthesis.—Procedures based upon chiral Schiff bases have been developed further, with use of an L-amino-acid t-butyl ester [41] as starting material for the asymmetric synthesis of D-phenylglycine (Scheme 1).[41a] This interesting route reveals clearly the progress which still has to be made towards an economical asymmetric synthesis of α-amino-acids. There is a limitation imposed by the oxidation–decarboxylation cleavage step (the cleavage of the intermediate secondary amine causes destruction of the chiral starting material), and little can be learnt from the relationship between structure and optical yield expected through this route. There is a clue in the fact that of the various L-amino-acid

[36] P. Lindberg, R. Bergman, and B. Wickberg, *J.C.S. Chem. Comm.*, 1975, 946; *J.C.S. Perkin I*, 1977, 604; G. M. Hatfield and J. P. Schaumberg, *Lloydia*, 1975, **38**, 489.
[37] W. Keller-Schierlein and J. Widmer, *Helv. Chim. Acta*, 1976, **59**, 2021.
[38] C. Keller-Juslén, M. Kuhn, H. R. Loosli, T. J. Petcher, H. P. Weber, and A. von Wartburg, *Tetrahedron Letters*, 1976, 4147.
[39] B. K. Park, A. Hirota, and H. Sakai, *Agric. Biol. Chem.*, 1976, **40**, 1905.
[40] S. Ito, G. Nardi, and G. Prota, *J.C.S. Chem. Comm.*, 1976, 1042.
[41] (*a*) S. Yamada and S. Hashimoto, *Chem. Letters*, 1976, 921; (*b*) *Tetrahedron Letters*, 1976, 997.



Reagents: i, HCN; ii, acid hydrolysis; iii, Bu^{t}OCl; iv, ZCl; v, deprotection

Scheme 1

t-butyl esters tried, the valine derivative gives the highest optical yield (62%) of D-phenylglycine,[41a] suggesting that limited conformational mobility may be linked with high optical yield. L-Alanine is obtained in 65—76% optical yield in a corresponding route starting with methyl pyruvate, and employing H_2–Pd/C for asymmetric reduction to give a secondary amine corresponding to that in Scheme 1.[41b]

A quite different use of chiral Schiff bases has been reported from Yamada's laboratory.[42] Condensation of glycine t-butyl ester with a chiral ketone, (1*S*,2*S*,5*S*)-2-hydroxypinan-3-one, in the presence of BF_3–Et_2O gives the corresponding imine which, on reaction with an alkyl halide after carbanion formation with Pr^i_2NLi, gives a diastereoisomer mixture from which the corresponding D-amino-acid t-butyl ester is released by hydrolysis. Moderate chemical and optical yields (50—79 and 66—83%, respectively) were obtained.[42]

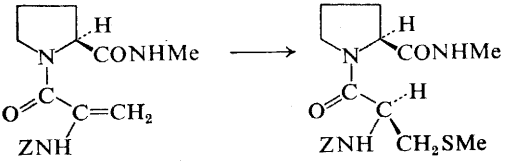
L-Alanine has been obtained in low optical yields by hydrogenolysis of the chiral imines formed by condensation of ethyl pyruvate with chiral amines;[43] in the best case, an optical purity of 46.5% was achieved and this was the case where a bulky amine, S-bornylamine, was used.

Asymmetric catalytic hydrogenation of prochiral acylaminocrotonates $R^1CONHC(=CR^2R^3)COR^4$ is represented in several papers in the recent literature,[44–48] all involving studies of rhodium complexes carrying chiral ligands as homogeneous catalysts. A broad study of the effects of structure of the

[42] S. Yamada, T. Oguri, and T. Shioiri, *J.C.S. Chem. Comm.*, 1976, 136.
[43] S. Kiyooka, K. Takeshima, H. Yamamoto, and K. Suzuki, *Bull. Chem. Soc. Japan*, 1976, **49**, 1897.
[44] V. A. Pavlov, E. I. Klabunovskii, G. S. Barysheva, L. N. Kaigorodova, and Y. S. Airapetov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1975, 2374.
[45] N. Takaishi, H. Imai, C. A. Bertelo, and J. K. Stille, *J. Amer. Chem. Soc.*, 1976, **98**, 5400.
[46] T. Hayashi, T. Mise, S. Mitachi, K. Yamamoto, and M. Kumada, *Tetrahedron Letters*, 1976, 1133.
[47] G. Gelbard, H. B. Kagan, and R. Stern, *Tetrahedron*, 1976, **32**, 233.
[48] K. Achiwa, *J. Amer. Chem. Soc.*, 1976, **98**, 8265.

N-acyl moiety and stereochemistry about the C=C bond on optical yields[47] provides useful source material for the continuing attempts at interpretation of the factors controlling the stereochemical course of this process, using the *O*-isopropylidene-2,3-dihydroxy-bis(diphenylphosphino)butane–RhCl catalyst. A polymer-bound analogue of this particular catalyst has been tested,[45] and in one of two other studies involving phosphorus-containing ligands[46, 48] for the asymmetric synthesis of phenylalanines, optical yields up to 91% have been achieved[48] in the synthesis of dopa and its analogues. Studies of the validity of earlier interpretations of n.m.r. data for the assignment of stereochemistry to 2-acylaminocrotonates have been reported.[49, 50]

Asymmetric induction in favour of the D,L-dipeptide is observed in nucleophilic addition of MeSH to the dehydroalanyl-L-prolinamide (11).[51]



(11)

General Methods of Synthesis of α-Amino-acids.—This section in previous volumes has provided a brief survey of classical methods of amino-acid synthesis, side-by-side with newer methods. The general picture in the 1976 literature is of consolidation of the use of recently-introduced methods, and of continuing use of diethyl acetamidomalonate[52–54] (refs. 128, 131, 134, 246), hydantoins,[55–57] modified Strecker synthesis,[58, 59] β-chloroalanine,[60, 61] and isonitriles ($CNCH_2CO_2Et + RR^1CBrCO_2R^2$ followed by hydrolysis gives $H_3\overset{+}{N}CH(CRR^1CO_2H)CO_2^-$).[62]

A conventional method for the introduction of an α-amino-group into an aliphatic carboxylic acid involves the azido-group as an intermediate, *e.g.* Al–Hg reduction of ethyl 2-azido-2-alkenoates,[63] but it is worth pointing out that the handling of azides can be particularly hazardous under certain circumstances.

[49] A. Srinivasan, K. D. Richards, and R. K. Olsen, *Tetrahedron Letters*, 1976, 891.
[50] R. Glaser and M. Twaik, *Tetrahedron Letters*, 1976, 1219.
[51] U. Schmidt and E. Öhler, *Angew. Chem.*, 1976, **88**, 54; *Angew. Chem. Internat. Ed.*, 1976, **15**, 42.
[52] A. Tun-Kyi and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2181.
[53] H. Gross and T. Gnauk, *J. prakt. Chem.*, 1976, **318**, 157.
[54] Y. Kataoka, Y. Seto, M. Yamamoto, T. Yamada, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1976, **49**, 1081.
[55] C. Perier, M. C. Ronziere, A. Rattner, and J. Frey, *J. Chromatog.*, 1976, **125**, 526.
[56] T. G. Tsarkova, I. A. Avrutskaya, M. Y. Fioshin, E. P. Krysin, and I. I. Gubenko, *Electrokhimiya*, 1975, **11**, 1803; T. G. Tsarkova, I. A. Avrutskaya, M. Y. Fioshin, and E. P. Krysin, *ibid.*, 1976, **12**, 902.
[57] H. Maehr, L. Yarmchuk, and M. Leach, *J. Antibiotics*, 1976, **29**, 221.
[58] R. F. Eizember and A. S. Ammons, *Org. Prep. Proc. Internat.*, 1976, **8**, 149.
[59] N. Sarda, A. Grouiller, and H. Pacheco, *Tetrahedron Letters*, 1976, 271.
[60] C. De Marco, A. Rinaldi, S. Dernini, and D. Cavallini, *Gazzetta*, 1975, **105**, 1113.
[61] B. Weinstein, K. G. Watrin, J. H. Loie, and J. C. Martin, *J. Org. Chem.*, 1976, **41**, 3634.
[62] M. Bochenska and J. F. Biernat, *Roczniki Chem.*, 1976, **50**, 1195.
[63] C. Shin, Y. Yonezawa, and J. Yoshimura, *Chem. Letters*, 1976, 1095.

Thus,[64] azidoacetic acid, $N_3CH_2CO_2H$, detonates at 90 °C under visible light, and at 25 °C in the presence of Fe or Fe salts.

Improved methods for anion formation from simple glycine derivatives have been developed, so that alkylation studies might then follow to open up general amino-acid syntheses. Preliminary studies of alkylation of the hippuric acid trianion, formed with Pr^i_2NLi and TMEDA, and the correspondingly-formed ethyl hippurate dianion, indicate predominant mono-*C*-alkylation.[65] Mono- or di-alkylation occurs with *N*-benzylideneglycine ethyl ester,[66] while an independent study [42] of Schiff base anion alkylation has taken the general process further in showing that a chiral alkylidene moiety may be mono-alkylated with a small degree of asymmetric induction (see preceding section). Alkylation of the trimethylsilyl *NN*-bis(trimethylsilyl)glycinate anion has been illustrated further (see ref. 123).

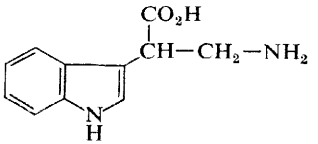
Further results in the application of *N*-acyl α-hydroxyglycines in the synthesis of *C*-aryl- and -heteroaryl-glycines have been reported.[67]

Only 0.08% yield of phenylalanine could be obtained from a reaction mixture including n-octyl phenylthiolacetate $PhCH_2COSC_8H_{17}$, with CO_2, NH_3, $Na_2S_2O_4$, and Schrauzer's iron–sulphur complex $(FeS_4C_4Ph_4)_2$,[68] although higher yields were obtained starting from the keto-acid which is assumed to be the reaction intermediate in this process.

Synthesis of β-Amino- and γ-Amino-acids.—Coverage of amino-acids other than α-amino-acids is justified by the co-occurrence of the various classes of amino-acid in a number of natural products. However, synthetic methods are usually straightforward and difficult to generalize, and only brief mention is possible.

(*S*)-4-Amino-2-hydroxy-n-butyric acid has been synthesized from the corresponding di-amino-acid [69] and from 3-acetoxy-2-methoxy-1-pyrroline.[70] A synthesis of the homologue (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid has been announced.[71]

The tryptophan isomer (12) has been synthesized from indole-3-acetonitrile.[72]



(12)

Prebiotic Synthesis: Model Reactions.—Continuing studies of a familiar type have been described, concerning the formation of nine amino-acids together with

[64] S. J. Borowski and K. Kamholz, *Chem. Eng. News*, 1976, **54**, 5.
[65] A. P. Krapcho and E. A. Dundulis, *Tetrahedron Letters*, 1976, 2205.
[66] G. Stork, A. Y. W. Leong, and A. M. Touzin, *J. Org. Chem.*, 1976, **41**, 3491.
[67] D. Ben-Ishai, I. Sataty, and Z. Bernstein, *Tetrahedron*, 1976, **32**, 1571.
[68] I. Tabushi, Y. Yabushita, and T. Nakajima, *Tetrahedron Letters*, 1976, 4343.
[69] Y. Horiuchi, E. Akita, and T. Ito, *Agric. Biol. Chem.*, 1976, **40**, 1649.
[70] Y. Yamada and H. Okada, *Agric. Biol. Chem.*, 1976, **40**, 1437.
[71] R. Steulmann and H. Klostermeyer, *Annalen*, 1975, 2245.
[72] V. S. Rozhkov, Y. I. Smushkevich, and N. N. Suvorov, *Zhur. org. Khim.*, 1976, **12**, 1076.

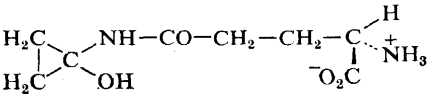
urea in CH_4–NH_3–H_2O mixtures subjected to electric discharge.[73, 74] Amino-acid synthesis resulting from shock waves in HCN–aldehyde–H_2O mixtures proceeds *via* aldimines and α-amino-nitriles as intermediates.[75] An interesting possibility that amino-acids arise, phoenix-like, from volcanic eruptions has been tested in a model system, in which lava at temperatures up to 1050 °C in contact with CH_4 (or CO)–NH_3–H_2O mixtures has been shown to initiate the synthesis of amino-acids and other organic compounds.[76] Other environmental factors which have been considered are transition metal ions in brine (a model for prebiotic sea),[77] and catalysis of condensation reactions by clay.[77, 78] Contrary to previous claims, montmorillonite shows only weak catalytic properties for the polymerization of activated amino-acids.[78]

A late stage in a plausible route for the abiotic synthesis of α-amino-acids, the hydrolysis of amino-malonotrile or acrylonitrile–electrophile adducts, has been shown to proceed under very mild conditions, and to lead to glycine, β-hydroxyaspartic acid, glutamic acid, threonine, and allothreonine.[79]

An extensive review of the prebiotic synthesis of amino-acids, and their detection in meteorites and rock samples, has appeared.[80]

Protein and Other Naturally Occurring Amino-acids.—Several of the papers cited in a preceding section covering general methods of synthesis describe the exploration of new methods for the synthesis of well-known amino-acids. A good deal of effort has been put into developing routes to γ-carboxyglutamic acid, and derivatives suitable for use in peptide synthesis; protected dehydroalanines participate in Michael addition reactions with di-t-butyl malonate,[81] and protected β-chloroalanines give mono-alkylated products with dibenzyl malonate,[61] leading to correspondingly-substituted γ-carboxyglutamic acids. Schwyzer and co-workers have continued their chemical studies of γ-carboxyglutamic acid,[82, 83] with syntheses of γγ′-di-t-butyl ester derivatives and establishment of absolute configuration by chemical correlation.[83]

Syntheses of L-2-oxalylamino-3-aminopropionic acid, an isomer of the neurotoxin from seeds of *Lathyrus sativus*,[84] and of coprine (13), an α-amino-acid from the mushroom *Coprinus atramentarius*,[36] have been announced.

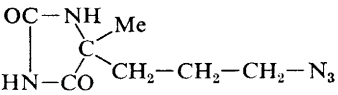


(13)

[73] C. Simionescu, R. Mora, N. Olaru, and E. Ionanid, *Compt. rend.*, 1976, **282**, 679.
[74] A. Constantinescu and C. Liteanu, *Rev. Chim. (Bucharest)*, 1976, **27**, 906.
[75] I. Barak and A. Bar-Nun, *Origins Life*, 1975, **6**, 483.
[76] L. M. Mukhin, V. B. Bondarev, V. I. Kalinichenko, E. N. Safonova, and Y. S. Petrenko, *Doklady Akad. Nauk S.S.S.R.*, 1976, **226**, 1225.
[77] M. Ventilla and F. Egami, *Proc. Japan Acad.*, 1976, **52**, 21 (*Chem. Abs.*, **34**, 117287).
[78] A. Brack, *Clay Miner.*, 1976, **11**, 117 (*Chem. Abs.*, **85**, 187961).
[79] J. W. Thanassi, *J. Mol. Evol.*, 1975, **7**, 65.
[80] A. T. Soldatenkov and I. A. Sytinskii, *Uspekhi Khim.*, 1976, **45**, 329.
[81] S. Bajusz and A. Juhasz, *Acta Chim. Acad. Sci. Hung.*, 1976, **88**, 157.
[82] W. Maerki, M. Oppliger, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 901.
[83] W. Maerki and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 1591.
[84] G. Wu, S. B. Bowlus, K. S. Kim, and B. E. Haskell, *Phytochemistry*, 1976, **15**, 125.

NN-Disubstituted aspartates may be synthesized by Stevens rearrangement of corresponding quaternary ammonium salts $RR'\overset{+}{N}(CH_2CO_2Me)_2X^-$,[85] while $\beta\beta$-disubstituted analogues have been prepared by alkylation of ethyl isonitriloacetate with α-bromo-esters followed by conventional hydrolysis procedures.[61]

α-Alkyl Analogues of the Protein Amino-acids.—Synthesis of α-methyl-L-arginine *via* the ornithine analogue has been achieved using a hydantoin route frequently adopted previously for similar cases, conventional functional group manipulation procedures being applied to (14).[57] Enzymic conversion into α-methyl-D-arginine and α-methyl-L-ornithine was a late step in the synthesis of a newly-discovered [29] antibiotic from *Streptomyces*.



(14)

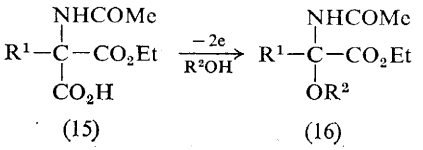
Side-chain Halogenated Analogues of the Protein Amino-acids.—Further details have been published dealing with electrochemical conversion of γγγ-trichloro-L-butyrine derivatives into the dichloro-analogues without racemization (see Vol. 8, p. 10).[86] Also arising from this project are βγ-unsaturated-γγ-dichloro-L-butyrine and γγ-dichloro-L-threonine derivatives.[86]

Photofluorination is demonstrated to be an efficient method for the synthesis of 3-fluoro-D-alanine and its 2-^{2}H analogue from the D-alanines without racemization.[87] Threonine derivatives give 2-amino-3-fluoro-butyric acid analogues on treatment with SF_4 in HF;[88] bromofluorination of 2-amino-pent-4-enoic and -hex-5-enoic acid derivatives followed by conventional aminolysis procedures leads to 4-fluoro-ornithine, -arginine, and -citrulline, and 5-fluoro-lysine, respectively.[89]

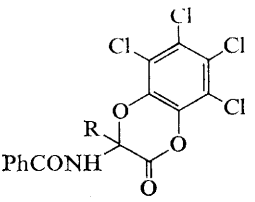
α-Hydroxy-, -Alkoxy-, -Amino-, and -Alkylthio-analogues of the Protein Amino-acids.—α-Amino-acids carrying a hetero-atom at the α-position are of continuing interest not only because of the presence of the $-NH-\underset{|}{C}(X)-CO-$ moiety in gliotoxins and related natural products, but because of their synthetic applications, *e.g.* in the synthesis of dehydro-amino-acids. Mention has been made in a preceding section of continuing studies of the use of α-hydroxyglycine derivatives as starting materials in amino-acid synthesis.[67] *N*-Chlorination of Boc-Val-OMe with Bu^tOCl and treatment with NaOMe gives *N*-t-butoxycarbonyl-α-methoxy-valine methyl ester in 94% yield [90] (see Scheme 2).

A new route to α-alkoxy- and α-acetoxy-α-amino-acids from α-acetamido-malonate half esters, (15) → (16), involves electrolysis of solutions in alcohols or

[85] A. T. Babayan, S. T. Kocharyan, and S. M. Ogandzhanyan, *Armyan. khim. Zhur.*, 1976, **29**, 456.
[86] T. Iwasaki, Y. Urabe, Y. Ozaki, M. Miyoshi, and K. Matsumoto, *J.C.S. Perkin I*, 1976, 1019.
[87] J. Kollonitsch and L. Barash, *J. Amer. Chem. Soc.*, 1976, **98**, 5591.
[88] R. S. Loy and M. Hudlicky, *J. Fluorine Chem.*, 1976, **7**, 421.
[89] V. Tolman and J. Benes, *J. Fluorine Chem.*, 1976, **7**, 397.
[90] H. Poisel and U. Schmidt, *Angew. Chem.*, 1976, **88**, 295; *Angew. Chem. Internat. Edn.*, 1976, **15**, 294.



(15) (16)



(17)

in acetic acid.[91] An unexpected bonus from studies of oxazol-5(4*H*)-ones (already known to be useful intermediates for the activation of the α-C-atom of an α-amino-acid) follows[92] from reaction of *o*-chloranil oxidation products (17) with nucleophiles to give α-alkoxy- (16) and analogous -benzylthio- and -anilino-amino-acids.[92]

Aliphatic α-Amino-acids Carrying Hydroxy-groups and Ether Groups in Side-chains.—5-Hydroxybutylhydantoin serves as starting material for the synthesis of ε-hydroxynorleucine.[55] α-Amino-β-phenyoxypropionic acid has been obtained through a modified Strecker reaction.[59]

Stereoisomers of γ-hydroxyisoleucine isolated as their lactones from photochlorination of D-allo-isoleucine followed by hydrolysis are (2*R*,3*R*,4*R*)-2-amino-3-methyl-4-hydroxyvaleric acid, and its 4*S*-epimer, with 2*S*-epimers of these being obtained by $Ba(OH)_2$ epimerization.[93]

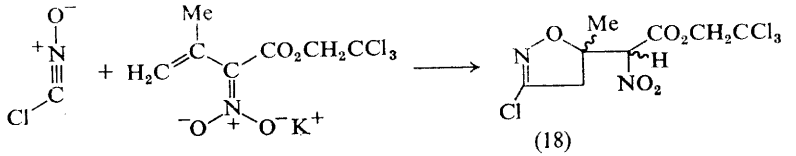
Aromatic and Heterocyclic Amino-acids.—Routine syntheses from aromatic aldehydes leading to *p*-methoxy- and *p*-hydroxyphenylglycine,[58] and to phenylalanine and tryptophan,[56] have been reported, the interest in the latter study laying particularly in the electroreduction of 4-arylidenehydantoins.

β-Methylphenylalanine has been obtained through the acetamidomalonate route,[54] a point of interest being the stereoselectivity observed in the decarboxylation step (the L-erythro-isomer occurs in bottromycin). ββ-Disubstituted phenylalanines may be obtained from corresponding α-cyano-esters $ArCMe_2CH(CN)CO_2Et$ by conventional elaboration,[94] or from ketones $ArCMe_2COMe$ *via* the keto-acids.[95] The latter route has been applied in the synthesis of ββ-dimethyl-dopa.[95]

Manipulation of the aryl moiety in a phenylalanine has been a well-worked operation over the years, and novel procedures are still being established; *N*-acetyl-3-iodo-L-tyrosinamide gives the thyronine analogue by coupling with di(4-methoxyphenyl)iodonium tetrafluoroborate followed by deprotection with HBr–AcOH, and procedures for further iodination leading to 3,3′-di-iodo- and 3,3′,5′-tri-iodo-L-thyronines have been described.[96]

Isoxazolines are being found in increasing numbers in various classes of natural products, including amino-acids, and a synthesis of the 3-chloroisoxazolin-

[91] H. Horikawa, T. Iwasaki, K. Matsumoto, and M. Miyoshi, *Tetrahedron Letters*, 1976, 191.
[92] J. M. Riordan and C. H. Stammer, *Tetrahedron Letters*, 1976, 1247.
[93] M. Hasan, D. Georgopoulos, and T. Wieland, *Annalen*, 1976, 781.
[94] N. A. Jonsson and L. Mikiver, *Acta Pharm. Suec.*, 1976, **13**, 75.
[95] N. A. Johsson and L. Mikiver, *Acta Pharm. Suec.*, 1976, **13**, 65.
[96] P. Block, *J. Medicin. Chem.*, 1976, **19**, 1067.



(18)

5-ylglycine (18) has been achieved [97] to reward the vigilance of its creators in seeking new applications of cycloaddition reactions for relevant natural products syntheses. The initial exploration of the route involved the methyl α-nitrocrotonate, but difficulty was experienced in hydrolysing the ester at the end of the synthesis while retaining the intact side-chain. The configuration of a crystalline isomer isolated from the diastereoisomeric product mixture was not established.

***N*-Substituted Amino-acids.**—A considerable body of papers has been published concerning particularly the *N*-methyl homologues of the basic amino-acids, as part of a *Proceedings* volume.[14] N^{ε}-Monomethyl-L-lysine is the major reaction product from L-lysine and formaldehyde in dilute neutral solutions, conditions under which no reaction is observed with L-arginine.[14b] A new procedure for obtaining *N*-monomethyl derivatives of simple amino-acids uses conventional reagents (MeI–NaH–THF) after conversion of the amino-acid into the phosphinamide $Ph_2PONHCHRCO_2H$.[98]

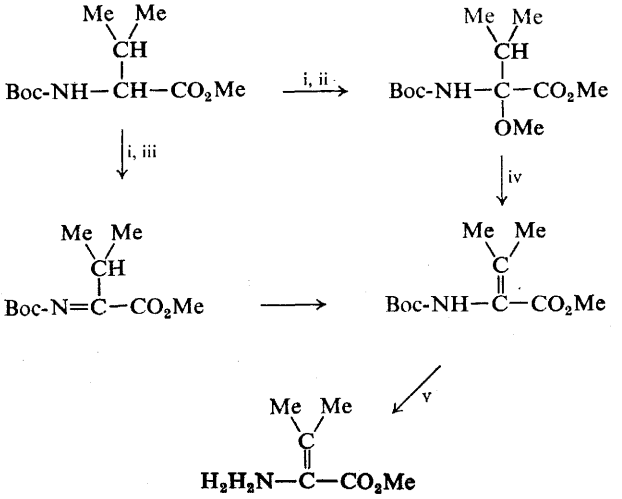
α-Hydrazino-acids may be obtained from amino-acids *via* α-diazo-esters [99] or *via* α-bromo-acids,[100] the latter route being illustrated in the synthesis of 'D-α-hydrazino-ornithine' from L-ornithine.[100, 101]

Amino-acids with Unsaturated Side-chains.—Conventional methods have been used for the synthesis of L-propargylglycine $H_3\overset{+}{N}CH(CH_2C{\equiv}CH)CO_2^-$ (from diethyl acetamidomalonate and propargyl bromide) and its resolution.[52]

Further illustration of the route announced last year (Vol. 8, p. 11) for the conversion of an α-amino-acid into its dehydro-analogue is provided [90] by the alternative reaction sequences in Scheme 2. Methyl dehydrovalinate, perhaps better named methyl 2-amino-3,3-dimethylacrylate, is obtained in 86% yield *via* the α-methoxy-α-amino-acid intermediate. There are alternative routes to these unsaturated α-amino-acid esters, and further examples of their preparation from 2-azido-2-alkenoates by Al–Hg reduction,[63] and of the synthesis of *N*-acyl analogues from *N*-acyl-2-thiono-oxazolidine-4-carboxylates,[102] have been published.

Novel synthetic methods have been described, one [103] exploiting the 'ene' addition pathway in leading to γδ-unsaturated amino-acids from an alkene and an *N*-(arenesulphonyl)imine, p-$MeC_6H_4SO_2N{=}CHCO_2Bu^n$; and the other [104]

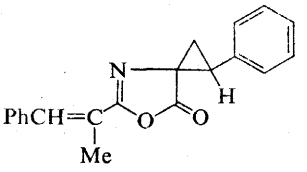
[97] J. E. Baldwin, C. Hoskins, and L. Kruse, *J.C.S. Chem. Comm.*, 1976, 795.
[98] S. Coulton, G. A. Moore, and R. Ramage, *Tetrahedron Letters*, 1976, 4005.
[99] N. Takamura and S. Yamada, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 800.
[100] T. Sawayama, H. Kinugasa, and H. Nishimura, *Chem. Pharm. Bull.*, 1976, **24**, 326.
[101] J. G. Johansson, R. M. Parkhurst, and W. A. Skinner, *Indian J. Chem.*, 1976, **14B**, 209.
[102] D. Hoppe and R. Follmann, *Chem. Ber.*, 1976, **109**, 3062.
[103] O. Achmatowicz and M. Pietraszkiewicz, *J.C.S. Chem. Comm.*, 1976, 484.
[104] J. W. Hines, E. G. Breitholle, M. Sato, and C. H. Stammer, *J. Org. Chem.*, 1976, **41**, 1466.



Reagents: i, Bu^{t}OCl; ii, NaOMe; iii, DABCO; iv, HCl; v, NH_3

Scheme 2

unexpectedly yielding DL-styrylglycine rather than a cyclopropylphenylalanine on acid hydrolysis of the oxazolone (19). A reliable synthesis of styrylglycine [104] through the α-amino-nitrile route was developed to provide authentic material for comparison with the hydrolysis product from (19).

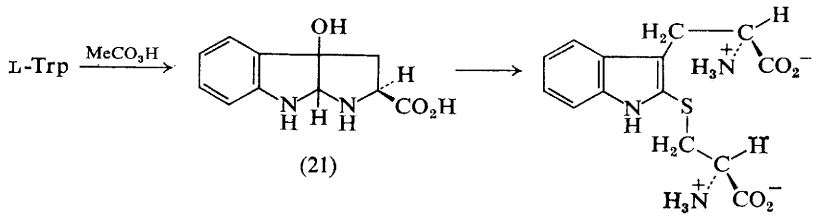


(19)

Amino-acids Containing Sulphur or Selenium.—Addition of thiols to protected amino-acids carrying unsaturated side-chains gives corresponding sulphides.[104, 105] The alternative approach, in which cysteine *S*-alkylation leads to analogous products, is illustrated in a novel synthesis of tryptathionine (20) involving a useful modified tryptophan (21).[106]

Routine studies of the preparation and resolution of sulphoxides of *S*-alkyl- and -propenyl-cysteines [107] and of *S*-(2-methylprop-1-enyl)-L-cysteine [108] are supplemented by interesting studies of cyclization of alkenylcysteine sulphoxides to thiazine-*S*-oxides.[108] Homolanthionine sulphoxides and sulphones are formed from homocystine by treatment with H_2O_2 in acid solution.[109]

[105] J. T. Snow, J. W. Finley, and M. Friedman, *Internat. J. Peptide Protein Res.*, 1976, **8**, 57.
[106] W. E. Savige and A. Fontana, *J.C.S. Chem. Comm.*, 1976, 600.
[107] G. G. Freeman and R. J. Whenham, *Phytochemistry*, 1976, **15**, 521.
[108] J. F. Carson and R. E. Lundin, *J.C.S. Perkin I*, 1976, 1195.
[109] P. Clopath, and K. S. McCully, *Anal. Biochem.*, 1976, **73**, 231.



(21) (20)

Thioasparagine is formed from β-cyanoalanine derivatives by conventional thioamide synthesis,[110] with benzyl thioaspartates $ArCH_2SCOCH_2CH(\overset{+}{N}H_3)\text{-}CO_2^-$ being formed as a result of *S*-alkylation of the thioamide followed by hydrolysis, in procedures involving deblocking of certain *N*-protected intermediates.[110]

Independent studies leading to the synthesis of L-4-selenalysine, $H_2NCH_2\text{-}CH_2SeCH_2CH(\overset{+}{N}H_3)CO^-$, from β-chloro-L-alanine have been reported,[60, 111] and followed up by a synthesis of 4-selena-homolysine.[112] Selenocysteine, used in the latter synthesis [112] and in an increasing number of studies in plant biochemistry,[113] is easily prepared from selenocystine by $NaBH_4$ reduction.[113]

Phosphorus-containing Amino-acids.—The title of this small section is to be interpreted to refer to amino-carboxylic acids with phosphorus-containing functional groups in side-chains. DL-Phosphinothricin, $HOP(O)MeCH_2CH_2\text{-}CH(\overset{+}{N}H_3)CO_2^-$, has been synthesized by alkylation of diethyl acetamidomalonate with $ClCH_2CH_2OP(O)MeCH_2CH_2Cl$, formed from ethylene oxide and $MePCl_2$ followed by rearrangement.[53]

Further examples of amino-acids of this class are listed in the next section.

A List of Amino-acids which have been Synthesized for the First Time.—In addition to several new amino-acids mentioned elsewhere in the chapter, the following compounds have been synthesized recently for the first time.

Compound	*Ref.*
L-*o*-Carboranylalanine, $B_{10}C_2CH_2CH(\overset{+}{N}H_3)CO_2^-$	114
Selenium analogue of L-tryptophan (Se in place of NH^{indole})	115
Phosphonate analogues of L-aspartic acid	116
Phosphinate analogues of L-aspartic acid	116
5-Aza-analogue of L-arginine	117
5-Aza-analogue of 4,5-dehydro-L-arginine	117
N-Substituted γ-glutamic acid hydrazides	117

[110] C. Ressler and S. N. Banerjee, *J. Org. Chem.*, 1976, **41**, 1336.
[111] T. Sadeh, M. A. Davis, and R. W. Giese, *J. Pharm. Sci.*, 1976, **65**, 623.
[112] C. DeMarco, S. Dernini, A. Rinaldi, and D. Cavallini, *Gazzetta*, 1976, **106**, 211.
[113] A. Shrift, D. Bechard, C. Harcup, and L. Fowden, *Plant Physiol.*, 1976, **58**, 248.
[114] O. Leukart, M. Caviezel, A. Eberle, E. Escher, A. Tun-Kyi, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2184.
[115] L. Liatem and L. Christiaens, *Bull. Soc. chim. France*, 1975, 2294.
[116] M. Soroka and P. Mastalerz, *Roczniki Chem.*, 1976, **50**, 661.
[117] V. Cavrini, A. Chiarini, L. Garuti, G. Giovanninetti, and L. Franchi, *Il Farmaco, Ed. Sci.*, 1976, **31**, 599.

Compound	*Ref.*
p-(Diethyleneimidophosphoramido)phenylalanine	118
β-(1-Chloro-2-naphthyl)-DL-alanine	119
β-(1-Bromo-2-naphthyl)-DL-alanine	119
S-Adenosyl-homocysteine homologues	120, 121
S-Adenosyl-methionine homologues	121
2-Amino-ω-(uracil-1-yl)-n-alkanoic acids	122
2-Amino-ω-(thymin-1-yl)-n-alkanoic acids	122
L-3-(3-Deoxy-1,2:5,6-di-*O*-isopropylidene-α-allofuranos-3-yl)alanine	123
DL-3-(β-D-Ribofuranosyl)alanine	124
(2*R*)-L-Arabino-tetrahydroxybutyl)-(4*R*)-thiazolidinecarboxylic acid, and (2*S*)-epimer	125
	125

Labelled Amino-acids.—Further studies of the selective deuteriation of cobalt(III) complexes $[Co(en)_2(L\text{-amino-acidato})]^{n+}X_n^-$ [126, 127] and $[Co(NH_3)_4(L\text{-amino-acidato})]^+X^-$ (X = Cl, I, or NO_3) have been published (see Vol. 6, p. 20). Separation by ion-exchange chromatography of the diastereoisomeric complexes and α-^{2}H exchange at pH 9.6 led to further diastereoisomeric pairs, which on chromatographic separation and $NaBH_4$ reduction gave optically-pure D- and L-α-^{2}H labelled amino-acids.[126] The stereochemical course of the deuterium exchange reaction is controlled by the amino-acid side-chains since greater stereoselectivity was observed with aspartic and glutamic acid complexes than with the corresponding complexes of the amides of these amino-acids.[127] α-^{2}H-*S*-Benzyl-DL-cysteine has been prepared from benzyl chloromethyl sulphide and diethyl acetamidomalonate with the use of ^2HCl–^2H_2O as reagent for the hydrolysis–decarboxylation step;[128] [ββ-$^2H_2$]- and [αββ-$^2H_3$]-analogues were also prepared *via* the acetamidomalonate route. (β*R*)[β-^{2}H]-L-Homoserine, and (γ*R*)[γ-^{2}H]-DL-homoserine and its (γ*S*)-isomer, have been prepared by routes starting with *cis*-addition of NH_2OH to cinnamic acid, and with enantiomers of $Ph(CH_2)_2CH^2HOH$, respectively.[129] DL-[γ-^{2}H]-Leucine may be prepared by hydroboration of isobutene with $B_2{}^2H_6$, with conventional elaboration and modified acetamidomalonate synthesis.[130] A route providing nearly equal amounts of (2*S*,3*R*)- and (2*R*,3*R*)-[β-^{2}H]-phenylalanines has been developed (Scheme 3).[131] Condensation of 3-(^{2}H-formyl)-indole with hippuric acid and routine elaboration gives (2*S*,3*R*)-[3-^{2}H]-tryptophan.[132] Aromatic

[118] R. Poskiene, K. Karpavicius, A. Puzerauskas, O. V. Kildisheva, and I. L. Knunyants, *Izvest. Akad. Nauk. S.S.S.R., Ser. khim.*, 1976, 407.
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[124] A. Rosenthal and A. J. Brink, *Carbohydrate Res.*, 1976, **47**, 332.
[125] R. Bognar, Z. Gyoergydeak, L. Szilagyi, G. Horvath, G. Czira, and L. Radics, *Annalen*, 1976, 450.
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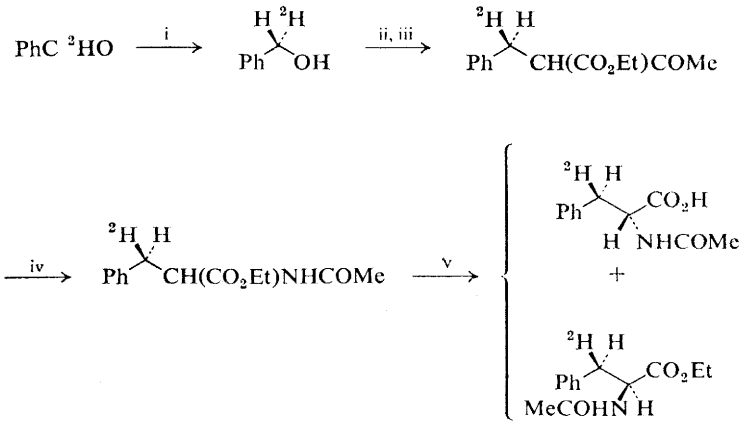
[128] D. A. Upson and V. J. Hruby, *J. Org. Chem.*, 1976, **41**, 1353.
[129] D. Coggiola, C. Fuganti, D. Ghiringhelli, and P. Grasselli, *J.C.S. Chem. Comm.*, 1976, 143.
[130] J. A. Sogn, W. A. Gibbons, and S. Wolff, *Internat. J. Peptide Protein Res.*, 1976, **8**, 459.
[131] U. Nagai and J. Kobayashi, *Tetrahedron Letters*, 1976, 2873.
[132] S. Sawada and M. Kitagawa, *Bull. Koyoto Univ. Educ., Ser. B*, 1975, **47**, 19 (*Chem. Abs.*, **85**, 160484).

amino-acids labelled in the aryl moiety, *viz.* DL-tyrosine-[2′-^{3}H] [133] and 3-(4-azido-3,5-$^3H_2$-phenyl)-L-alanine,[134] have been prepared *via* the benzaldehyde [133] and by catalytic tritiation,[134] respectively. Distribution of the label in generally-



Reagents: i, yeast; ii, TsCl; iii, AcCHNaCO$_2$Et; iv, HN$_3$,H$^+$; v, α-chymotrypsin

Scheme 3

^{3}H-labelled amino-acids may be determined by ^{3}H-n.m.r. spectrometry (see ref. 191).

A range of familiar chemical operations has been used to create ^{11}C-labelled amino-acids; $^{11}CH_3I$ (from $^{11}CO_2$) has been used to synthesize methyl-labelled L-methionine [135] and DL- or L-ethionine,[136] while carboxyl-^{11}C-labelled DL-phenylglycine and DL-phenylalanine have been prepared by carboxylation of α-lithioisocyanides with $^{11}CO_2$.[137] Some idea of the potential of these compounds in *in vivo* studies is provided by data for methionine-^{11}C, produced by irradiation of methionine in an electron accelerator equipped with a tungsten target; the product is a γ-emitter with half-life 20 min.[138]

S-Ethylthiouronium-^{14}C bromide has been employed in the synthesis of labelled L-arginine from L-ornithine.[139]

Biological synthesis of ^{15}N-labelled amino-acids for n.m.r. studies has been achieved by algae cultured on $^{15}NO_3^{2-}$-containing media.[140] A related approach

[133] P. W. Jeffs, N. Johns, and D. B. Johnson, *J. Labelled Comp. Radiopharm.*, 1976, **12**, 133.
[134] W. Fischli, M. Caviezel, A. Eberle, E. Escher, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 873.
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[138] G. Gundlach, E. L. Sattler, W. Trampisch, and U. Wagenbach, *Z. Naturforsch., Biosci.*, 1976, **31C**, 377; A. Donnerhack, E. L. Sattler, and W. Trampisch, *Strahlentherapie*, 1976, **151**, 240.
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[140] A. Severge, F. Juttner, E. Breitmayer, and G. Jung, *Biochim. Biophys. Acta*, 1976, **437**, 289.

using a high-methionine-producing mutant of *Saccharomyces cerevisiae* cultured on an $Na_2{}^{75}SeO_3$-containing medium leads to ^{75}Se-selenomethionine.[141]

Resolution of Amino-acids.—The main threads of the various aspects available for study under this heading, which have made up this section of this chapter in recent years, continue to be unravelled further. The chiral macrocyclic polyether project, in which the factors responsible for high chiral recognition of enantiomers of amino-acid perchlorates or amino-acid ester perchlorates [142] are being sought, has been extended to optically-pure 22-crown-6 polyethers bonded to polystyrene;[143] the project has been reviewed.[144] Several other chromatographic resolution procedures have been described, one [145] taking a similar approach to Cram's in bonding dibenzo-18-crown-6 ethers to ion-exchange resins and employing h.p.l.c. techniques, and others using chiral metal complexes, D-$[Co(en)_3]^{3+}$ on a weak acid cation exchange resin [146] and *N*-benzyl-L-leucinatocopper(II) bound to polystyrene,[147] as stationary phases. Copolymers of *N*-acryloyl-L-phenylalanine esters with ethylenediacrylate furnish cross-linked acrylamide-type polymers suitable for the resolution of DL-mandelic acid and related compounds;[148] this method could be tried for the resolution of analogous DL-amino-acid derivatives.

Gas–liquid chromatographic resolution of volatile amino-acid derivatives on an *N*-acyl-L-valinamide [149] on *N*-acyl-L,L-dipeptide ester [150, 151] as stationary phase is represented by further papers from innovators in this field. Higher resolution factors may be achieved when the *N*-acyl moiety of the stationary phase is a bulky alkanoyl grouping.[149] The mechanism of the differential interaction of a chiral stationary phase with enantiomeric amino-acid derivatives has been assumed to be predominantly due to different hydrogen bonding equilibrium constants, but this may need to be re-thought since some resolution can still be achieved with stationary phases incapable of hydrogen bonding.[152]

Stereospecific complex formation forms the basis of a procedure for the resolution of DL-aspartic or glutamic acids, in which combination with (L-argininato)$Cu(ClO_4)_2$ followed by crystallization and destruction of the complex with H_2S gives the amino-acid of moderate optical purity, but in low overall yield.[153]

Stereospecific complex formation of partly-resolved amino-acids with cobalt complexes is reflected in easily measured o.r.d. and c.d. spectra, from which the

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[151] W. A. Koenig, *Chromatographia*, 1976, **9**, 72.
[152] K. Stoelting and W. A. Koenig, *Chromatographia*, 1976, **9**, 331.
[153] T. Sakurai, O. Yamauchi, and A. Nakahara, *J.C.S. Chem. Comm.*, 1976, 553.

optical purity of the amino-acids may be calculated for samples of about 1 mg [154] (see also Vol. 7, p. 21).

Conventional resolution procedures have been described for 3,4,5-trimethoxyphenylglycine (resolved as its *Z*-derivative using a chiral base),[155] *N*-acylprolines [156] and 3-(3,4-methylenedioxyphenyl)-2-methylalanine hydrochloride [157] (resolved through preferential crystallization), and *N*-benzoyl-amino-acids (use of papain in stereoselective formation of *N*-benzoyl-amino-acid anilides).[158] Novel asymmetric transformation procedures in which more than 50% yield of one enantiomer can be obtained from a racemic amino-acid have been reported for phenylglycine esters, using (+)-tartaric acid and a carbonyl compound,[159] and for the formation of L-lysine from DL-α-amino-ε-caprolactam, based on the preferential crystallization of the L-enantiomer with $NiCl_2$ and EtOH accompanied by the Ni^{II}-catalysed racemization of residual D-enantiomer.[160]

Further studies of preferential adsorption by quartz of one enantiomer from DL-alanine hydrochloride in DMF use an extremely sensitive test system; the 'racemate' is a mixture of ^{3}H-labelled L-alanine and ^{14}C-labelled D-alanine.[161] The conclusion that *l*-quartz preferentially adsorbs L-alanine under these conditions appears to be soundly based, since a discrimination factor larger than would be expected for the different isotopic formulations of the two enantiomers was demonstrated.[161]

Analytical resolution of DL-amino-acids is covered also in section 6 of this chapter.

4 Physical and Stereochemical Studies of Amino-acids

Crystal Structures of Amino-acids and their Derivatives.—A review has appeared [162] of the solid-state hydrogen-bonding patterns for amino-acids established through neutron diffraction studies. A statistical treatment of available data from neutron diffraction studies has been published, which will be useful for computations of preferred conformations within amino-acid residues in peptides.[163]

A substantial amount of the 1976 literature concerns simple compounds, often repeating earlier *X*-ray studies. In this category papers describing L-isoleucine hydrochloride monohydrate (crystal form II),[164] D-allo-isoleucine hydrochloride monohydrate,[165] L-leucine,[166] histidine dihydrochloride,[167] DL-allo-threonine

[154] Y. Fujii, S. Hirasawa, and S. Takahashi, *Chemistry Letters*, 1976, 817.
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[163] R. Balasubramanian, *Indian J. Biochem. Biophys.*, 1976, **13**, 7.
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hydrobromide,[168] α-methyl-DL-tyrosine,[169] and allo-4-hydroxy-L-proline,[170] have appeared. 2′,3′-Dimethyl-3,5-di-iodo-DL-thyronine[171] and 3′-isopropyl-3,5-di-iodo-L-thyronine[172] have been subjected to *X*-ray crystal analysis and other physical studies; the latter compound is particularly interesting since it is the most potent known thyromimetic agent.[172]

The absolute configuration (2*S*,3*R*) has been assigned to 3-amino-2-hydroxy-4-phenyl-butanoic acid, present in bestatin, through *X*-ray crystal analysis of its methyl ester hydrobromide.[173] *X*-Ray studies similarly leading to stereochemical information for uncommon amino-acids have been carried out with ibotenic acid,[174] quisqalic acid,[175] oxypinnatanine,[31] and DL-1-aza-3-thiacyclohexane-6-carboxylic acid monohydrate,[176] while derivatives of common amino-acids which have been studied include *N*-carboxy-anhydrides of glycine[177] and alanine,[178] *N*-acetyl-L-glutamine,[179] N^{α}-toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride,[180] and the cysteine derivatives (2*R*,4*R*)-*S*-carboxymethyl-cysteine sulphoxide,[181] the corresponding sulphone,[182] and α-(*S*-cysteinyl)-thymine.[183]

N.M.R. Spectrometry.—General trends in n.m.r. studies appearing in the literature of 1976 are reflected in papers on amino-acids, where more confident exploration of nuclei other than ^{1}H and ^{13}C is being undertaken. Continuing consolidation of well-established areas of study is also represented in the 1976 literature.

Conformational studies of protein amino-acids in aqueous solutions are reaching something like a point of culmination, with a mass of data now available relating to the torsion angles about α-CH—β-CH_2 bonds. The three minimum energy staggered conformations for this bond are represented in different proportions for the various β-substituted alanines, and component vicinal coupling constants for the rotamers have been determined.[184] A similar objective is represented in studies of the three-carbon carboxyl-C to β-H coupling constants for aspartic acid and for derivatives of histidine and of cysteine;[185]

[168] P. Swaminathan and R. Srinivasan, *J. Cryst. Mol. Struct.*, 1975, **5**, 203.
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[184] J. Feeney, *J. Magnetic Res.*, 1976, **21**, 473.
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values 1.3 ± 0.3 Hz for the gauche conformation and 9.8 ± 0.3 Hz for the anti conformation lead to a conclusion that the carboxylate anion–β-substituent relationship is predominantly anti in aqueous solutions of these amino-acids.[185]

Useful generalizations have been reported for ^{13}C-chemical shift data for amino-acids.[186] The pH-dependence of ^{14}N-linewidths has been determined for glycine, alanine, lysine, and proline,[187] and temperature-dependence [188] and pH-dependence [140, 188] of ^{13}C—^{15}N coupling constants for ^{15}N-amino-acids have been assessed.

A routine use of ^{1}H-n.m.r. for the precise analysis of mixtures of phenylglycine with its dihydro-, tetrahydro-, and hexahydro-analogues has been described,[189] using tetramethylammonium bromide as internal standard. ^{3}H-N.m.r. spectra of ^{3}H-phenylalanine obtained through the Pt-catalysed $^{3}H_2O$ exchange procedure show an average 26% exchange in the side-chain, mostly at the β-position, with 74% exchange of the phenyl protons, fairly equally distributed.[190] This spectrometric assay has also been applied to other generally-3H-labelled amino-acids.[190]

Fundamental studies of polycrystalline amino-acids at 130—500 K point to origins of spin–lattice relaxation in reorientation of protonated amino-groups and this results in the relaxation of other protons by spin-exchange.[191] Perturbation of spin-lattice relaxation times of lanthanide(III) ion binding can be exploited in conformational assignments to L-hydroxyproline in $^{2}H_2O$.[192] Further studies of ^{2}H and ^{14}N pure quadrupole resonance in amino-acids have been reported.[193]

Amino-acid derivatives studied by n.m.r. include *N*-acetyl-L-proline deuteromethyl ester (^{1}H and ^{13}C-n.m.r. data interpreted in terms of rotational barriers in support of previous conclusions),[194] N^{α}-acetyl-L-prolinamide (^{1}H n.m.r. conformational studies),[195] and Boc-amino-acid esters of glycine, alanine, and phenylalanine (continuing ^{1}H and ^{13}C-n.m.r. studies of conformational equilibria involving the amide moiety).[196]

Interaction between aromatic amino-acids and nucleic acid models has already been extensively studied and is now supplemented by studies of solutions containing adenosine-5′-phosphate and phenylalanine,[197] tryptophan,[197] or *O*-methyl-tyrosine,[198] and the solution behaviour of adenin-(5′-*N*)-yl derivatives

[186] J. C. MacDonald, G. C. Bishop, and M. Mazurek, *Canad. J. Chem.*, 1976, **54**, 1226.
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[198] S. V. Zenin, *Studies Biophys.*, 1976, **55**, 175.

of phenylalanine, tyrosine, and tryptophan methyl esters.[199] Related studies [200] show that lysine and arginine form complexes with DNA, polynucleotides, nucleosides, and nucleic acid bases.

Optical Rotatory Dispersion and Circular Dichroism Spectra.—A positive Cotton effect at 215 nm in the c.d. spectrum of *N*-(γ-glutamyl)-2-amino-3-hexanone, isolated from the mushroom *Russula ochroleuca*, allows the assignment of the L-configuration to the chiral centre in the glutamyl moiety, but the opportunity was not taken to interpret the negative Cotton effect at 280 nm for the assignment of absolute configuration to the other chiral centre in this compound;[30] the (*S*)-configuration for this chiral centre shown in (3) might be assigned on the basis of the octant rule, but support is required from data for closely similar compounds when semi-empirical assignments are made to acyclic ketones.

The alternative to the use of the 215 nm carboxylate Cotton effect for the assignment of absolute configuration to an α-amino-acid is the introduction of an appropriate chromophore, usually by *N*-substitution, which results in diagnostic Cotton effect behaviour, and further advocacy of the use of fluorescamine derivatives for this purpose (see also Vol. 8, p. 19) has been published.[201] *N*-(3-Methyl-2-quinoxaloyl)amino-acids are new representatives of chromophore-substituted amino-acids, but these appear to lack a consistent relationship between sign of Cotton effect and absolute configuration.[202]

An investigation of the chirospectroscopic properties of the selenide chromophore has been made, in the framework of a c.d. study of seleno-amino-acids.[203]

Chromophore–chromophore interaction effects arise in studies of the extrinsic Cotton effect contribution to the c.d. of 3,5-di-iodo-L-tyrosine near 320 nm induced by binding to bovine serum albumin,[204] and in the c.d. of adenin-(5′-*N*)-yl derivatives of phenylalanine, tyrosine, and tryptophan methyl esters,[199] interpreted in the latter case to show coplanarity of adenine and aromatic moieties in these compounds.

There are few examples of the effect of isotopic substitution on optical rotation data, but a new result, that ^{15}N-alanine shows lower o.r.d. amplitudes at corresponding wavelengths than its ^{14}N counterpart, implies surprisingly large differences.[205]

Mass Spectrometry.—Chemical ionization techniques continue to show their relative advantages in mass spectrometric analysis of amino-acids, with H_2 as a more suitable reactant gas than CH_4 since it gives lower abundance of M + 1 ions and increased abundance of fragment ions with advantages, therefore, in structure determination.[206] This study included β-, γ-, and higher homologous amino-acids as well as 14 common α-amino-acids.[206] Free amino-acids have

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[205] W. Darge, I. Laczko, and W. Thiemann, *J. Radioanalyt. Chem.*, 1976, **30**, 521.
[206] C. W. Tsang and A. G. Harrison, *J. Amer. Chem. Soc.*, 1976, **98**, 1301.

been studied also by secondary ion mass spectrometry, which leads to high abundance secondary ion–parent peaks in mass spectra.[207] Parallel studies of chemical ionization mass spectrometry in this area [208, 209] and of field desorption [210] and negative ion [211] techniques are mostly concerned with derivatives of amino-acids (thiohydantoins [209, 210] and *N*-nitrobenzoyl amino-acid methyl esters [211]).

Derivativization has, of course, been the first stage in nearly all previous studies of amino-acids, and the more routine papers in the 1976 literature continue this tradition with studies of methylthiohydantoins,[212] fluorescamine derivatives,[213] *N*-acetyl amino-acid *p*-nitrobenzyl esters,[214] *N*-dimethylamino-methylene amino-acid methyl esters,[215] and *N*-pentafluoropropionyl amino-acid hexafluoropropyl esters.[216]

Further illustration of the sensitivity of the g.c.–m.s. combination is provided [216] by a study of the determination of glutamic acid and γ-aminobutyric acid in rat brain nuclei, based upon *ca.* 50 μg protein samples (see also ref. 318).

Other Physical and Theoretical Studies.—A study of crystalline amino-acids by photoelectron spectroscopy has been reported,[217] indicating extensive intermolecular hydrogen bonding between zwitterionic tautomers; i.r. spectra of α-amino-acids are also usually interpreted in terms of zwitterionic structures, but γ-aminobutyric acid has been reported [218] to be capable of adopting the alternative uncharged form in the solid state. Far i.r. spectra (10—150 cm^{-1}) of α-amino acids at temperatures 80 and 300 K contain characteristic peaks for all common amino-acids except glycine.[219] Raman study of ^{15}N-glycine has been described.[220]

Gas-phase ionization energies of simple molecules are readily determined by He^{I} photoelectron spectroscopy, but only those amino-acids with alkyl side-chains were found to be sufficiently stable for study by this technique.[221]

Semi-empirical m.o. calculations indicate non-planar structures for *N*-acetyl-L-alanine *N*-methylamide and MeCONHMe in the solid state,[222] and comparable studies [223] for nineteen α-amino-acids predict three-dimensional structures corresponding closely with those determined by *X*-ray crystal structure and n.m.r. analysis.

[207] A. Benninghoven, D. Jaspers, and W. Sichtermann, *Appl. Phys.*, 1976, **11**, 35.
[208] D. Issacher and J. Yinon, *Clinica Chim. Acta*, 1976, **73**, 307.
[209] T. Suzuki, K.-D. Song, Y. Hagaki, and K. Tuzimura, *Org. Mass Spectrom.*, 1976, **11**, 557.
[210] H. R. Schulten and B. Wittmann-Liebold, *Analyt. Biochem.*, 1976, **76**, 300.
[211] B. J. Stapleton and J. H. Bowie, *Org. Mass Spectrom.*, 1976, **11**, 429.
[212] J. Lindeman and R. E. Lovins, *Analyt. Biochem.*, 1976, **75**, 682.
[213] J.-J. Shieh, K. Leung, and D. M. Desiderio, *Org. Mass Spectrom.*, 1976, **11**, 479.
[214] C. L. Brown and C. L. Chan, *J. Amer. Chem. Soc.*, 1976, **98**, 2682.
[215] I. Horman and F. J. Hesford, *Biomed. Mass Spectrom.*, 1974, **1**, 115.
[216] L. Bertilsson and E. Costa, *J. Chromatog.*, 1976, **118**, 395.
[217] D. T. Clark, J. Peeling, and L. Colling, *Biochim. Biophys. Acta*, 1976, **453**, 533.
[218] P. V. Huong and J. C. Cornut, *J. Chem. Phys.*, 1976, **65**, 4748.
[219] X. Gerbaux and A. Hadni, *Compt. rend.*, 1976, **282**, 181, 397.
[220] H. Steinback, *J. Raman Spectroscopy*, 1976, **5**, 49.
[221] L. Klasinc, *J. Electron. Spectrosc. Related Phenomena*, 1976, **8**, 161.
[222] V. Renugopalakrishnan and R. Rein, *Biochim. Biophys. Acta*, 1976, **434**, 164.
[223] A. A. Akhrem, V. P. Golubovich, S. G. Galaktionov, and G. V. Nikiforovich, *Vestsi Akad. Navuk Belarusk. S.S.R., Ser. khim. Navuk*, 1976, 77.
[224] H. D. Belitz and H. Wieser, *Z. Lebensm.-Untersuch.*, 1976, **160**, 251.

One of the simplest possible physical studies forms the basis of the report [224] that the sweet or bitter taste of amino-acids and peptides may be related to their three-dimensional arrangement of polar and hydrophobic groups.

5 Chemical Studies of Amino-acids

Racemization.—The application of adventitious racemization for dating fossils and corresponding biological samples has been surveyed in this section in preceding volumes of this report. The optical purity of amino-acids existing in these samples, either in the free state or as constituents of residual collagen, is related to the age of the sample, and confident use of this fact has been made after racemization rate constants appropriate for the particular site have been determined. Further application of the method has been reported for 4220-year-old Bristlecone pine and fossil wood from Kalambo Falls, Zambia, with data obtained on 13 amino-acids extracted from the samples.[225] Racemization rate constants derived from these data, for proline and hydroxyproline, lead to a date >110 000 years for the Acheulian–Sangoan transition at the site, based on the optical purity of amino-acids found in contemporary samples.[225] The possible sources of error in the method have been well appreciated, and studies of fossil coral samples of known age from Pleistocene sites [226] indicate the inadequacy of the method, based on epimerization and racemization of L-isoleucine, when leaching of free amino-acids from samples, or later contamination, or variations in the mineral content leading to variable racemization rates, can be suspected to have occurred. Meanwhile, several laboratories are using the method, a further example being a report of preliminary studies of the state of the amino-acids comprising the collagen of mammoth bone.[227]

Novel systems which are effective in the racemization of L-alanine in alkaline aqueous solutions are copper(II) salts with nitrosophenols [228] and copper(I) oxide.[229] In the latter case, copper(II) ions are probably also the effective species, formed from Cu_2O during aerial oxidation of the amino acid.

Reactions of Amino-acids Involving Amino- and Carboxy-groups.—There is still scope for the formation of novel functional derivatives in the amino-acid series, current examples being the synthesis of DL-phenylalanine ortho-esters [$Ph(CH_2)_3CN \rightarrow PhCH_2CH_2C(=NCl)OEt \rightarrow NH_2CH(CH_2Ph)C(OEt)_3$],[230] and formation of *NN*-dichloro-amino-acids.[231] In the latter example, a quantitative yield of $Cl_2NCH_2CO_2H$ is obtained from the amino-acid by treatment with Bu^tOCl in MeOH at 0 °C (an analogous product is formed from β-alanine), but the tendency for these derivatives to explode at temperatures not much above room temperature should be noted. Factors determining the stability of chloro-derivatives of amino-acids have been assessed.[232]

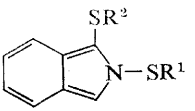
[225] C. Lee, J. L. Bada, and E. Peterson, *Nature*, 1976, **259**, 183.
[226] J. F. Wehmiller, P. E. Hare, and G. A. Kujala, *Geochim. Cosmochim. Acta*, 1976, **40**, 763.
[227] G. Dungworth, A. W. Schwartz, and L. Van De Leemput, *Comp. Biochem. Physiol.*, 1976, **53**(4B), 473.
[228] K. Hirota, H. Koizumi, Y. Hironaka, and Y. Izumi, *Bull. Chem. Soc. Japan*, 1976, **49**, 289.
[229] A. Tai, K. Okada, T. Masuda, and Y. Izumi, *Bull. Chem. Soc. Japan*, 1976, **49**, 310.
[230] J. Zemlicka and M. Murata, *J. Org. Chem.*, 1976, **41**, 3317.
[231] J. Vit and S. J. Barer, *Synth. Comm.*, 1976, **6**, 1.
[232] J. J. Kaminski, N. Bodor, and T. Higuchi, *J. Pharm. Sci.*, 1976, **65**, 553.

Quaternization of amino-acids may be achieved under mild conditions using MeI and $KHCO_3$ in MeOH.[233]

Thermal degradation of amino-acids is the subject of continuing studies, with recent emphasis on aspartic acid[234] and sulphur-containing amino-acids.[235, 236] Under N_2 at temperatures between 350—650 °C, succinic acid is the major condensable product (12%) with dimethylmaleic anhydride, from aspartic acid,[234] while S is lost as CS_2 or COS when the common sulphur-containing amino-acids are heated at 850 °C under N_2 to give degradation products of a similar type to those obtained from simple aliphatic amino-acids.[235] A variety of sulphur-containing products is formed from cysteine derivatives heated in soya bean oil at 200 °C.[236]

Strecker degradation of α-amino-acids with phenalene-1,2,3-trione hydrate, giving the corresponding aldehyde, NH_3, and CO_2, has been studied from the structure–reactivity viewpoint.[237] A similarly well-known reaction of amino-acids, nitrous acid deamination, has been studied for its stereochemical consequences with isoleucine derivatives.[238]

Colour reactions of amino-acids of particular current interest, formation of fluorescent products with fluorescamine,[239] and with *o*-phthalaldehyde and a thiol,[240] have been discussed. In the latter case, the 1-(alkylthio)-2-substituted iso-indoles (22) are the surprising products whose intense fluorescence allows the picomole level determination of α-amino-acids.[240]



(22)

L-Amino-acids and derivatives employed in asymmetric synthesis and in stereoselective synthesis are: L-amino-acid t-butyl esters used in asymmetric transamination with ketones,[241] aldehydes,[41] and keto-esters,[41] boron hydride–L-leucine methyl ester complex for the asymmetric reduction of ketones,[242] L-glutamic acid in the enantioselective synthesis of epoxy-ketones,[243] and similar use of L-lysine in new syntheses of L-pipecolic acid and of (*S*)-(+)-coniine.[244]

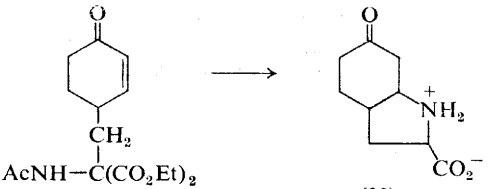
Reactions Involving Side-chains of α-Amino-acids.—In this section, papers are brought together covering reactions of side-chain functional groups in amino-

[233] F. C. M. Chen and N. L. Benoiton, *Canad. J. Chem.*, 1976, **54**, 3310.
[234] A. W. Fort, J. M. Patterson, R. Small, B. K. Bandlish, and W. T. Smith, *J. Org. Chem.*, 1976, **41**, 3697.
[235] J. M. Patterson, C.-Y. Shille, and W. T. Smith, *J. Agric. Food Chem.*, 1976, **24**, 988.
[236] F. Ledl, *Z. Lebensm.-Untersuch.*, 1976, **161**, 125.
[237] W. I. Awad, S. Nashed, S. S. M. Hassan, and R. F. Zakhary, *J.C.S. Perkin II*, 1976, 128.
[238] W. Kirmse and G. Rauleder, *Annalen*, 1976, 1333.
[239] H. Nakamura and J. J. Pisano, *J. Chromatog.*, 1976, **121**, 33, 79.
[240] S. S. Simons and D. F. Johnson, *J. Amer. Chem. Soc.*, 1976, **98**, 7098.
[241] S. Yamada, N. Ikota, and K. Achiwa, *Tetrahedron Letters*, 1976, 1001.
[242] M. F. Grundon, D. G. McCleery, and J. W. Wilson, *Tetrahedron Letters*, 1976, 295.
[243] S. Yamada, N. Oh-hashi, and K. Achiwa, *Tetrahedron Letters*, 1976, 2557, 2561.
[244] K. Aketa, S. Terashima, and S. Yamada, *Chem. Pharm. Bull.*, 1976, **24**, 621.

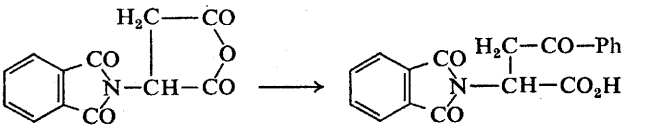
acids. An attempted synthesis of a β-cyclohexenonyl-alanine *via* the acetamidomalonate route led instead to the indolinonecarboxylate (23).[245]

Alkylation rates for methionine derivatives and related model compounds indicate the high nucleophilicity of the methionine sulphur atom; among common nucleophiles, only the thiolate anion has higher nucleophilicity.[246]

Friedel–Crafts acylation of benzene with phthalylaspartic anhydride and $AlCl_3$ gives 3-benzoyl-2-phthalimidopropionic acid (24), consistent with anhydride



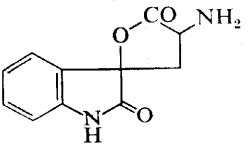
(23)



(24)

ring-opening in the predicted direction (opposition by the phthalimido-group of build-up of charge at the neighbouring carbon atom),[247] although the isomeric structure had been assigned earlier to the reaction product.

Reactions of tryptophan derivatives involving the indole grouping include the formation of β-3-oxindolylalanine during the hydrolysis of proteins in 6M hydrochloric acid, if dithioglycollic acid or cystine is present,[248] the same product is formed from tryptophan itself.[248] L-Tryptophan gives two diastereoisomers of the spirolactone (25) on treatment with t-butyl hydroperoxide and $FeSO_4$.[249]



(25)

Inorganic radical anion oxidation of tryptophan has been studied.[250] Condensation products are formed from tryptophan and α-keto-acids *via* 1,2,3,4-tetrahydroharman-1,3-dicarboxylic acid and its homologues.[251]

[245] P. Sawlewicz, I. Barska, M. Smulkowski, J. Gumieniak, T. Czarnomska, M. Dzieduszycka, and E. Borowski, *Roczniki*, 1976, **50**, 1005.

[246] G. A. Rogers, N. Shaltiel, and P. D. Boyer, *J. Biol. Chem.*, 1976, **251**, 5711.

[247] W. G. Reifenrath, D. J. Bertelli, M. J. Micklus, and D. S. Fries, *Tetrahedron Letters*, 1976, 1959.

[248] T. Nakai and T. Ohta, *Biochim. Biophys. Acta*, 1976, **420**, 258.

[249] G. Stoehrer, *J. Heterocyclic Chem.*, 1976, **13**, 157.

[250] M. L. Posener, G. E. Adams, P. Wardman, and R. B. Cundall, *J.C.S. Faraday I*, 1976, **72**, 2231.

[251] N. T. Chu and F. M. Clydesdale, *J. Food Sci.*, 1976, **41**, 891.

Specific Reactions of Amino-acids Related to Biochemical Processes.—The decarboxylation of L-threonine by a di-aquo-cobyric acid–thiol complex has been investigated.[252] Continuing studies of the condensation of pyridoxal with amino-acids to form Schiff bases provide comparisons of the reactivity of α-amino-acids, α,ω-di-amino acids, and ω-amino-acids.[253]

Effects of Electromagnetic Radiation on Amino-acids.—A larger number of references than usual has been collected for this section, and rather than leave particular areas of current study unrepresented, there has been even greater restraint on the amount of space used for each reference. Except for a study of the formation of methyl radicals by u.v. photolysis of *N*-methyl- and *N*-acetyl-amino-acids at 77 K,[254] the photochemical studies in the recent literature are concerned with the aromatic amino-acids. The effect of sodium alginate on the rates of dye-sensitized photo-oxidation of tyrosine and tyramine has been studied,[255] and the products of irradiation of phenylalanine at 253.7 nm in H_2O_2 have been shown to be aspartic acid, serine, alanine, ammonia, and a trace of lysine.[256] Effects of solvent polarity, metal ions, and sulphur compounds on the phosphorescence and fluorescence of tryptophan at 77 K have been assessed.[257] Decay rates and spin-lattice relaxation rates for the lowest excited triplet states of tyrosine and tryptophan at 1.34 K have been measured;[258] and flash photolysis products of aqueous solutions of tryptophan and tryptophan-containing peptides have been studied.[259]

γ-Irradiation of aqueous solutions of amino-acids [260–263] gives products of H-abstraction resulting from secondary effects of the formation of hydroxyl radicals;[260] penicillamine gives thiyl radicals in de-aerated 1M $HClO_4$,[261] and proline gives products of de-amination or ring cleavage together with hydroxy-proline,[262] while peroxyglycine radicals are formed in irradiated glycine solutions under O_2.[263] γ-Irradiated solid glycine [264] and L-valine [265] undergo partial degradation. These results are supplemented by results from *X*-irradiation of cysteine hydrochloride [266] and aspartic acid hydrochloride [267] leading to products from primary radical formation; the e.s.r. spectra of *X*-irradiated solid L-amino-acids differ in nearly every case from those of corresponding DL-amino-acids.[268] M.o. calculations show that loss of an electron under ionizing radiation would be expected to occur from the carboxy-grouping of aliphatic amino-acids, while for tyrosine and tryptophan the source of the excited electron is the aromatic

[252] S. H. Ford and H. C. Friedmann, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 1077.
[253] A. M. Der Garabedian and M. A. Der Garabedian, *F.E.B.S. Letters*, 1976, **72**, 87.
[254] G. H. Schepel and H. Neubacher, *Radiation Environ. Biophys.*, 1976, **13**, 49.
[255] G. R. Seely and R. L. Hart, *Photochem. Photobiol.*, 1976, **23**, 1, 7.
[256] A. S. Ansari, S. Tahib, and R. Ali, *Experientia*, 1976, **32**, 573.
[257] L. A. King and J. N. Miller, *Biochim. Biophys. Acta*, 1976, **446**, 206.
[258] K. W. Rousslang and A. L. Kwiram, *Chem. Phys. Letters*, 1976, **39**, 226.
[259] H. Templer and P. J. Thistlethwaite, *Photochem. Photobiol.*, 1976, **23**, 79.
[260] T. Masuda, S. Nakano, K. Yoshihara, and M. Kondo, *J. Radiation Res.*, 1976, **17**, 63.
[261] G. C. Goyal and D. A. Armstrong, *J. Phys. Chem.*, 1976, **80**, 1848.
[262] N. A. Duzhenkova and J. Kopoldova, *Khim. vysok. Energii*, 1976, **10**, 351.
[263] S. Abramovitch and J. Rabani, *J. Phys. Chem.*, 1976, **80**, 1562.
[264] P. R. Crippa, C. Giori, and A. Vacli, *Radiat. Eff.*, 1976, **29**, 143.
[265] D. G. Howitt, R. M. Glaeser, and G. Thomas, *J. Ultrastruct. Res.*, 1976, **55**, 457.
[266] W. W. H. Kon and H. C. Box, *J. Chem. Phys.*, 1976, **64**, 3060.
[267] S. M. Adams, E. E. Budzinski, and H. C. Box, *J. Chem. Phys.*, 1976, **65**, 998.
[268] H. Shields and P. J. Hamrick, *J. Chem. Phys.*, 1976, **64**, 263.

moiety;[269] for phenylalanine, there is roughly equal probability of electron abstraction from carboxy and phenyl groupings.[269]

Reference has been made in the preceding paragraph to the selective radiolysis of L- and DL-amino-acids, and for solid samples the differences in crystal structures account for these results. However, the interest which has been stimulated recently (Vol. 7, p. 25; Vol. 8, p. 16) in the enantioselective destruction of racemic amino-acids by irradiation of solutions has been expressed by the appearance of papers from workers new to the field. While no support for the notion that positrons react at different rates with D- and L-tryptophan has been secured, since positronium yields from the interaction of ^{22}Na positrons with each enantiomer are identical,[270] a relative enrichment of *ca.* 19% has been reported for the D-enantiomer as a result of 12 weeks' irradiation of a 0.005% aqueous solution of DL-tryptophan by ^{32}P β-radiation (0.57 MeV) resulting in 33% decomposition of the sample and indicating preferential decomposition of the L-enantiomer.[271] γ-Radiolytic decarboxylation of D-phenylalanine-1-^{14}C occurs *ca.* 2.7 times faster than for the L-enantiomer,[272] an extraordinary example of the selective destruction of one enantiomer by non-polarized radiation.

6 Analytical Methods

Reviews of sample preparation [273] and determination [273, 274] of amino-acids in biological samples have been published.

Gas–Liquid Chromatography.—Quantitative analysis of amino-acids by g.l.c. has been reviewed.[275]

Conversion of amino-acids into volatile derivatives is the unavoidable preliminary stage in their analysis by g.l.c., and perfluoroalkyl derivatives remain the most widely used, with, as usual, strong advocacy of alternative new types. *N*-Trifluoroacetyl amino-acid n-butyl esters [276–279] remain in wide use, together with analogous ethyl [14c] and trimethylsilyl [280, 281] esters. *N*-Heptafluorobutyryl amino-acid isobutyl esters of fifty amino-acids have been studied by g.l.c.;[282] analogous iso-amyl esters have been included in a broad study.[276] *N*-Pentafluoropropionyl-γ-aminobutyric acid hexafluoroisopropyl ester and the analogous

[269] D. A. Dixon and W. N. Lipscomb, *J. Biol. Chem.*, 1976, **251**, 5992.
[270] W. Brandt and T. Chiba, *Phys. Letters*, 1976, **57A**, 395.
[271] W. Darge, I. Laczko, and W. Thiemann, *Nature*, 1976, **261**, 522.
[272] O. Merwitz, *Radiat. Environment. Biophys.*, 1976, **13**, 63.
[273] E. Reid, *Analyst*, 1976, **101**, 1.
[274] J. E. Hammond and J. Savory, *Ann. Clin. Lab. Sci.*, 1976, **6**, 158.
[275] S. V. Vitt, M. B. Saporovskaya, G. V. Avvakumov, and V. M. Belikov, *Uspekhi. Khim.*, 1976, **45**, 548.
[276] G. E. Otter and L. Taylor, *J. Inst. Brew., London*, 1976, **82**, 264.
[277] L. A. Appelqvist and B. M. Nair, *J. Chromatog.*, 1976, **124**, 239.
[278] K. Samukawa, T. Nagai, and S. Takahashi, *Radioisotopes*, 1976, **25**, 135.
[279] V. Amico, G. Oriente, and C. Tringali, *J. Chromatog.*, 1976, **116**, 439.
[280] M. Schwarz and G. Michael, *J. Chromatog.*, 1976, **118**, 101.
[281] M. Donike, *J. Chromatog.*, 1975, **115**, 591.
[282] R. J. Siezen and T. H. Mague, *J. Chromatog.*, 1977, **130**, 151.
[283] P. Felker, *Anal. Biochem.*, 1976, **76**, 192.
[284] I. M. Moodie and R. D. George, *J. Chromatog.*, 1976, **124**, 315.
[285] M. Makita, S. Yamamoto, and M. Kono, *J. Chromatog.*, 1976, **120**, 129; M. Makita, S. Yamamoto, K. Sakai, and M. Shiraishi, *ibid.*, 1976, **124**, 92; M. Makita and S. Yamamoto, *Yakugaku Zasshi*, 1976, **96**, 777; *ibid.*, 1976, **96**, 813.

L-glutamic acid derivative form the basis of a g.c.–m.s. analysis of amino-acid transmitters in rat brain nuclei.[216]

N-Acetyl amino-acid methyl [283] and n-propyl esters [284] are also advocated as volatile derivatives for the g.l.c. analysis of amino-acids, and several papers have been published [285] describing the use of *N*-isobutyloxycarbonyl amino-acid methyl esters for the same purpose.

Husek introduced 1,3-dichloro-1,1,3,3-tetrafluoropropanone as a reagent in 1974 for derivatization of amino-acids for g.l.c. analysis (Vol. 7, p. 26), and has reported further [286] on the merits of the resulting cyclic stable derivatives. Ongoing study of g.l.c. separation of *N*-phenylthiohydantoins is represented by independent reports [287] establishing conditions for resolution of leucine and isoleucine PTH's.

Mention has been made in a preceding section of the use of g.l.c. for the determination of the optical purity of amino-acids, in which volatile derivatives are separated on optically-active stationary phases;[149–152] the alternative approach, in which diastereoisomeric derivatives of the amino-acid are formed, is illustrated for aspartic acid, for which *N*-trifluoroacetylation followed by esterification with (+)-3-methyl-2-butanol gives a pair of diastereoisomeric derivatives which may be separated on normal g.l.c. stationary phases.[288]

Ion-exchange Chromatography.—The main categories into which papers could be located under this heading are advances in instrumentation, and improvements in ion-exchange separation of particular amino-acids as part of a quantitative analysis routine. Opportunities to reduce the time taken in automated amino-acid analysis of mixtures have been studied;[289, 290] in the determination of phenylalanine and tyrosine, the use of anisylalanine as internal standard has been proposed,[290] and a similar use of 5-methyltryptophan in quantitative analysis of tryptophan has been suggested.[291]

Attention has been given to the separation of asparagine and glutamine from other amino-acids in the analysis of biological fluids.[292] A broad study has been made [293] of the automated amino-acid analysis of sulphur-containing amino-acids and their derivatives, and conditions for separation of *S*-carboxymethyl cysteine from its Se analogue have been established.[294]

Further illustration of the potential of the *o*-phthalaldehyde–mercaptoethanol system for automated fluorimetric amino-acid analysis has been provided in the determination of nanomole quantities of amino-acids in blood plasma samples.[295]

High Performance Liquid Chromatography.—In terms of cost, equipment for h.p.l.c. can compete with that for g.l.c., but in terms of speed in the provision

[286] P. Husek, *Ergeb. Exp. Med.*, 1976, **20**, 24 (*Chem. Abs.*, **85**, 58973).
[287] P. D. Van Wassenaar and R. B. Iyengar, *J. Chromatog.*, 1976, **118**, 99; J. P. Van Eerd, *Anal. Biochem.*, 1976, **71**, 612.
[288] W. Rahn, H. Eckstein, and W. A. Koenig, *Z. physiol. Chem.*, 1976, **357**, 1223.
[289] R. S. Ersser, *Med. Lab. Sci.*, 1976, **33**, 257.
[290] R. S. Ersser, *Med. Lab. Sci.*, 1976, **33**, 57.
[291] M. Wilkinson, G. A. Iacobucci, and D. V. Myers, *Analyt. Biochem.*, 1976, **70**, 470.
[292] S. Gross and M. L. Maskaleris, *Clin. Chem.*, 1976, **22**, 1233.
[293] L. Bowie, J. C. Crawhall, N. Gochman, K. Johnson, and J. A. Schneider, *Clin. Chim. Acta*, 1976, **68**, 349.
[294] A. Rinaldi, P. Cossu, and C. DeMarco, *J. Chromatog.*, 1976, **120**, 221.
[295] M. Roth, *J. Clin. Chem. Clin. Biochem.*, 1976, **14**, 361.

of data, the former technique is a clear leader; the scope for use of inexpensive components has been considered in a report [296] of the separation of taurine, γ-aminobutyric acid, and 5-hydroxytryptophan at <50 pmole levels from brain tissue samples, requiring between 3 and 7 minutes for each analysis. Dansyl-amino-acids may be readily separated by h.p.l.c.;[297] the literature on h.p.l.c. of PTH's has been boosted considerably during 1976,[298–302] and includes a reasoned comparison of g.l.c. and h.p.l.c. for the identification of PTH's.[302] The speedier h.p.l.c. technique should be considered to complement g.l.c. rather than replace it, since resolution of all amino-acids is not yet possible but those difficult to resolve by g.l.c. are readily resolved by h.p.l.c. (and *vice versa*).

Thin-layer Chromatography.—A new edition of a standard textbook includes an extensive survey of the chromatography of amino-acids and peptides.[303]

Analytical separation of particular classes of amino-acid by t.l.c. has been described for side-chain methylated lysines and arginines,[14d] and for the resolution of DL-tryptophan into its enantiomers.[304] The use of microcrystalline cellulose for separation of enantiomeric amino-acids is not new, but although several tryptophan analogues were also successfully resolved, this system is not suitable for other common amino-acids.[304]

Further experience in the use of fluorescamine [305] and the (cheaper) *o*-phthalaldehyde–mercaptoethanol [306, 240] spray reagents in quantitative t.l.c. of amino-acids has been described. The detection limit for fluorescent derivatives located through the *o*-phthalaldehyde procedure is in the range 50—100 pmoles.[306]

Improvements in technique have been reported for separations of dansylamino-acids,[307] methylthiohydantoins (two-dimensional t.l.c. of 23 derivatives),[308] and phenylthiohydantoins (use of formamide-impregnated paper,[309] separation of PTH's of basic amino-acids on polyamide layers [310]).

Other Analytical Methods.—Although many of the papers mentioned in this section have links with some of the preceding sections, their scope is rather broader. Analysis of actinomycin hydrolysates, which contain imino-acids as well as amino-acids, is best achieved through combinations of colorimetric and fluorimetric procedures.[311] The well-established fluorimetric assay for tryptophan

[296] J. L. Meck, *Analyt. Chem.*, 1976, **48**, 375.
[297] E. Bayer, E. Grom, B. Kaltenegger, and R. Uhmann, *Analyt. Chem.*, 1976, **48**, 1106.
[298] K. Muramoto, H. Kawauchi, Y. Yamamoto, and K. Tuzimura, *Agric. Biol. Chem. (Japan)*, 1976, **40**, 815.
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[301] C. Bollett and M. Caude, *J. Chromatog.*, 1976, **121**, 323.
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[303] A. Niederweiser, in 'Chromatography', ed. E. Heftmann, 3rd ed., Van Nostrand-Reinhold, New York, 1975, p. 393.
[304] R. L. Munier, A. M. Drapier, and C. Gervais, *Compt. rend.*, 1976, **282**, 1761.
[305] J. C. Touchstone, J. Sherma, M. F. Dobbins, and G. R. Hansen, *J. Chromatog.*, 1976, **124**, 111.
[306] E. Lindberg and G. Gunnar, *J. Chromatog.*, 1976, **117**, 439.
[307] M.-L. Lee and A. Safille, *J. Chromatog.*, 1976, **116**, 462.
[308] K. D. Kulbe and Y. M. Nogueira-Hattesohl, *Analyt. Biochem.*, 1976, **72**, 123.
[309] E. Soczewinski, J. Iskierko, and J. Klimek, *Chromatographia*, 1976, **9**, 323.
[310] S. Bose and H. B. Brewer, *Analyt. Biochem.*, 1976, **71**, 42; T. P. Hopp, *ibid.*, 1976, **74**, 638.
[311] A. M. Felix, J. W. Westley, and J. Meienhofer, *Analyt. Biochem.*, 1976, **73**, 70.

has been automated;[312] it has been found [313] that sucrose quenches the fluorescence generated in this assay, but since the effect is concentration-dependent it can be compensated. Comparative studies of dansyl-[1-^{14}C]-leucine with its mansyl analogue [mansyl = 6-(*N*-methylanilino)-2-naphthylsulphonyl] show that the latter derivative generates slightly greater fluorescence in some solvents, but not in others.[314]

A new electrophoretic method (omegaphoresis) has been applied to the quantitative analysis of amino-acids and peptides in mixtures, allowing the estimation of nmole to pmole levels in less than 5 minutes.[315]

Determination of Specific Amino-acids.—Reviews of the assay of hydroxy-L-proline in urine have been published.[316] Proline can be determined in samples containing hydroxyproline and other amino-acids using the isatin colour reaction and spectrophotometric (598 nm) quantitation.[317] Other aliphatic amino-acids for which appropriate chemical or physical techniques have been explored are: γ-amino-butyric acid in brain tissue (silylation [318] or *N*-pentafluoropropionylation and hexafluoropropyl ester formation [216] followed by m.s. determination at μg levels), L-canavanine (magenta colour reaction with photo-activated trisodium pentacyanoammonioferrate),[319] 2,6-di-aminopimelic acid,[320] and glycine and taurine at picomole levels (even in the presence of several thousand-fold excess of other amino-acids) using established [^{14}C]-dansyl chloride methods.[321] Fluorescence methods employed for the assay of aromatic amino-acids have been reported for tryptophan,[322] histidine and its derivatives,[323] and phenylalanine.[324] The fluorescamine derivative of histidine remains intensely fluorescent after heating in acid solutions, whereas the fluorescence of other correspondingly labelled compounds disappears;[323] exploitation of this fact allows the development of a method for the assay of imidazoles at *ca.* 0.01 nmole levels.[323] An unusual method developed for the assay of L-phenylalanine [324] in serum involves a specific fluorescence-forming reaction with ninhydrin in the presence of leucylalanine.[324]

Biochemical assays through which the amounts of γ-aminobutyric acid in brain tissue samples may be estimated have been reviewed.[325] Enzymic assays,

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[318] F. Cattabeni, C. L. Galli, and T. Eros, *Analyt. Biochem.*, 1976, **72**, 1.
[319] G. A. Rosenthal, *Analyt. Biochem.*, 1976, **77**, 147.
[320] V. C. Mason and S. Bach-Andersen, *Z. Tierphysiol., Tierernaehr. Futtermittelkd.*, 1976, **36**, 221 (*Chem. Abs.*, **84**, 132218).
[321] E. Schulze and V. Neuhoff, *Z. physiol. Chem.*, 1976, **357**, 593.
[322] P. Pajot, *European J. Biochem.*, 1976, **63**, 263.
[323] H. Nakamura and J. J. Pisano, *Arch. Biochem. Biophys.*, 1976, **177**, 334.
[324] U. Grimm, *Zentralbl. Pharm., Pharmacother Laboratoriumsdiagn.*, 1976, **115**, 959 (*Chem. Abs.*, **86**, 13402); G. Machill, *ibid.*, 1976, **115**, 949 (*Chem. Abs.*, 86, 13458).
[325] S. J. Enna and S. H. Snyder, in 'Proceedings of the 6th International Congress of Pharmacology', ed. E. Klinge, Pergamon Press, Oxford, 1976.

including new examples of immobilized enzyme reactor electrodes for electrochemical techniques, have been established for L-leucine (L-amino-acid oxidase immobilized, with catalase, on glass),[326] L-glutamic acid,[327] L-arginine,[328] and L-lysine [328] (employing the respective decarboxylases and CO_3^{2-}-selective electrodes), and L-asparagine and L-arginine (immobilized asparaginase and arginase–urease electrodes, respectively, with NH_4^+-selective electrode).[329] L-Glutamine in serum may be estimated through use of an *Escherichia coli* glutamate synthase [330] or through use of an L-glutamine-binding protein from the same source.[331]

[326] G. Johansson, K. Edstrom, and L. Ogren, *Analyt. Chim. Acta*, 1976, **85**, 55.
[327] S. J. Yao, S. K. Wolfson, J. M. Tokarsky, and S. B. Weiner, *Bioelectrochem. Bioenerg.*, 1976, **3**, 106.
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[329] N. T. Tjien, *Canad. J. Biochem.*, 1976, **54**, 62.
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2

Structural Investigations of Peptides and Proteins

BY I. D. WALKER, L. RYDÉN, G. WINTER, A. DELL, H. MUIRHEAD, AND R. H. PAIN

PART IA: Isolation, General Properties, and Amino-Acid Sequence Analysis *by I. D. Walker and L. Rydén*

1 Introduction

The following review of material reported in 1976 in the field of protein chemistry is not a comprehensive one. Our estimate is that at least one thousand and perhaps nearly twice as many reports on the isolation of various proteins were published during the year. As previously, our emphasis is on new techniques and approaches but, in addition, results in areas that we feel to be particularly novel, exciting or interesting are discussed. The two previous Reports have contained a comprehensive tabulation of the use of affinity methods. This year only some new developments in affinity chromatography are mentioned, and instead a more extensive discussion of the isolation and study of membrane-bound proteins is provided.

The increased use of microtechniques based on preparative gel electrophoresis allows today's protein chemist to obtain information on molecular weight, primary structure, functional and immunological properties *etc.*, on microgram quantities of protein, sometimes isolated in a single step. These approaches are gaining in importance as the roles of very minor components of the cell are being studied. Major portions of the Sequencing sections are concerned with the development and application of methods for examining trace quantities of purified proteins. This emphasis reflects the recent interest in obtaining sequences of precursor proteins and transplantation antigens. The advantages and limitations of the various approaches to microsequencing are discussed at length.

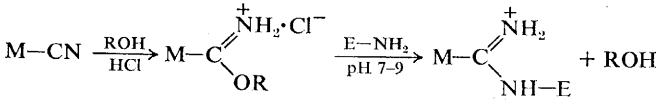
The gap between detailed knowledge of individually isolated proteins and the supramolecular assemblies of which they often form a part is continuously being bridged. This is illustrated by discussions on the composition and topology of soluble multimeric complexes, functional assemblies of low dissociation constants, and polyfunctional proteins where the activities all appear as different domains on a single long polypeptide chain. The association of membrane-bound proteins with lipids and with other protein constituents is reviewed, as is the year's contribution to our understanding of their way of being arranged in the functional membrane.

2 Protein Isolation Methodology

Chromatographic Methods.—In addition to the long established methods of gel filtration, ion exchange chromatography, adsorption chromatography, and precipitation, affinity chromatography, using enzyme–substrate analogues,

protein cofactors, antibodies, *etc.*, is today in widespread use. The ubiquitous initial ammonium sulphate precipitation step is rarely efficient, and batch-wise adsorption on, for example, DEAE-cellulose is often a useful alternative for both preliminary purification and, sometimes, thousand-fold reduction in volume.[1, 2] Another precipitation method involves the use of low molecular weight poly-(ethylene glycols), which is reported to be very selective.[3] Impressive resolution is achieved by the proper use of cellulosic ion exchangers, as demonstrated with the separation of eukaryotic[4, 5] and prokaryotic[6] ribosomal proteins. The resolution in gel filtration can be considerably improved when dry elutriated gel beads with a narrow size range are employed.[7] Adsorption of the protein on a top layer of ion exchanger on the gel bed, and then starting the elution with a zone of high salt, is a useful trick to obtain narrow application zones.[8]

Affinity Chromatography. Coupling methods. The cyanogen bromide coupling technique is the favourite method for immobilization of ligands, and agarose is the preferred support. Drawbacks of CNBr coupling are introduction of positive charges and slow leakage of the immobilized ligand in some buffers (see discussion in last year's Report). If amino-group-containing supports, such as amino-alkylated glass beads, are used, the CNBr method will give a guanido type linkage that does not leak.[9] A similar linkage is obtained by using fibres of polymerized acrylonitrile as support and coupling protein amino-groups *via* an imidoester intermediate (Scheme 1).[10]



Scheme 1

An alternative to agarose is a copolymer of agarose and polyacrylamide.[11] It is more stable towards mechanical damage and has both amino- and hydroxy-groups available for coupling. In some cases it is advantageous to create aldehyde functions in the agarose by periodate oxidation and to couple amines *via* a Schiff base intermediate that is subsequently reduced. This also allows introduction of spacer arms by use of alkyl diamines, an approach that was favoured in coupling of carboxypeptidase Y.[12] The optimal use of CNBr coupling for preparing immunosorbents has been investigated.[13] A radically different approach is to entrap macromolecules or even cells into beads as

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[12] F. A. Liberatore, J. E. McIsaac, jun., and G. P. Royer, *F.E.B.S. Letters*, 1976, **68**, 45.
[13] S. Comoglio, A. Massaglia, E. Rolleri, and U. Rosa, *Biochim. Biophys. Acta*, 1976, **420**, 246.

they are formed by polymerization, either with cross-linked acrylamide [14] or poly(ethylene glycolmethacrylate).[15]

General ligand affinity chromatography. As used with immobilized co-enzyme NADH [16] or blue dextran [17] for nucleotide-binding proteins, this technique has been developed also for coenzyme A-dependent enzymes [18] and DNA-binding proteins.[19] A simple way to achieve specific purification is to perform a gel filtration with the macromolecular ligand, here blue dextran, present so that the enzyme elutes in the void volume, and repeat the procedure after dissociation.[20] When using a general ligand, specificity can be provided by the eluent.[21] Along similar lines antibodies, raised against an easily available antigen, could be used for immunosorption of cross-reacting material [22] that might be difficult to purify otherwise, or be present in small amounts.

Elution. In general, for enzymes binding several substrates and/or allosteric effectors, new possibilities arise in affinity chromatography.[23] Alcohol dehydrogenase was adsorbed on an inhibitor column in the presence of its coenzyme, NADH, and eluted when ethanol was added to the buffer.[24] Isoleucine-specific tRNA synthetase was eluted from a blue dextran column at different ATP concentrations depending on the presence of isoleucine in the medium,[25] and a similar approach was used in purification of thymidylate synthetase.[26] Cytoplasmic actin was purified by ion exchange chromatography only in the presence of liganded ATP.[27] A different parameter, the temperature, influenced the binding of *Bothrops atrox* venom protease to an affinity column, pointing to the importance of hydrophobic effects.[28] Proteins that are bound to immunosorbents, are often eluted by including denaturants, such as urea or chaotropic ions, in the eluent. Considerably less drastic conditions will suffice for elution if neither the immobilized antibody or the antigen is chemically modified to a suitable extent before the chromatography, thereby diminishing the strength of the antibody–antigen complex.[29] Sometimes increased resolution is achieved if a protein that is bound to an affinity column is eluted with a concentration gradient of the specific eluent. The interpretation of the chromatographic profiles obtained in such experiments is, however, not always straightforward, as is discussed in a recent report.[30] A new volume of *Methods in Enzymology* deals with applications

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[29] R. F. Murphy, A. Iman, A. E. Huges, M. J. McGucken, K. D. Buchanan, J. M. Coulon, and D. T. Elmore, *Biochim. Biophys. Acta*, 1976, **420**, 87.
[30] L. S. Gennis, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3928.

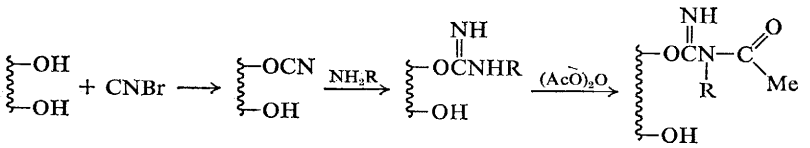
in affinity chromatography.[31] A recent volume of *Methods in Enzymology* deals with applications in affinity chromatography.[31]

A relatively new approach is that of *affinity partitioning*, where the specific ligand is covalently bound to the polymer in one phase of a two-phase system, usually poly(ethylene glycol)-dextran. When the polymers are modified by introduction of charges – quaternary ammonium ions and sulphonic acid groups, respectively – the partitioning changes with pH since it depends directly on the net charge of the protein.[32] When one polymer contains fatty acid side-chains the partitioning depends on the hydrophobicity of the protein,[33] and can even be used to get a quantitative measure of that.[34] Even whole cells or membrane fragments can be fractionated in this system, as shown in a study of acetylcholine receptors from *Torpedo californica*.[35]

In *covalent chromatography* thiol-containing proteins are bound to an activated thiolated matrix by thiol–disulphide exchange. Alternatively, an immobilized organomercurial is used. Elution is achieved by reducing conditions. Excellent results were obtained by these methods in the purification of factor XIII from platelets,[36] type III collagen,[37] and band 3, the major transmembrane protein, from human erythrocytes.[38] The recently introduced methods of *chelating chromatography*[39] and *charge transfer chromatography*[40] offer new possibilities, but have so far no reported applications.

Hydrophobic Chromatography. Matrices. Chromatography on hydrophilic matrices (mostly agarose) with introduced hydrophobic groups is commonly called *hydrophobic chromatography*, although hydrophobic effects are certainly not the only ones responsible for binding. Ligand coupling by the CNBr method introduces charged groups in the matrix. These can be estimated quantitatively with *p*-nitrophenyl acetate, which reacts with the isourea linkage,[41] and neutralized by acetylation with acetic anhydride (Scheme 2).[42]

The binding of α-lactalbumin to butyl, pentyl, and hexyl agarose failed after neutralization but not before pointing to the importance of ionic forces.[42] In



Scheme 2

[31] *Methods Enzymol.*, 1976, Vol. 34.
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[40] J. Porath and K. Dahlgren-Caldwell, *J. Chromatog.*, 1977, **133**, 180.
[41] M. M. Werber, *Analyt. Biochem.*, 1976, **76**, 177.
[42] M. Wilchek and T. Miron, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 108.

fact, charges are most often introduced deliberately by using ω-amino alkyl agarose as adsorbent. Omission of the amino-group can give irreversible adsorption,[43] as does the use of too long alkyl chains.[44–46] Especially if multipoint binding occurs [47, 48] it is desirable to work close to conditions of sorption–desorption equilibrium. Variation of the length of the alkyl chain is often useful to obtain a desired specificity, as demonstrated in the preparations of *Clostridium* proteases [46] and *E. coli* acetyl transferases.[45] The terminal amino-group reacts with alkylsuccinic anhydrides [49, 50] or can be replaced by a carboxy-group [51] to obtain new types of adsorbents, sometimes with advantage. Yeast alcohol dehydrogenase was thus purified in a single step on 10-carboxydecyl-Sepharose.[51]

In most cases, *elution* from hydrophobic columns is achieved by increasing the ionic strength of the buffer,[43–46, 52] testifying to the importance of ionic forces. In some procedures proteins are adsorbed at high salt concentrations and eluted by a reverse salt gradient,[51, 53–56] *i.e.* true hydrophobic chromatography occurs. Decreasing ammonium sulphate concentrations were used in the purification of milk galactosyltransferase on norleucine-Sepharose [53] and immunoglobulin A on phenylalanine-Sepharose,[54] respectively. Alternatively, elution is achieved by increasing the hydrophobicity of the eluent by alcohols *etc.*, or by the addition of detergents to the buffer.[50] Cytochrome P450 was eluted from octylamine-Sepharose by non-ionic detergents,[57, 58] and alcohol dehydrogenase was eluted from amino- and carboxy-alkyl-Sepharose by increasing ethanol concentrations.[51]

Hydrophobic effects in other types of chromatography. Hydrophobic effects often play a role in affinity chromatography, as illustrated by the dramatic influences of the length of the spacer arm on adsorption of electric eel acetylcholine esterase [59] and nucleotide receptor protein [60] to their affinity columns. In both cases elution was achieved by specific ligand. An interesting case is that of interferon, which has been extremely difficult to handle and purify. This protein is very hydrophobic. A step forward was in the use of 0.1% sodium dodecyl sulphate (SDS) to keep it in a disaggregated and active form.[61] It binds to lectin-Sepharose, but is not eluted by specific ligand but rather by increased concentration

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[59] J. Massoulié and S. Bon, *European J. Biochem.*, 1976, **68**, 531.
[60] J. Ramsayer, C. B. Kantstein, G. M. Walton, and G. N. Gill, *Biochim. Biophys. Acta*, 1976, **446**, 358.
[61] E. T. Tönmä and K. Paucker, *J. Biol. Chem.*, 1976, **251**, 4810.

of ethylene glycol [62, 63] or propane-1,2-diol.[63] Likewise it can be bound to blue dextran agarose and then eluted by ethylene glycol.[64] The proper use of hydrophobic columns, such as octyl-Sepharose, tryptophan-Sepharose,[62] or di-tryptophan-Sepharose,[65] eluted with, *e.g.*, ethylene glycol, resulted in a 3000-fold purification of human fibroblast interferon [62, 63] and 10 000-fold purification of rabbit interferon.[62] Halophilic effects in immunoadsorption are illustrated by the purification of platelet microtubule protein, where the protein was eluted by 0.05% Triton X-100, a non-ionic detergent.[66] The elution of enzymes, obtained from extremely halophilic bacteria by a reverse salt gradient of ammonium sulphate, starting at 2.5 mol l^{-1}, from both Sepharose and cellulose ion exchangers, seems to be a particularly clean case of salting-out chromatography.[67, 68] In the case of a glass-adhering protein from macrophage plasma membrane, the protein attached to glass beads remained after rinsing with detergents and could only be released by electrophoresis and was then completely pure.[69]

Chromatography in organic solvents. The extremely hydrophobic proteolipids that are extracted in methanol–chloroform mixtures are not handled on the alkylated agaroses but rather on gels that swell in that medium, such as Sepharose LH-20 [70] or acetylated Sephadex G-200.[71] The carbodi-imide-reactive protein of the *E. coli* energy transducing system was chromatographed in methanol–chloroform–water, 3 : 3 : 1, on hydroxypropyl-Sephadex G-50.[72]

Electrophoretic Methods.—Electrophoresis and electrofocusing in polyacrylamide gels are mostly used as analytical tools but are gaining use as preparative methods. In most procedures microgram quantities are recovered, but this suffices for, *e.g.*, raising of antiserum in rabbits [73] or the study of *N*-terminal amino-acid sequences with either intrinsically labelled proteins [74] or the use of radiolabelled reagents [75] (see Section 5). Analytically, the two-dimensional methods, combining isoelectric focusing and electrophoresis in SDS, are impressively powerful (see discussion in last year's Report), able to resolve about one thousand components, as exemplified by studies on radiolabelled non-histone chromosomal protein [76] and membrane proteins from bacterial and eukaryotic cells.[77] Other procedures employ electrophoresis in two different dissociation conditions [78] or the use of a dissociating buffer only in the second dimension to obtain information on subunit composition.[79]

[62] M. W. Davey, E. Sulkowski, and W. A. Carter, *J. Biol. Chem.*, 1976, **251**, 7620.
[63] M. W. Davey, E. Sulkowski, and W. A. Carter, *Biochemistry*, 1976, **15**, 704.
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[71] J. Monreal, *Biochim. Biophys. Acta*, 1976, **427**, 15.
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[73] E. A. Neuwelt, J. J. Frank, and C. C. Levy, *J. Biol. Chem.*, 1976, **251**, 5753.
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[77] G. Ferro-Luzzi Ames and K. Nikaido, *Biochemistry*, 1976, **15**, 616.
[78] M. J. Condrand and J. T. Penniston, *J. Biol. Chem.*, 1976, **251**, 253.
[79] F. Iborra and J.-M. Buhler, *Analyt. Biochem.*, 1976, **74**, 503.

New Gels and Staining Procedures. Gel supports. A few alternatives to the traditional acrylamide–methylenebisacrylamide system have been explored. Using the cross-linkers *NN'N''*-triallylcitric triamide or *NN'*-(1,2-dihydroxyethylene)bisacrylamide, gels with more open structures were obtained.[80] As these were soluble in dilute periodic acid, scintillation counting with good efficiency was possible. Cross-linking with *NN'*-diallyltartardiamide gives transparent gels with good mechanical stability.[81] The mentioned supports are quite macroporous and thus suitable for isoelectric focusing. A third very macroporous bed for this purpose is obtained by cross-linking Sephadex G-75 beads with 4% acrylamide–0.13% bisacrylamide in a thin layer.[82] An alternative support for SDS electrophoresis with molecular sieving properties is obtained by soaking cellulose acetate foils in 1—10% solutions of poly(ethylene glycols).[83] This support allows easy elution of peptides but some resolution is lost as compared to the conventional procedures. Gradients of polyacrylamide are useful for obtaining zone sharpening in analytical electrophoresis. A very simple way of forming such gels consists of adding two layers of different concentrations of acrylamide in a glass tube, tilting the tube to a desired angle and rotating it during polymerization.[84]

Detection of proteins. Coomassie Brilliant Blue–perchloric acid, by far the most widely used stain, is still being improved. Its sensitivity was increased three-fold by including a wash with 5% acetic acid, and as little as 5 ng of human serum albumin was detectable.[85] Alternative stains include Fast Green,[86] which permits faster staining but at the sacrifice of sensitivity. In SDS gels a solution of 1M-KCl in 10% acetic acid will make the SDS–protein complexes visible as opaque bands within 30 min.[87] The cationic carbocyanine dye called 'Stains all' gives colourful gels with red proteins, blue sialoglycoproteins, and yellow-orange lipids, but at the loss of some sensitivity as compared to standard procedures.[88] More sensitive procedures employ fluorescent probes, such as fluorescamine[89] or MDPF [2-methoxy-2,4-diphenyl-3(2*H*)-furanone],[90] and detect the bands either by direct inspection or with the aid of a fluorescence scanner. Proteins, labelled before denaturation with SDS and electrophoresis, still move according to their molecular weights permitting their estimation.[90] Labelling of serum albumin with fluorescamine after denaturation in SDS did not work well, suggesting that the lysyl side-chains were not available for reaction in the complex.[91] Carbohydrates in glycoproteins can be selectively dansylated by treatment with dansylhydrazine after oxidation by periodate.[92] This method detects *ca.* 40 ng of

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[81] G. Baumonn and A. Chrambach, *Analyt. Biochem.*, 1976, **70**, 32.
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[83] H. J. Bode, *F.E.B.S. Letters*, 1976, **65**, 56.
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[86] M. J. Bertolini, D. L. Tankersley, and D. D. Schroeder, *Analyt. Biochem.*, 1976, **71**, 6.
[87] L. P. Nelles and J. R. Bamburg, *Analyt. Biochem.*, 1976, **73**, 522.
[88] L. E. King, jun., and M. Morrison, *Analyt. Biochem.*, 1976, **71**, 223.
[89] S. J. Tata and G. F. J. Moir, *Analyt. Biochem.*, 1976, **70**, 495.
[90] B. O. Barger, F. C. White, J. L. Pace, D. L. Kemper, and W. L. Ragland, *Analyt. Biochem.*, 1976, **70**, 327.
[91] S.-I. Tu and L. Grosso, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 9.
[92] A. E. Eckhardt, C. E. Hayes, and I. J. Goldstein, *Analyt. Biochem.*, 1976, **73**, 192.

carbohydrate. The most sensitive methods, of course, rely on radioactively labelled proteins. Tritiated proteins can be detected conveniently by autoradiography if the gel is first washed with a solution of scintillator.[93] Carbohydrate in sialoglycoproteins can be tritiated selectively by borohydride after sialidase and galactose oxidase treatment, as exemplified by a study on human erythrocyte glycoproteins.[93] A direct means of observing the protein zones, even during the run, is to visualize the refractive index differences in the gel by darkfield techniques[94] or an optical set-up based on the Schlieren technique.[95] Some of the gel-staining techniques have been adopted for the sensitive assay of proteins in solution.[96, 97]

It is interesting to note that some proteins escape detection by the standard techniques of Coomassie staining, u.v.-scanning, and iodination.[98] In the case referred to, the protein was detected as a contaminant when sequencing was attempted. How common this is, is of course left to speculation.

Preparative Gel Electrophoresis. The analytical one-dimensional systems in either rods or slabs can be used also for preparative purposes. Detection of the components are mostly done on either a narrow strip of a slab gel or a rod run in parallel, respectively. The use of radiolabelled or fluorescently marked proteins makes staining superfluous. Some special equipment has been developed for large-scale preparative runs either with or without detergents present.[99–101]

Elution after electrophoresis. For recovery, the sections of the gels containing the protein of interest are cut out, minced, and then eluted by stirring in buffer. The composition of the buffer is critical to obtain a satisfactory recovery. After SDS electrophoresis low concentrations of SDS (0.1%) are mostly employed.[102] In the preparation of histones quantitative recovery of microgram quantities was claimed.[103] Elution in detergent-free buffers has been used with success after SDS electrophoresis of erythrocyte membrane proteins.[104] For gels run in urea-containing buffer, urea is used for the elution.[105, 106] A useful alternative is provided by electrophoretic elution, where the minced gels are placed in a membrane-enclosed cell.[107, 108] In a different set-up the gel slices are placed on top of a small column of hydroxyapatite and electrophoresed into the column, to be eluted later with phosphate buffer containing 0.1% SDS.[109] The often slow

[93] C. Gahmberg, *J. Biol. Chem.*, 1976, **251**, 510.
[94] A. Elliott, *Biochem. J.*, 1976, **159**, 743.
[95] E. Fries, *Analyt. Biochem.*, 1976, **70**, 124.
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[102] G. H. de Vries, L. F. Eng, D. L. Lewis, and M. G. Hadfield, *Biochim. Biophys. Acta*, 1976, **439**, 133.
[103] C. G. Goff, *J. Biol. Chem.*, 1976, **251**, 4131.
[104] S. Bhakdi, O. J. Bjerrum, and H. Knüfermann, *Biochim. Biophys. Acta*, 1976, **446**, 419.
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[106] J. M. Andreu, J. Carreira, and E. Muñoz, *F.E.B.S. Letters*, 1976, **65**, 198.
[107] L. G. Abood, J. S. Hong, F. Takeda, and A. M. Tometsko, *Biochim. Biophys. Acta*, 1976, **443**, 414.
[108] I. Possner, *Analyt. Biochem.*, 1976, **72**, 491.
[109] B. R. Ziola and D. G. Scraba, *Analyt. Biochem.*, 1976, **72**, 366.

removal of SDS from the proteins by dialysis after these procedures can be improved by adding DEAE-cellulose to the surrounding medium.[110] A two-step dialysis procedure, where the first buffer contained 6M-urea and reducing agent and the second did not, was used to recover 50% of the activity of the lactose repressor prepared by centrifugation in the presence of SDS.[111]

Isoelectric Focusing and Isotachophoresis. Preparative *isoelectric focusing* with sucrose gradients as anticonvection medium, as commonly performed, has

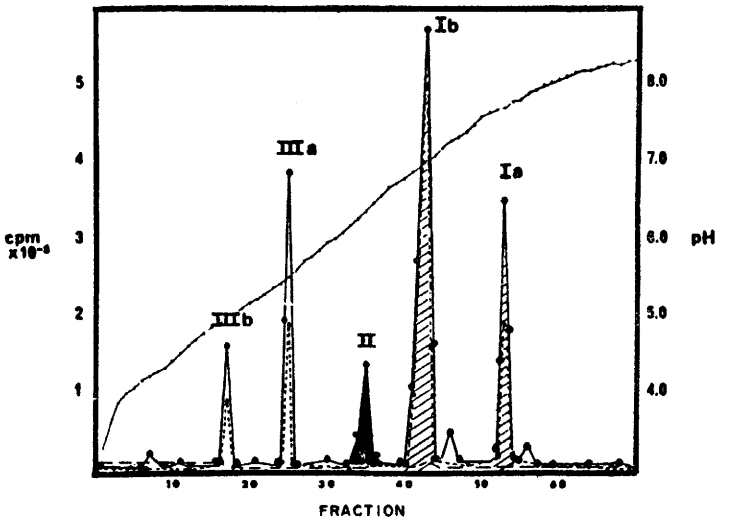

Figure 1 *Isoelectric focusing in a* 2.5 × 20 cm *column of Sephadex G-15. The sample was a preparation of RNA polymerase from 6-day-old soybean hypocotyl chromatin. Fractions of* 0.5 ml *were collected and assayed for RNA polymerase activity in standard conditions* (●), *in the absence of DNA* (- - - -), *and in the presence of α-amantin* (○) (Reproduced by permission from *Analyt. Biochem.*, 1976, **76**, 311)

several drawbacks, such as convectional dilution during elution, spreading of precipitated protein, and sucrose in the recovered fractions. Some of these have been overcome recently. In a modified Uniphor electrophoresis apparatus, equipped with an elution chamber, the current can be left on during elution to allow the proteins to be recovered in extremely sharp zones.[112] In a preparative gel electrophoresis apparatus the components can be electrophoresed out after the anode or cathode solution has been replaced by amphoteric buffer species with proper isoelectric points.[113] In these procedures a polyacrylamide gel replaces the sucrose gradient as anticonvection medium. In two new small-scale preparative set-ups the support was a flat bed of 4 mm thick polyacrylamide gels[114] or a 2 mm thick layer of Sephadex G-75.[114a] These two

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[111] J. R. Sadler and M. Tecklenburg, *Biochemistry*, 1976, **15**, 4353.
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[113] A. McCormick, L. E. M. Miles, and A. Chrambach, *Analyt. Biochem.*, 1976, **75**, 314.
[114] D. Graesslin, H. C. Weise, and M. Rick, *Analyt. Biochem.*, 1976, **71**, 492.
[114a] A. I. Coffer and R. J. B. King, *Analyt. Biochem.*, 1976, **72**, 172.

systems have a capacity of *ca.* 0.5 mg[114a] or a few milligrams[114] of protein. Elution is particularly simple when Sephadex beads are used as supporting medium. Very good results are obtained by a procedure when the focusing takes place in a 2.5 × 20 cm column of Sephadex G-15[115, 116] (Figure 1). A small layer of polyacrylamide gel was placed on top of the Sephadex, under the electrode solution. Focusing, performed in the presence of 0.15% ampholytes and 10% glycerol, was essentially complete after 5 h when the column was eluted. In a small load most components were recovered in close to 1 ml of eluent, but for a bigger load (the maximum amount was 300 mg of protein) the peak was up to 8 ml wide.[115]

The rather novel method of *isotachophoresis* has so far few applications in preparative protein chemistry. An attraction of this method is that results are generally improved for increased protein loads, in particular if spacer ions are lacking between some components. A successful application is the isolation of human growth hormone components on a gram scale using a macroporous polyacrylamide gel as support in a preparative apparatus.[117] Selected buffers were found to be superior to ampholines for providing spacer ions. As an analytical method isotachophoresis run in polyacrylamide gels proved superior to isoelectric focusing in a study of immunoglobulin heterogeneity.[118] Also in this application ampholine was omitted. In fact, selected buffers could replace ampholine both in electrofocusing and isotachophoresis in polyacrylamide gels.[119]

Solubilization of Membrane-bound Proteins.—*Extraction by Detergents. Choice of detergent.* In the great majority of cases membrane-bound proteins are brought into solution by the addition of detergents, even when these need not be present in later steps (see below). The choice of detergent and the concentration used influences selectivity, recovery, and maintenance of activity, the important parameters being the hydrophile–lipophile balance (HLB) and critical micelle concentration (CMC). A high CMC is advantageous both in the solubilization and subsequent removal of detergent. A higher HLB or lower concentration often gives better selectivity but less good recoveries. Cholate was found to be superior to Triton X-100 in the solubilization of mitochondrial membranes, due to its higher CMC.[120] The alkylglucosides constitute a class of both efficient and easily removed detergents as demonstrated in a study on bovine rhodopsin.[121] To solubilize *b*-type cytochromes from various sources by detergents of the Triton series, Triton X-100 was more efficient, but the hydrophilic Triton X-405 more selective.[122] For some membranes, such as the mitochondrial inner membrane, selectivity had to be sacrificed to get an acceptable recovery. Similarly for erythrocyte ghosts, deoxycholate was most efficient but cholate and Triton X-100 more selective, extracting preferentially peripheral and integral

[115] T. J. O'Brien, H. H. Liebke, H. S. Cheung, and L. K. Johnson, *Analyt. Biochem.*, 1976, **72**, 38.
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[117] G. Baumann and A. Chrambach, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 732.
[118] A. Ziegler and G. Köhler, *F.E.B.S. Letters*, 1976, **71**, 142.
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[120] R. W. Egan, M. A. Jones, and A. L. Lehninger, *J. Biol. Chem.*, 1976, **251**, 4442.
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[122] E. Slinde and T. Flatmark, *Biochim. Biophys. Acta*, 1976, **455**, 796.

(total ?) proteins, respectively.[123] Success in using Triton X-100 – or presumably any detergent – depends on the lipid composition of the membrane.[124] The choice of detergent also influences the activity of membrane-bound enzymes in a complicated way as demonstrated in a study on adenylate cyclase.[125] Lysolecithin is an alternative selective detergent for extraction of proteins from membranes. At carefully chosen concentrations selective solubilization of different components from mitochondrial[126] and microsomal[127] membranes was achieved. Addition of concentrated $MgCl_2$ to Triton X-100 solution conferred selectivity in extraction of proteins from the chicken erythrocyte nuclear envelope.[128]

Influence of detergent concentration. At low enough detergent concentration some proteins can be extracted before lysis of the membrane occurs. A few proteins were solubilized by 0.01% Nonidet P-40, a non-ionic detergent, from plasma membranes.[129] Data on the influence of deoxycholate concentration on the Semliki Forest virus membrane tell a very instructive story:[130] the membrane lysis occurred at 0.9 mM (= 0.04%) detergent, proteins started to be liberated at 1.5 mM, and they were free of phospholipids at 2.0 mM. The dissociation of the spike glycoprotein polypeptides finally did not occur until 2.3 mM deoxycholate. Some membrane-bound activities are also influenced by very low concentrations of detergent, as exemplified by the proline transport system of *E. coli* which was already 80% inactivated in 0.1 mM deoxycholate.[131] This effect was reversed by the addition of serum albumin which bound the detergent.

Other procedures. Various other ways to obtain selectivity have been used. Extensive washing of human erythrocytes with non-detergent-containing media left behind mainly the major transmembrane protein, band 3.[132] Extensive washing of *E. coli* outer membrane with SDS-containing solutions left only a single transmembrane protein that was solubilized by trypsin and lysozyme digestion.[133] This protein, as is the case with erythrocyte band 3 protein, appeared to be involved in transport of saccharides. Pretreatment of the plasma membrane of the slime mould *Discoidea discoideum* with concanavalin A to obtain cross-linkages made it stable to 0.2% Triton X-100.[134]

Extraction and Handling in Detergent-free Media. Extraction. Many membrane-bound proteins can be quite easily extracted in ordinary buffers or buffers containing either chelating agents, such as edta, or salts. Examples are offered by an epithelial basement membrane glycoprotein[135] or angiotensin-converting enzyme from rat lung.[136] In the latter case a deoxycholate treatment was replaced by prolonged extraction in phosphate buffer. Some investigators make the

[123] R. Coleman, G. Holdsworth, and J. B. Finean, *Biochim. Biophys. Acta*, 1976, **436**, 38.
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[130] A. Helenius, E. Fries, H. Garoff, and K. Simons, *Biochim. Biophys. Acta*, 1976, **436**, 319.
[131] S. Mizushima, *Biochim. Biophys. Acta*, 1976, **419**, 261.
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[134] R. W. Parish and U. Müller, *F.E.B.S. Letters*, 1976, **63**, 40.
[135] L. D. Johnson and J. Warfel, *Biochim. Biophys. Acta*, 1976, **455**, 538.
[136] J. J. Lanzillo and B. L. Fanburg, *Biochim. Biophys. Acta*, 1976, **439**, 125.

detergent-free extraction more efficient by additional treatments such as sonication,[137] heating[138] (60° C for 20 min), freezing and thawing[139] or osmotic shock.[140, 141] Release of membrane-bound proteins with proteases, although a long established method, has definite drawbacks if the structure of the integral protein is of interest, although water-soluble, active forms of membrane-associated enzymes can be prepared in this way. A different enzymatic treatment, namely digestion with pancreatic lipase, efficiently solubilized NAD glycohydrolase from calf spleen,[142] but the presence of trypsin in the lipase preparation makes this method questionable.

Protein fractionation. When a membrane-bound protein has been initially brought into solution by detergents, these can sometimes be omitted in the purification steps to follow. The proteins from *Acholeplasma laidlawii* cell membrane, originally extracted in presence of Tween 20, could be electrophoresced without detergent.[143] The Tween-20-extracted proteins from human erythrocytes membrane were chromatographically purified in ordinary buffers.[55] Membrane-associated enzymes from several sources, all originally solubilized with 0.1% Triton X-100, could be handled in detergent-free buffer.[144–146] When chromatography on affinity columns, in particular those containing immobilized proteins, such as lectins or antibodies, is attempted, it is essential that detergents inducing protein unfolding are avoided. Affinity chromatography in the presence of non-ionic detergents or cholate or deoxycholate is, however, less risky. For example, microsomal β-glucuronidase, solubilized in 0.26% deoxycholate, was successfully bound to an immunosorbent and later eluted in detergent-free media.[147] Membrane fragments or vesicles, obtained after disruption of membranes in the absence of detergents, are usually handled by centrifugation techniques, but some other methods are available. ATPase in microsomal vesicles from hog stomach was purified by free-flow electrophoresis in a Hannig apparatus in the absence of detergents.[148] Similarly, receptor-containing membrane fragments were handled by affinity partitioning in the absence of detergents.[35]

Properties of the proteins. A few careful physicochemical studies on membrane proteins in detergent-free media indicate that the proteins in solution might form defined polymers. The spike complexes from Semiliki Forest virus membrane, dissolved in Triton X-100, form octamers when the detergent is removed by sucrose gradient centrifugation,[149] and spectrin from human erythrocyte plasma membrane forms tetramers.[150]

[137] H. Tuppy and G. Sperk, *European J. Biochem.*, 1976, **68**, 13.
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Heterogeneity: Real or Artefactual.—Many proteins are isolated in several different forms, and the possibility that the heterogeneity is caused by the preparation procedure has then to be considered. Sometimes it is evidently not the case. Microsomal and mitochondrial cytochrome P450 have been isolated in several forms, four of them from liver microsomes,[151] all of which possess a different substrate specificity.[151–154] A detailed study of myelin basic protein demonstrated that microheterogeneity was due to partial phosphorylation of specific threonine and serine side-chains and partial deamidation of a specific glutamine residue.[155] The latter might be due to the preparation conditions, but this is less likely with the former.

Evidence for Proteolysis. Proteolysis during the preparation has repeatedly been shown to give rise to complex subunit patterns and electrophoretic microheterogeneity of proteins. Recent examples of this are provided by studies of basement membrane collagen, where more than 20 components were separated,[156] and of mouse submaxillary gland nerve growth factor, where the multiple forms of the γ-subunit[157] were demonstrated to arise from proteolysis.[158, 159] In a few cases one form of a protein has actually been converted into another by limited controlled proteolysis, as, *e.g.*, for milk galactosyl transferase[160] and rat liver citrate lyase,[161] and in others a protease has been isolated from the same source as the original protein. Particularly intriguing is the isolation of a chromatin-bound protease, still active in standard dissociating conditions, *i.e.* 2—3M-NaCl, 5M-urea, from a variety of eukaryotic cells.[162, 163] The large polypeptide chain subunits in eukaryotic RNA polymerases[164, 165] and DNA polymerase[166] have mol. wts. of about 200 000 or above if properly isolated. These are converted into a form with mol. wt. 170—180 000, also found in some preparations, by a factor isolated from the same source. Amino-acid sequencing of the different forms can also provide convincing proof of their origin by proteolysis and has been applied to human plasminogens[167] and porcine neurophysins.[168]

Means to avoid Proteolysis. Increased awareness of the problems of proteolysis has led many investigators to use methods designed to avoid this, particularly when a very large protein is to be isolated. Such methods repeatedly proved

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effective in reducing heterogeneity and several proteins were isolated in an 'un-nicked' form for the first time. The use of fresh tissue gave single peptide chain forms of glial fibrillary acid protein from rat brain,[169] acetylcholinesterase from electric eel,[170] and ceruloplasmin from human serum.[171] Gel filtration of yeast extract was used to remove the protease in the preparation of pyruvate kinase.[172] Grinding instead of autolysis of yeast cells produced one form instead of, as earlier, five forms of phosphoglyceromutase.[173] Rapid preparation at low temperatures gave the single monomeric form of rat tendon collagen.[174] The use of a protease inhibitor, usually phenylmethylsulphonyl fluoride and less often DFP, proved essential for the preparation of intact old yellow enzyme,[175] cytochrome c_1 of yeast mitochondria,[177] rabbit hepatocyte myosin,[178] and 19 S thyroglobulin from pig or sheep.[179] The thyroglobulin peptide chain was in this study reported to have an approximate mol. wt. of 330 000, much higher than previous estimates. In some cases the possibility remains that the conversion is of physiological significance, although it certainly is very remote in most of the examples cited. Mimicking of the nicking (*i.e.* controlled proteolysis) might, however, be extremely useful in structural studies (see, *e.g.*, ref. 180).

3 Protein Characterization Methodology

By gel electrophoretic methods an isolated protein is conveniently studied with regard to homogeneity, isoelectric point, subunit composition, and molecular weight and enough material for quantitative analysis of constituent amino-acids and carbohydrates can be prepared on a microscale. These methods are, however, not without pitfalls as will be discussed below. Developments in the field of amino-acid analysis are described in Section 5.

Homogeneity and Subunit Composition.—*Gel Electrophoresis as a Homogeneity Criterion.* In most cases the one band–one protein (or peptide chain in SDS-gel electrophoresis) correspondence is valid, but some notable exceptions have been reported. Beef heart cytochrome *c* oxidase was previously believed to contain six subunits as revealed by SDS–polyacrylamide gel electrophoresis, but in a modified system containing 6M-urea, seven subunits were separated.[181] When two peptides of mol. wt. 24 000 and 21 000 were resolved in the latter system the beef heart enzyme finally proved similar to the yeast enzyme, with regard to number and size of subunits. Two components in the SDS-gel electrophoretic analysis of human erythrocyte ghosts, PAS 1 and PAS 2, have previously been shown to be interconvertible, and both correspond to the

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sialoglycoprotein, glycophorin. Both of these forms were transformed into a third component, PAS 4, by increased ionic strength, a conversion that was complete in 0.25 M buffer.[182] In a suitable discontinuous buffer system, PAS 1 is resolved into two components and PAS 2 into three components (mol. wts. 35 000, 38 000, and 47 000).[183] The interrelation of these forms and the role of the carbohydrate in the heterogeneity are questions that need to be considered. The apoprotein of the Folch-Pi proteolipid from bovine brain, homogeneous in free boundary electrophoresis and in the ultracentrifuge, appeared as three bands in SDS-gel electrophoresis.[184] As these bands all had the same *N*-terminal amino-acid sequence and *C*-terminal amino-acid, the heterogeneity is puzzling.

Stoicheiometry of Oligomeric Proteins. The analysis of protein subunit composition by SDS-gel electrophoresis allows a calculation of the stoicheiometry if dye binding is strictly proportional to mass. This is certainly not always the case (see ref. 98), but densitometric tracing of Coomassie Brilliant Blue stained gels often gives stoicheiometries close to the expected as exemplified by the series of haptoglobin 2-1 polymers[186] or for peptides derived from collagen by cyanogen bromide cleavage,[187] cases where independent estimates were available. A similar way to estimate stoicheiometry is to measure the fluorescence of fluorescamine-tagged peptides after elution from the gel.[188] SDS-gel electrophoresis of oligomeric proteins containing chemically identical subunits, suitably crosslinked by a bifunctional reagent, provide information on the symmetry of the protein, *i.e.* if the subunits are identical or quasi-identical. This was shown by theory and borne out in practice in a study of lactate dehydrogenase and aldolase.[189] Chromatographic methods for the study of these problems are also available.[190]

Molecular Weight and Shape: Association Equilibria and Lipid Binding.—*Molecular Weight from SDS-gel Electrophoresis.* In most instances the determination of protein molecular weight by SDS-gel electrophoresis gives correct values as judged by independent methods, but this is not invariably so. The use of a Ferguson plot after gel electrophoresis in a series of gel concentrations is a safeguard against incorrect molecular weight estimates due to anomalous behaviour caused by bound carbohydrate or unusual detergent binding.[191] This method has been applied in several cases.[192–194] In an investigation concerned with influenza virus hemagglutinin,[194] the apparent molecular weight of the heavy subunit varied from 92 000 to 51 000 as the gel concentration increased from 3 to 15%. A value of 47 000 was obtained from gel filtration and sedimentation

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equilibrium in 6M-guanidine. A different approach to the molecular weight estimation of protein–SDS complexes is gradient gel electrophoresis.[195]

Molecular Weight from Gel Filtration in the presence of Denaturants. The molecular weight estimation of protein in SDS requires that all proteins assume the same conformation – which is thought to be a rod-like SDS–protein complex – in the detergent, so that the measured parameter is strictly a function of peptide chain length. A different way to normalize conformation is to use concentrated guanidine HCl as solvent, in which (nearly ?) all proteins form random coils. The molecular weight is then conveniently estimated by gel filtration. A drawback of the method is that it has a low resolving power as compared to SDS-gel electrophoresis, but it seems often to be more reliable as exemplified by the work on the virus haemagglutinin [194] mentioned above. Gel filtration is also used to measure size (or molecular weight) of protein–SDS complexes. For large proteins this method gives an underestimate of size possibly due to end-on insertion in the gel beads of the SDS–protein rod.[196] The original model of the SDS–protein complex as a rather inflexible rod has been questioned by recent physicochemical and optical measurements.[197, 198]

Molecular Weight from Sedimentation Equilibrium in the presence of Detergents. The measurement of the molecular weight of proteins in the presence of a detergent, such as Triton X-100, where binding or conformation is not normalized, is less straightforward. One possibility still is to perform gel electrophoresis at different gel concentrations,[199] but hydrodynamic methods, in particular sedimentation equilibrium, are preferable. This approach is also required if unequivocal molecular weights of proteins solubilized in SDS are sought. These methods require that the degree of detergent binding is precisely known or that the contribution from the detergent cancels. Detergent binding can be assessed by measuring excess detergent in the protein peak, as compared to buffer, in a gel filtration run,[200] or, less precisely, after gel electrophoresis.[201] SDS can be estimated by spectrophotometry after binding to methylene blue [200] or basic fuchsin [202] and subsequent extraction in chloroform, although the use of radiolabelled SDS might be preferred. Triton X-100 binding can be most accurately measured by using radiolabelled detergent.[201, 203] A different possibility is to estimate detergent binding from the partial specific volume of the complex as obtained from sedimentation in H_2O–D_2O solutions.[204] Knowledge of detergent binding (δ_D), and the partial specific volumes of the detergent ($\bar{v}_D$) and the protein ($\bar{v}_P$) will lead to an estimation of the molecular weight of the protein

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(M_P) from the equation

$$M_P(1 - \phi'\rho) = M_P[(1 - \bar{v}_P\rho) + \delta_D(1 - \bar{v}_D\rho)]$$

where $M_P(1 - \phi'\rho)$ is obtained from the sedimentation equilibrium run.[200] If lipid is still associated to the protein a third term, $\delta_L(1 - \bar{v}_L\rho)$, has to be included on the right-hand side. The symbol ρ refers to the specific weight of the solvent (with detergent). A straightforward way to avoid the estimation of detergent binding is to measure the partial specific volume of the protein in the detergent solution at equilibrium conditions so that ϕ' is obtained. This will give the molecular weight of only the protein moiety in the complex directly from the

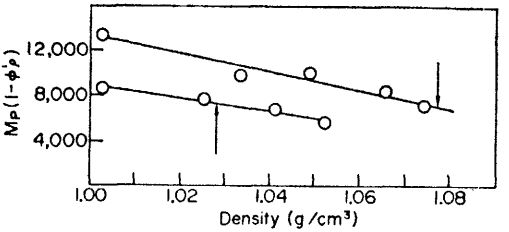

Figure 2 *Reduced molecular weights, $M_P(1 - \phi'\rho)$, of the apoprotein of lipoprotein AI solubilized in Lubrol WX (upper line) and $C_{12}E_8$ (lower line) at different densities in H_2O–D_2O mixtures. Total detergent concentrations were close to* 5 mmol l^{-1}, *which is a factor of more than* 50 *above the c.m.c. for $C_{12}E_8$ and a factor of more than* 500 *for Lubrol WX. The protein concentration was ca.* 5 μmol l^{-1}. *Arrows indicate the value of $\rho = 1/\bar{v}_D$ for each detergent*
(Reproduced by permission from *Proc. Nat. Acad. Sci., U.S.A.*, 1976, **73**, 4467)

sedimentation equilibrium plot.[193] A more manageable method is to adjust the specific weight of the solvent to precisely the inverse of the partial specific volume of the detergent, thereby making the second term on the right-hand side equal to zero.[205] In practice this can be done by the use of deuteriated water. If equilibrium runs are done at several values of ρ, the best value of $M_P(1 - \phi'\rho)$ is obtained from a linear or near-linear plot (Figure 2).

Molecular Weights of Proteins soluble in Normal Buffers. Such measurements are performed by sedimentation equilibrium. A note of caution should be added to the general use of partial specific volumes calculated from amino-acid compositions, as significant deviations between calculated and experimentally obtained values occur.[206] Molecular size, as indicated by Stokes radius or diffusion coefficient, is measured by gel filtration. Correct or near-correct molecular weights are obtained only if the unknown sample and the reference proteins are of similar shape, but gel filtration remains the most used method for estimation of molecular weights. In the case of long rod-like molecules even the Stokes radius measurements fail (*i.e.* underestimates are obtained), as shown by independent hydrodynamic data.[196]

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Protein Association Equilibria. These can be studied by established hydrodynamic methods or by gel filtration using frontal analysis, *e.g.* reports on the dimerization of nerve growth factor,[207] and polymerization of apolipoprotein AI [208, 209] and cytochrome b_5.[210] Some recent developments in the area of ligand–protein associations have been reported.[211]

4 Proteins Isolated, and Some of their General Properties

Isolated Proteins.—*Membrane-bound Proteins.* The number of proteins that have been reported isolated during the year is so great that complete coverage is not feasible. Instead a comprehensive tabulation of isolated membrane-bound proteins is provided in Table 1, and a few others are mentioned below in connection with the topics of membranes, organization of subunits, and polyfunctionality of peptide chains. Few receptor proteins are included in Table 1, since these have almost exclusively been studied when still associated with the membrane. In some cases partial purification was achieved by centrifugation techniques, as exemplified by gonadotropin receptors [277] and receptors for nerve growth factor.[246] Bacterial chemoreceptors seem not always to be true membrane-bound proteins but rather dissolved in the periplasmic space (*e.g.*, the *E. coli* galactose-binding protein).[278]

Nucleic Acid-associated Proteins. Several chromatin-associated proteins have been obtained in pure form, such as some steroid receptors [279–281] or the nuclear receptors for thyroid hormones,[282] which earlier had proved difficult to isolate. Other proteins of interest in this area of investigation are the DNA unwinding proteins [19] and the DNA-dependent RNA polymerases.[116, 283–286] The complexity of the eukaryotic chromatin non-histone proteins indicates that the characterization of these proteins will take a long time. In a survey of the HeLa cell chromatin by two-dimensional electrophoresis 450 proteins were detected, most of them present in less than 10 000 copies per cell.[76] The involvement of histones other than H1 in the formation of the nu-bodies is well known. A study of the virus SV-40 minichromosome showed that addition of histone H1 induced further condensation of these structures.[287] The investigations on the bacterial ribosomal proteins will soon have reached the stage of a complete knowledge of their covalent structure (see Section 6), while their approximately 70 eukaryotic counterparts have only recently become available in pure form.[4, 5, 288]

Other Proteins. The purification of *human interferon* has been tackled more successfully since the very hydrophobic nature of this protein has been recognized, and preparations from both leucocytes [61] and fibroblasts [63] have been obtained by, in particular, hydrophobic chromatography (see discussion under this section). The fascinating story of the *calcium-binding proteins* each one with a different origin and function, but all belonging to a single family of homologous proteins, continues to unfold. Further members of the family have been isolated from adrenal medulla [289, 290] and crayfish muscle.[291] Studies on the cyclic nucleotide

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Table 1 *Purifications of membrane-bound proteins reported during* 1976. *Conditions for extraction from membrane and crucial steps in the purification scheme are given under comments. The following abbreviations are used: AffCh = affinity chromatography; ConACh = chromatography on concanavalin A-Sepharose; HCh = hydrophobic chromatography; GelCh = gel chromatography (gel filtration); DEAE = ion exchange chromatography on DEAE-cellulose or Sephadex; IEF = isoelectric focusing; PAGE = polyacrylamide gel electrophoresis; chrom. = chromatography; prec. = precipitation; cent. = centrifugation; prep. = preparative; det. = detergent; SDS = sodium dodecyl sulphate; PEG = poly(ethylene glycol)*

Protein	*Source*	*Conditions for solubilization*	*Comments on purification etc.*	*Ref.*
Enzymes				
Glycerol-esterhydrolase	Rat intestinal mucosa	Triton X-100	AffCh, prep. PAGE	212
β-Glucoronidase	Rat liver microsomes and lysosomes	Deoxycholate	Immunosorpt. in det. free buffer	147
Maltase	Rat intestinal brush border	Triton X-100		213
Nicotinamide dinucleotide glycohydrolase	Calf spleen	Pancreatic lipase	DEAE	142
Penicillinase	*Bacillus licheniformis* plasma membrane	Triton X-100	GelCh, DEAE	214
Angiotensin I converting enzyme	Rat lung	Standing in phosphate buffer		136
Proparathyroid hormone converting enzyme	Bovine parathyroid gland	Triton X-100		214*a*
γ-Glutamoyl transpeptidase	Human kidney brush border	Deoxycholate	ConACh	215
γ-Glutamoyl transpeptidase	Rat kidney brush border	Triton X-100 or papain-treatment		216
γ-Glutamoyl transpeptidase	Rat kidney brush border	Lubrol WX. Disaggregated with bromelain-treatment	ConACh	217
Peptidases	Rat intestinal brush border	Triton X-100	prep. PAGE	218
Aminopeptidase	Rat intestinal brush border	Triton X-100		213
DD-Carboxypeptidase	*Proteus mirabilis*	Genopol X-100	AffCh	219
5′-Nucleotidase	Rat liver plasma membrane	Deoxycholate		220
Nucleoside phospho-transferase	*Erwinia herbicola*	Triton X-100	IEF	221
Adenylate cyclase	Rat brain	Lubrol PX	AffCh (Blue dextran Sepharose)	222
Adenylate cyclase	Dog kidney medulla	Lubrol PX	GelCh	223

Guanylate cyclase	Sea urchin sperm	Triton X-100 or Lubrol PX or Lubrol WX (most useful)	AffCh, DEAE, and GelCh in det. free buffer	224
Proline reductase	*Clostridium sticklandii*	Triton X-100	HCh	225
Nitrate reductase	*E. coli*	Heat-treatment (60 °C, 20 min)	GelCh in det. free buffer	138
3-Hydroxy-3-methylglutaryl coenzyme A reductase	Rat liver	Freezing and thawing	Det. free buffers. Pure enzyme binds lipids	139
Thyroid peroxidase	Pig thyroid particulate fraction	Triton X-100 or Brij 58	GelCh	226
Protoporphyrinogen oxidase	Mammalian mitochondria	Triton X-100. Sonication	GelCh in det. free buffers	227
NADPH-cyt *c* (cyt P450) reductase	Rat and pig liver microsomes	Polyethoxynonylphenylether, cholate	AffCh	228
NADH-dehydrogenase	*E. coli*	Triton X-100	Det. or phospholipids required for activity	229
Dopamine β-hydroxylase	Bovine adrenal medulla	Triton X-100	Prec. by PEG in det. free buffer	144
Monoterpene hydroxylase (cyt P450 dependent)	*Vinca rosea* (green plant)	Cholate		230
Prostaglandin cyclo-oxygenase	Sheep vesicular gland	Tween 40	AffCh	231

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Table 1 (*cont.*)

Protein	*Source*	*Conditions for solubilization*	*Comments on purification etc.*	*Ref.*
Carbamoyl phosphate synthetase	Rat liver mitochondria	Triton X-100	DEAE	232
Vitamin K-dependent carboxylase	Rat liver microsomes	Triton X-100		233
Phosphatidyl-glycerophosphate synthetase	*Bacillus licheniformis*	Triton X-100	AffCh	234
Phosphatidyl-glycerophosphate synthetase	*E. coli*	Triton X-100	AffCh	235
Collagen glucosyltransferase	Chick embryo	Triton X-100	Later purification steps in det. free buffer	146
Na^+, K^+-activated ATPase	Duck nasal salt gland microsomes	Low concentration SDS	Gradient cent.	238
Cation-activated ATPase	Hog stomach microsomes	No detergent	Sucrose gradient cent. Free-flow electrophoresis (vesicles separated)	148
F_1-ATPase	Rat liver mitochondria inner membrane	Prewash in phosphate buffer, then deoxycholate (other det. partially inactivates)		236
F_1-ATPase	Wheat seedling sub-mitochondrial particles	Sonication	GelCh and DEAE in det. free buffer	137
F_1-ATPase	*Micrococcus* sp.	Osmotic shock	AffCh in det. free buffer	140
F_1-ATPase	*E. coli*	EDTA, 2-mercaptoethanol	PEG prec., DEAE	237
Carbodi-imide-reactive protein subunit of F_1-ATPase	*E. coli*	Chloroform–methanol (2 : 1)	DEAE and GelCh on OH-propyl-Sephadex G-50 in $CHCl_3$–CH_3OH–H_2O (3 : 3 : 1)	72
Cytochromes				
Cyt *c* oxidase	*Rhodopseudomonas palustris*	Triton X-100/1M-KCl		239
Cyt *c* reductase (cyt c_1-*b* complex)	Yeast mitochondria	1M-KCl/deoxycholate		240
Cyt *c* reductase	Yeast mitochondria	Cholate	Cyt c_1 purified	177
Cyt P450	Mouse liver microsomes	Cholate		151
Cyt P450	Rabbit liver microsomes	Cholate	HCh, elution with Emulgen 913	58

Cyt P450	Rabbit liver microsomes	Cholate	PEG prec., DEAE	241
Cyt P450	Rabbit lung microsomes	Cholate	DEAE, two forms separated	242
Cyt P450	Rat liver microsomes	Deoxycholate	DEAE, 10 different forms isolated	154
Cyt P450	Bovine adrenal cortex mitochrondria	Cholate	HCh, elution with cholate and Emulgen 913	57
Membrane transport proteins				
Saccharide-transporting protein	*E. coli* outer membrane	Pretreatment with SDS, then treatment with lysozyme and trypsin	Reconstituted in liposomes	133
Glucose-transporting protein (Na^+-dependent)	Rabbit kidney brush border	Triton X-100	Reconstitution possible	243
Glucose-transporting protein (band 3)	Human erythrocytes	Pretreatment with buffers and acylation removes 65% of protein. Band 3 remains		132
Band 3	Human erythrocytes	Pretreatment with 2,3-di-methyl maleic anhydride. Triton X-100	Covalent chrom. on thiol-Sepharose	38
Glucose-transporting protein	Human erythrocytes	Triton X-100 or 30 mM octylglucoside (better selectivity)	Reconstituted in liposomes	244
Methionine-binding protein	*Aspergillus nidulans* mycelium	Triton X-100	AffCh	244*a*

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Table 1 (*cont.*)

Protein	*Source*	*Conditions for solubilization*	*Comments on purification etc.*	*Ref.*
Membrane-bound receptors				
Nerve growth factor receptor	Rabbit superior cervical ganglia	Triton X-100, deoxycholate		245
Nerve growth factor receptor	13-day-old chick embryo brain	Osmotic shock of synaptosomal fraction		246
Nicotinic acetylcholine receptor	Electroplax from *Torpedo california* and *T. marmorata*	Triton X-100	Gradient cent.	204
Nicotinic acetylcholine receptor	*T. california*	No detergent	Affinity partitioning	35
Nicotinic acetylcholine receptor	*Electrophorus electricus*	Triton X-100	IEF	247
Nicotonic acetylcholine receptor	Rat brain	Triton X-100	AffCh	248
Nicotinic acetylcholine receptor	Rat brain	Triton X-100		249
Muscarinic acetylcholine receptor	Rat brain	2M-NaCl (det. destroys binding activity)	DEAE, GelCh	250
β-Adrenergic receptor	Frog erythrocytes	Digitonin		251, 252
Gonadotropin receptors	Rat testis	30% ethanol (det. gives less stable preparation)		253
Asialoglycoprotein receptors	Rat liver lysosomes, Golgi bodies, smooth microsomes and plasma membrane	Triton X-100	AffCh, GelCh	254
Cell surface antigens				
Histocompatibility antigens	Cultured human lymphoblastoid cells	Brij 99 : 97	AffCh	255
Histocompatibility antigens	Mouse lymphocytes	Nonidet P40	AffCh	256
Histocompatibility antigens	Cultured human lymphoblastoid cells	Deoxycholate	Lectin-Sepharose chrom. in 0.5% deoxycholate	257

Histocompatibility antigens	Chicken lymphocytes	Nonidet P40	Immunoprecipitation	258
Histocompatibility antigens	Mouse lymphocytes	Nonidet P40	Immunoprecipitation	259
Terminal complement complex	Target sheep erythrocytes	Triton X-100	Prep. IEF in polyacrylamide gel, GelCh	260
Cell membrane glycoproteins				
Glycocalicin	Human platelet membrane outer surface	No detergent		261
Intrinsic membrane proteins (glycophorin and band 3 protein)	Human erythrocytes	SDS	Prep. SDS-PAGE	104
Glycophorin	Human erythrocytes	Tween 20	HCh in det. free buffer	55
ABO-blood group active glycoproteins	Human erythrocytes	Triton X-100	IECh. Det. removed by chrom. on Sephadex LH20 and elution with EtOH–H_2O (1 : 1)	262
Glycophorin A	Human erythrocytes	SDS/high salt	Lectin-Sepharose chrom.	263
Thy-1 glycoproteins	Rat thymocytes and brain	Deoxycholate		264

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Table 1 (*cont.*)

Protein	*Source*	*Conditions for solubilization*	*Comments on purification etc.*	*Ref.*
Glycoprotein	Mouse kidney epithelial basement membrane	Phosphate buffer		135
Lectin	Bovine liver plasma membrane	Triton X-100		266
Glycoprotein	*Halobacterium salinarium* cell envelope	Aqueous phenol extraction after delipidation in chloroform–methanol	Contains Asn-linked complex oligosaccharide	267
Phosphoglycoprotein	Rat brain	Pretreatment with chloroform–methanol (2 : 1). Extraction in SDS	ConA-Sepharose, GelCh	268
Glycoprotein	Bovine peripheral nerve myelin membrane	Pretreatment with chloroform–methanol (2 : 1). Extraction in SDS	GelCh	269
Other membrane proteins				
Membrane proteins	*Acholeplasma laidlawii* plasma membrane	Tween 20	Agarose suspension electrophoresis in det. free buffer	143
Major protein	*E. coli*	Osmotic shock, no detergent	Suggested to be on plasma membrane inside	141
Major protein	Rat liver microsomes	1M-urea–1% 2-mercapto-ethanol	Prep. PAGE	105
Major protein	*Antirrhinum majus* chloroplasts	SDS–2% 2-mercaptoethanol	GelCh	270
Protein I	*E. coli* cell envelope outer membrane	SDS–1% 2-mercaptoethanol	SDS-PAGE, IEF	271
Spike complex	Semliki Forest virus membrane	Triton X-100	Gradient cent. removes det.	149
Spike and shell proteins	Phage PM2 membrane	Acetic acid	GelCh in SDS	272
Egasyn (glucoronidase-binding protein)	Mouse liver microsomes and lysosomes	Triton X-100	Immunoprecipitation with anti-glucoronidase	273
Other proteins	Cultured mouse fibroblasts	0.01% Nonidet P40 (no lysis of cells at this concentration)	A single protein extracted	129

Other proteins	Bovine brain synaptic membranes	SDS	Prep. SDS-PAGE	107
Glass-adhering protein	Rat peritoneal macrophage	Glass-bead chrom.	Electrophoresis from glass beads after pretreatment with 8M-urea–2% SDS–2% 2-mercaptoethanol	69
Phosphorylated protein	Human erythrocytes	SDS	GelCh on Sephadex LH20 in chloroform–methanol (2 : 1)	70
Protein synthesis inhibitory protein	Rabbit reticulocytes	Triton X-100		274
Peptidoglycan synthesis stimulation protein	*Bacillus megaterium* plasma membrane	Toluene/LiCl		275
Proteolipid apoprotein	Bovine heart, liver, and kidney	Chloroform–methanol	Several forms	276
Proteolipid apoprotein	Bovine brain white matter	Chloroform–methanol	SDS-PAGE	184
Proteolipid apoprotein	Dog brain white matter	Chloroform–methanol	GelCh on acetylated Sephadex G-200 in chloroform–methanol mixtures	71

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phosphodiesterase activator protein prove convincingly that it belongs to this family.[292, 293] Their homology is finally demonstrated by primary structure data and such are given for the better-known members, parvalbumins, troponin C, and myosin light chains, in Section 6.

Organization of Membrane-bound Proteins *in situ*.—*Attachment to Membrane. Evidence from proteolytic release.* The earlier widespread use of proteases to release membrane-bound proteins indicated that they were attached *via* a part of the peptide chain that served as a hydrophobic tail; this was later confirmed by sequencing studies. A further example of this arrangement is provided by acetylcholinesterase from *Torpedo california*, where the tail can be nicked off by collagenase thereby releasing the catalytically active head.[294] The *Bacillus licheniformis* penicillinase anchoring piece consists of covalently bound phosphatidylserine, where the serine presumably is the *N*-terminal amino acid residue in the peptide chain, since it is a part of an easily released *N*-terminal peptide.[214, 295] Evidence is given that covalently bound phosphatidylserine is present on several others of the enzymes bound to the *Bacillus* plasma membrane.[296] The aminopeptidase of pig intestinal brush border membranes has also been isolated in both an exoenzyme form by proteolysis and a detergent-solubilized form. The anchoring sequence in this case consists of *ca.* 80 amino-acid residues[213] and seems to penetrate through the membrane as it reacts with antibody-carried 4-fluoro-3-nitrophenyl azide on inside-out vesicles.[297] When the membrane ATPase from *Streptococcus faecalis* underwent limited chymotryptic digestion an icosapeptide was released from the largest subunit thereby destroying its membrane binding properties.[298] The other four kinds of subunit in the protein remained intact, but as the large subunit is present in two or three copies two or three anchoring sequences might be present.

Evidence from other methods. Among other methods used to investigate membrane-binding areas in proteins is a direct search for hydrophobic sequences

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in the primary structure. The phage M-13 coat protein has a hydrophobic middle portion which is a likely candidate for the transmembrane part in the bacterial plasma membrane-associated protein.[299] Searches for α-helices with polar and apolar surfaces can also be done if a primary structure is available.[300, 301] The use of antibodies to assess surface availability constitutes a different possibility. The earlier mentioned brush border aminopeptidase proved to have two out of 12 antigenic sites masked when incorporated into the brush border membrane.[302] Proteolytic dissection was used to localize the transmembrane region of the peptide of band 3 from human erythrocytes,[303, 304] which contains nearly 900 amino-acid residues. The results (identification of points of cleavage) give no detailed picture but suggest that the part of the long peptide chain that passes through the membrane is close to the middle of the chain. Neutron diffraction studies on rhodopsin demonstrated that a major part of this protein was embedded in the membrane,[305] indicating a very different situation from that of the enzymes that are easily released by proteolysis.

Distribution within the Membrane. Localization inside or outside the membrane. Different labelling techniques have been used to localize protein to the cytoplasmic or outer surface or both. Radioactive labelling with iodine demonstrated the ATPase to be on the outside and calsequestrin, the calcium-binding protein, to be on the inside of the sarcoplasmic reticulum.[306] Similar techniques, supplemented with proteolysis, demonstrated carbamylphosphatase to penetrate through the mitochondrial inner membrane in isolated mitoplasts.[307] A single protein was labelled on the cytoplasmic side of the myelin sheath by reduction with triated borohydride after treatment of the protein amino-groups with salicylaldehyde or pyridoxalphosphate.[308] Of these two reagents the first penetrates through the membrane, whereas the second does not. Photo-labelling was used to prove both subunits of the acetylcholine receptor to be accessible in the electric tissue of *Torpedo california*.[309] Immunological techniques have also been employed for localization purposes, as exemplified by studies on human erythrocytes [310] and leucocytes.[311] In the last work the actin-binding protein was shown to be on the cytoplasmic surface.

Distribution on the membrane surface. Membrane-bound proteins have previously been made visible in the electron microscope by the aid of ferritin-labelled antibodies. A promising alternative to this technique is provided by the use of

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an avidine–ferritin conjugate. This can be bound to membrane-associated macromolecules, which have previously been covalently coupled to biotin. In this way excellent micrographs of concanavalin A receptors on plasma membranes were obtained.[312] The new biophysical technique of fluorescence energy transfer has recently been used to study the distribution of the concanavalin A receptors on the surface of mouse fibroblasts.[313] Although no specific organization was demonstrated they appeared to be non-randomly distributed. In contrast, a different study on mast cell IgE receptors indicated that there were diffusely distributed.[314] Also the rotational movements have been studied and a rotational diffusion coefficient of the human erythrocyte transmembrane protein, band 3, has been determined.[315]

Shape-maintaining proteins. Some membrane-bound proteins have been suggested to be essential for the cellular shape, that is to provide some kind of cyto-skeleton. If the major protein component of the cell envelope of *Halobacterium salinarium*, a glycoprotein of mol. wt. 200 000, is removed by proteolysis, the cells lose their shape.[316] In *E. coli* mutants, missing two major proteins (mol. wts. 37 000 and 33 000) of the cell envelope, no physiological malfunction was observed but stable ghosts could not be prepared, suggesting these two proteins to have mainly a shape-maintaining role.[317] Spectrin from human erythrocytes forms aggregates with actin giving a mesh network on the cytoplasmic side of the membrane.[318] It has been suggested that spectrin forms the strings in these, but careful physicochemical work on the isolated spectrin tetramer did not confirm the old notion of this protein as a fibrous [319] one but rather suggested it to have an expanded, although ordered, structure.[150] The possibility remains that the strings in the mesh network are made up of F-actin polymers.

Protein–Lipid and Protein–Protein Interactions. Binding of phospholipids. Influence of the headgroup. Several studies on the interaction of membrane proteins with lipids suggest that these interactions have some degree of specificity and often are required for a functional protein conformation. The sarcoplasmic reticulum Ca^{2+}-dependent ATPase contains, when isolated in the absence of detergents, *ca.* 70 mol of lipids per mol of protein.[320] In low concentration of non-ionic detergents *ca.* 30 mol of lipid remain bound per mol of enzyme, which remains active.[321] By gel filtration in deoxycholate- or cholate-containing buffers all phospholipid was removed to give an inactive enzyme.[321–323] The readdition of phospholipid, phosphatidylethanolamine or phosphatidylcholine, restored the activity.[320, 322] The first 15 mol of phospholipids were bound co-operatively

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indicating a conformational change.[320] A completely parallel situation was reported for acetylcholinesterase, although here activity was recovered after addition of phosphatidylserine to the lipid-depleted enzyme.[324] In the case of cytochrome b_5, studied by fluorescence depolarization of dansylated phospholipids, the protein interacted strongly with phosphatidylcholine, but not with phospholipids with inositol or serine on the headgroup.[325] The gonadotropin receptors of bovine *corpus luteum* were 90% and 75% inactivated by treatment of membrane fragments with phospholipases A and C, respectively, pointing to the importance of lipids in stabilizing the active conformation of the receptor molecule.[277, 326]

The influence of the fatty acid moieties on the phospholipid molecules is highlighted by investigations on auxotrophic mutants of *E. coli*, where the fatty acid composition of the plasma membrane could be varied at will by modifications to the growth medium.[327] The membrane D-lactate oxidase was bound to membrane vesicles at all fatty acid compositions, but the enzyme activity varied dramatically. The *cis*-isomers of unsaturated fatty acids in particular were required for high activity. Also the activity of the Na,K-ATPase of Erlich ascites tumour cells varied with fatty acid composition, the general conclusion being that activity increased with the transition point of the membrane.[328] The binding of phospholipids with differing fatty acid compositions to the erythrocyte glycophorin was studied by ^{13}C n.m.r. spectroscopy, and again a preferential association of unsaturated side-chains to the protein was noted.[329]

Protein–protein interactions. These evidently occur in multisubunit membrane-bound proteins, just as in soluble protein complexes, but sometimes the interactions are too weak to permit the entire complex to be isolated as such, in particular if detergents are used. These interactions thus have to be studied when the proteins still are bound to the intact membrane, and several such studies have been reported. Cross-linking experiments indicate that the Na,K-ATPase occurs as a dimer in the membrane.[330] The smaller of the subunits, β, in the $\alpha_2\beta_2$ structure was not available to proteases.[331] The oubain inhibition (on the cytoplasmic side of the larger subunit) proceeded more readily when two loosely bound proteins (mol. wts. 70 000 and 30 000) were removed by rinsing in chelator-containing buffer, pointing to their probable interaction with the enzyme *in situ*.[332] The acetylcholine receptor of *Electrophorus* was isolated in vesicles together with the ATPase, indicating a specific interaction.[333] Glycophorin from erythrocyte membrane is always isolated partially as a dimer, even in the presence of SDS, suggesting a dimeric association in the intact membrane.[182, 334] The occurrence of site–site interactions among insulin receptors also provides

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evidence for specific interactions.[335] A different kind of association is exemplified by the binding of the glycolytic enzyme aldolase to band 3 polypeptide of the erythrocyte membrane,[336] similar to the previously known binding of glyceraldehyde-3-phosphate dehydrogenase to the cytoplasmic side of this membrane.

Reconstitution experiments will provide final proof of requirements for activity in terms of lipid and protein composition. Several such successful experiments have been reported: the sarcoplasmic calcium-dependent ATPase was incorporated into vesicles that worked as a calcium pump;[337] vesicles with the erythrocyte band 3 protein formed a glucose-transporting system;[338] vesicles containing a *Salmonella* outer membrane protein worked as a saccharide-transporting system;[339] vesicles with mitochondrial cytochrome *c* oxidase and ATPase could successfully carry out oxidative phosphorylation.[340]

The Architecture of Multisubunit Assemblies.—*Soluble Complexes. Subunit arrangement.* Cross-linking reagents and specific antibodies have been used to obtain information on the arrangement of subunits in multisubunit proteins. Examples are provided by bacterial enzymes such as pyrocatechuate 3,4-dioxygenase of *E. coli*, which has 32 polypeptide chains of two different kinds,[341] the citrate lyase of *Klebsiella pneumoniae*, which has 18 subunits of three different kinds,[342] and the transcarboxylase of *Propionobacterium shermanii*, which has 18 subunits of three different kinds.[343] In the last case specific antibodies were used successfully to probe the topology of the complex. Cholera toxin consists of five B subunits and one A subunit with enzymatic activity. The complex seems to consist of a ring of hydrophobic B subunits which can form a transmembrane channel to let the A part, originally bound in the centre of the ring, pass into the infected cell.[344, 345]

Quaternary structure of nucleic acid-associated enzymes. Several of the enzymes and other factors taking part in transcription and translation in eukaryotic cells have been isolated and found to have complex subunit compositions. Initiation factor IF-E3, prepared by high salt extraction of rabbit reticulocyte ribosomes, contained 11 subunits as judged by SDS-gel electrophoresis, none of which was a ribosomal protein.[346] The initiation factor IF-E2 from the same source was a trimer of three different subunits.[347] The three DNA-dependent RNA polymerases (designated I, II, and III), which differ in their sensitivity to the inhibitory peptide amanitin, seem to be present in all eukaryotic cells. In several

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laboratories 10—12 subunits have been identified in all three, isolated from yeast cells,[283, 348, 349] and in RNA polymerase III from mouse plasmacytoma,[284] and *Bombyx mori* silk glands.[285] Conflicting reports speak of only five major subunits in enzyme II from hen oviduct or liver [350] and enzyme I from rat liver nucleoli.[286] Three common subunits have been identified in the three yeast enzymes,[348, 351, 352] a result that is supported by genetic evidence.[353] The functions of the different subunits in these complex enzymes are almost totally unknown. Some information is obtained from studies on enzymes partially depleted of some of the subunits;[352] two of them could be removed from enzyme I, to leave an enzyme with *ca.* 50% activity, while the removal of a peptide of mol. wt. 24 000, believed to belong to the common subunits, precisely paralleled loss of activity. The amanitin-binding peptide in enzyme II was identified with radiolabelled amanitin.[354] Forms of enzyme III that were sensitive or insensitive, respectively, to ACTH regulation were identified in the guinea-pig adrenal gland.[355]

Functional assemblies with low association constants might be difficult to detect because the constituent components are usually isolated separately. Partitioning in a two-phase system, by counter-current distribution, provides a means to detect such interactions, as exemplified by a study on the weak complex of the cytoplasmic enzymes aspartate aminotransferase and malate dehydrogenase from pig heart muscle.[356] By gradient centrifugation, also suitable for maintaining weak interactions, a complex containing all the glycolytic enzymes could be isolated from *E. coli*.[357] The complex formed by yeast arginase and ornithine transcarbamyl transferase depends on the presence of allosteric effectors, and renders the transferase enzyme inactive.[358] The intriguing complex formed in *E. coli* by the RNA replicase of the Qβ phage, elongation factors EF-Tu and EF-Ts, and ribosomal protein S1, has been dissociated by urea and reconstituted,[359] but the EF-Tu-Ts part dissociates spontaneously as the Qβ RNA is added and replication commences.[360–362]

Membrane-bound Complexes. The Mitochondrial Respiratory Chain. It has long been possible to prepare particles from the mitochondrial inner membrane able to carry out respiratory phosphorylation (Figure 3). By mild detergent treatment these could be fractionated into complexes I to IV, the ATPase, cytochrome *c*, and lipid constituents. The continued examination of the complexes by recently developed techniques has provided a more detailed picture of the structure of the

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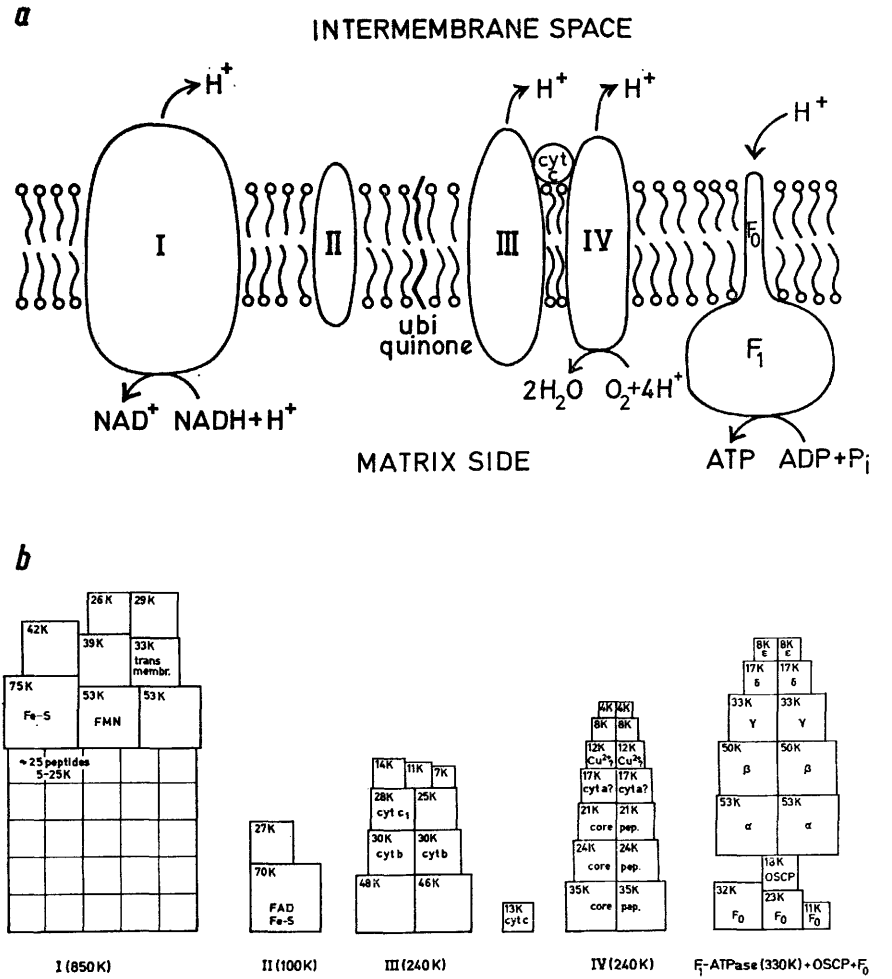

Figure 3 *Structural organization of the mitochondrial respiratory chain. (a) An overall view indicating the components; complexes* I—IV, *ubiquinone, cyctochrome* c *and the ATPase – and the basic reactions; proton translocation, ADP phosphorylation, NADH oxidation and reduction of molecular oxygen. (b) Subunit chart of the components as presently understood. The protein molecular weight of the complexes and peptides are given in kilodaltons* (K). *Peptides are believed to be involved in binding of prosthetic groups as indicated. In cytochrome* c *oxidase (complex IV), haeme and copper have been located only to the four smallest subunits. No attempts have been made to include the sparse information on subunit arrangement. Data have been taken mainly from references* 181, 370, 371a, and 379

mitochondrial electron-transport chain as consisting of close to 50 different peptide chains, organized in a specific way. The ATPase molecule contains five different peptides each in several copies. A collection of recent review articles provides an excellent summary of earlier work.[363–365] Respiratory or electron transport assemblies from thylakoid membrane and several prokaryotes all seem to be related structurally and functionally to the mitochondrial system. A discussion on the data from cytochrome *c* primary structures provides a basis for a possible evolution scheme of electron-transport systems in all living cells.[366]

Complex IV, or the cytochrome c oxidase, is at present the best known of the complexes. It contains seven subunits as isolated either from yeast [367] or bovine mitochondria.[181] By hydrophobic chromatography on leucine-Sepharose or gel filtration in 0.1% SDS, the three largest and most hydrophobic subunits (the core peptides), mol. wts. 35 000, 24 000, and 21 000, could be removed specifically, to yield an enzyme with two copies each of the smaller subunits (mol. wts. 14 600, 14 600, 12 300, and 10 600) as well as two haem *a* and two Cu^{2+} per mole.[368] By activating cysteine-107 in cytochrome *c* with Ellman's reagent, it was possible to create a disulphide bridge to subunit III, the smallest of the core peptides, pointing to a possible interaction between the two proteins in the functional complex.[369]

Complex III, the cytochrome c reductase, contains seven polypeptides as isolated from yeast.[240] An additional eighth small peptide is found in the beef heart preparation.[370] Cytochrome c_1, mol. wt. *ca.* 30 000, and cytochrome *b*, mol. wt. *ca.* 32 000, were identified and isolated from the yeast complex.[240] Two peptides, mol. wts. 14 000 and 11 000, co-purified with cytochrome *b*, pointing to a possible functional association of the three chains in the native complex. In cytochrome *c* reductase, the cytochrome *b* component was the only peptide synthesized on mitochondrial ribosomes,[371] while in cytochrome *c* oxidase all three of the largest peptide chains were synthesized in the mitochondria.

Complex I, NADH-Q reductase, together with complex II (the succinate dehydrogenase), is present in much smaller amounts than the two cytochrome-containing complexes, counted on a molar basis. Complex I is the largest protein component of the mitochondrial inner membrane, with an approximate protein mol. wt. of 850 000. A recently described preparation from beef heart mitochondria contains at least 16 peptide components separable by SDS–polyacrylamide gel electrophoresis.[371a] Two of these peptides (mol. wt. 53 000 and 26 000) contained the flavin prosthetic group, whereas one smaller (mol. wt. 33 000) and very hydrophobic peptide, tentatively was suggested to be the transmembrane proton translocator.

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The F_1-ATPase, coupling factor F_1, has been isolated from beef heart mitochondria,[372] chloroplasts,[373] and the prokaryotes *E. coli*[237] and *Streptococcus faecalis*.[374] All preparations contain two heavy (mol. wt. *ca.* 55 000) and three light (mol. wts. *ca.* 10 000, 20 000, and 35 000) subunits. The total mol. wt. is *ca.* 325 000, and studies with chemical cross-linking reagents, specifying $\alpha\alpha$ and $\alpha\beta$ contacts, suggest a stoicheiometry of $\alpha_2\beta_2\gamma\delta\varepsilon_2$ in the chloroplast enzyme[375] and $\alpha_2\beta_3\gamma_2\delta\varepsilon_2$ in the mitochondrial enzyme.[372] After dissociation of the *E. coli* enzyme by freezing and thawing and subsequent ion exchange chromatography, the α, γ, and ε subunits co-fractionated as a stoicheiometric complex, suggesting the original enzyme to be simply a dimer.[237] The question apparently needs unequivocal chemical methods to be settled. Limited chymotryptic digestion of the *S. faecalis* enzyme is said to remove a peptide of 20 amino-acid residues from the α subunit, thereby making the complex unable to bind to the membrane,[298] while the δ subunit is said to be responsible for membrane binding in the chloroplast-derived enzyme.[376] Irradiation of thylakoids in the presence of ATP makes ADP bind to one of the larger subunits.[377] α, γ, and ε all have accessible thiol groups, while the β subunit specifically reacts with the radiolabelled reagent 4-chloro-7-nitrobenzo-2-oxo-1,3-diazole.[372] A further peptide, or peptides, sometimes called the coupling factor F_0, is necessary for the proper binding of F_1-ATPase to the membrane and proton-driven phosphorylation of ADP. Such a peptide, of an approximate mol. wt. of 9000 was isolated from rat liver mitochondria, and shown to restore binding of the F_1-ATPase to the mitochondrial membrane.[378] The role of the so-called oligomycin sensitivity-conferring protein (OSCP), which previously was assumed to bind the enzyme to the membrane, is thus debated. The carbodi-imide reactive protein, an extremely hydrophobic peptide of 70 residues, was likewise isolated from the *E. coli* ATPase.[72] In any case a hydrophobic peptide, that might be the OSCP or not, is needed to induce proton transport in a reconstituted system containing the ATPase.[378a, 379] If cytochrome *c* oxidase is added to such a system, site 3 phosphorylation can take place.[379]

Polyfunctional Polypeptide Chains.—The concept of *multidomain, polyfunctional, polypeptide chains*, first established for the immunoglobulins, have proved to be applicable to many, in particular very large, proteins. As these proteins often are extremely susceptible to proteolytic enzymes, the new examples of this principle have been discovered as investigators have been more careful to avoid proteolysis during the preparations (see discussion in Section 2). Carefully isolated haemocyanin from *Helix pomatia* contained a single peptide chain, *ca.* 3300 amino-acid residues long.[380] Limited proteolysis and electron microscopy

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both indicated that the peptide chain was folded into seven domains each of mol. wt. *ca.* 50—55 000 and binding two copper ions. In contrast, haemocyanin from the horseshoe crab was reported to consist of eight different subunits, each of mol. wt. *ca.* 70 000.[381] How these proteins are related and, in particular, if the latter protein has been proteolytically nicked during preparation, remain to be seen. Fatty acid synthetase is a polyfunctional enzyme with an impressively long polypeptide chain. The goose uropygial gland enzyme was found to have a peptide chain of *ca.* 2500 residues,[382] and rabbit mammary gland enzyme *ca.* 3000,[383] while the yeast enzyme was reported to contain two different peptide chains each of *ca.* 1600 residues.[384] The presence of two distinct subunits was confirmed by genetic analysis. Controlled proteolysis was used to release the thioesterase activity from the enzyme.[385] Other proteins that have recently been prepared as long un-nicked chains include the 19 S thyroglobulin from pig or sheep of *ca.* 3000 residues,[179] filamin, an actin-binding protein from chicken gizzard of *ca.* 2200 residues,[386] and the RNA polymerases from eukaryotic cells where the largest subunit consists of *ca.* 2000 residues.[164–166]

A mammalian DNA polymerase that appears to be analogous to the two-headed prokaryotic enzyme has been isolated.[387] Several biosynthetic enzymes that have been suggested to have two[388–390] or three[391] activities on a single peptide chain have been isolated both from prokaryotic[388, 389] and mammalian[390, 391] cells. Several of the tRNA amino acyl synthetases have varied and complicated subunit structures and can also be two-headed, as demonstrated for the *E. coli* tryptophan-specific enzyme.[392] In this case it appears that the same active site appears twice due to internal duplication.

Precursor Proteins. Proteolysis products are sometimes not artefactual but of physiological significance. Examples of this are provided by the newly discovered precursor proteins, which are trimmed soon after synthesis, presumably after the propeptide has served a specific function, such as membrane transport, to give rise to the active proteins. The preproteins have often been isolable only from reconstituted protein-synthesizing systems where proteases are absent. Recently discovered examples of these are the preproteins of prolactin,[393, 394] adrenocorticotropic hormone,[395, 396] proparathyroid hormone,[397] proinsulin,[398]

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growth hormone,[399] and immunoglobulin light chain.[400] The enzyme responsible for the conversion of proparathyroid hormone was isolated from the parathyroid gland.[214a] In most of the mentioned cases, the proteins were shown to contain a relatively short presequence of *ca.* 25 amino-acid residues,[393, 397, 398, 400] but the adrenocorticotropic hormone was synthesized as a peptide of *ca.* 300 amino-acid residues, considerably larger than the active hormone.[395, 396]

5 Protein Sequencing Methods

Chain Cleavage and Separation of Peptides.—*Cleavage at Tryptophan Residues.* A potentially useful method for cleavage at tryptophanyl bonds in polypeptides has recently appeared. In a careful study Shechter *et al.*[401] have shown that *N*-chlorosuccinimide (NCS) cleaves peptide bonds *C*-terminal to tryptophan residues in a highly selective manner; they have developed conditions for the use of this reaction in generating large fragments suitable for sequence analysis. Treatment of several proteins tested with a ten-fold molar excess of NCS in 50% acetic acid resulted in cleavage yields of 20—50%, as determined by quantitative sequence analysis of peptide mixtures after NCS cleavage. No oxidation of histidyl residues or cleavage of tyrosyl bonds was detected. In contrast, *N*-bromosuccinimide, another reagent previously used for cleavage at tryptophan, is far less selective and its use results in major cleavages at both tryptophanyl and tyrosyl residues. Methionine and cysteine residues are oxidized by NCS to methionine sulphone and cystine, respectively, but neither modification is a serious disadvantage for the purpose of peptide purification and sequencing. Unfortunately no comparison was made in this work between NCS and BNPS-skatole, which has previously been used for cleavage at tryptophan and whose selectivity and efficiency seem to be comparable to NCS.

The species responsible for NCS cleavage of polypeptide chains is the chloronium ion, two equivalents of which are consumed per tryptophan. The indole nucleus is oxidized finally to a lactone which occurs at the *C*-terminal position of cleaved peptides. The lactone functions of CNBr peptides are commonly used as active groups to permit the peptide to be coupled to suitable aminated solid supports for sequencing, and it will be important to test whether this is also possible with NCS peptides.

Enzyme Cleavage at Proline Residues. The purification of an endopeptidase capable of cleaving at the *C*-terminal side of proline residues has been reported.[402] The specificity of the enzyme when tested on synthetic peptides was shown to be complete; only Pro-X bonds were cleaved, albeit at different rates depending upon the nature of residue X. When tested on a number of naturally occurring small peptides, the enzyme was shown to cleave at the expected points with the exception of a Pro-Pro bond in bradykinin-potentiating factor. However, larger polypeptides, even when denatured and rich in proline residues, were not susceptible to cleavage. The enzyme may be a useful addition to the repertoire of cleavage techniques for peptides already available to the protein chemist.

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[402] M. Koida and R. Walter, *J. Biol. Chem.*, 1976, **251**, 7593.

Fluorescamine and o-*Phthalaldehyde.* The sensitivities of fluorescamine and *o*-phthalaldehyde (OPA) for detecting amino-acids and peptides have been compared. One report [403] suggests that the former may be the reagent of choice for peptides whereas the latter may be preferable for amino-acids. Another report [404] claims that the two reagents are equally sensitive for detecting peptides on t.l.c. plates.

Benson [405] has described the design and operation of a peptide analyser using OPA for detection. The apparatus is simple and impressive; 300 pmol of a tryptic digest, when fractionated on a microbore ion exchange column, produced highly satisfactory fluorescence peaks. Buffer is pumped by nitrogen pressure through the microbore column and OPA, also under nitrogen pressure, is mixed with the eluent. The use of nitrogen pressure facilitates pulseless operation, a feature difficult to attain with mechanical pumps and which is necessary for stable fluorescence backgrounds.

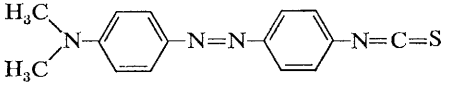
In addition to its greater sensitivity, the advantage of OPA over ninhydrin is that its reaction with peptides occurs rapidly at room temperature and no heating bath is required to generate the fluorescent product. OPA is soluble in aqueous buffers, which circumvents problems with the organic solvents which are necessary when fluorescamine detection of peptides is employed. The future prospects of OPA for microdetection of amino-acids and peptides are excellent: Benson points out that the sensitivity of detection of peptides may theoretically be increased by three orders of magnitude by using the fluorimeter at its most sensitive setting. If this is indeed possible, OPA detection of peptides will be extremely useful in circumstances where only subnanomolar amounts of protein are available for sequence studies.

Amino-acid Analysis.—Useful methods have recently become available for single-column amino-acid analysis of protein hydrolysates containing all common residues including cysteine derivatives and tryptophan. One report [406] recommends the use of 4M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole for hydrolysis to avoid destruction of tryptophan. The neutralized hydrolysate is then treated with dithiothreitol followed by sodium tetrathionate to convert cysteine and cystine residues into *S*-sulphocysteine. By this means all common residues may be determined in a single column run. However, there are still problems with tryptophan quantitation in glycoproteins, since this residue seems to be partially destroyed if carbohydrates are present during hydrolysis. Another report [407] describes a similar procedure for cysteine, cystine, and tryptophan analysis. Intact protein is first treated with 4-vinylpyridine to convert cysteine residues into their *S*-β-(4-pyridyl)ethyl derivatives which are more stable to acid hydrolysis than *S*-carboxymethyl derivatives. Hydrolysis is carried out with 4M methanesulphonic acid containing 0.2% tryptamine followed by conversion of cystine residues into *S*-sulphocysteine essentially as described in ref. 406.

[403] E. Mendez and J. G. Gavilanes, *Analyt. Biochem.*, 1976, **72**, 473.
[404] E. Gunnar and G. Lindberg, *J. Chromatog.*, 1976, **117**, 439.
[405] J. R. Benson, *Analyt. Biochem.*, 1976, **71**, 459.
[406] R. J. Simpson, M. R. Neuberger, and T. Y. Liu, *J. Biol. Chem.*, 1976, **251**, 1936.
[407] A. S. Inglis, D. T. W. McMahon, C. M. Roxburgh, and H. Takayanagi, *Analyt. Biochem.*, 1976, **72**, 86.

End-group Determination.—A new solvent, benzene–glacial acetic acid, 4.5 : 1, to replace the conventional solvent 2, has been introduced [408] for separating dansyl amino-acids on polyamide plates. The pairs, Ala/dansyl amide, Glu/Asp, Ser/Thr, and Arg/His, can be separated without recourse to a third solvent.

A new reagent, 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate (1) has been suggested [409, 410] as an alternative to dansyl chloride for determination of



(1)

N-terminal residues in peptides. Its structure combines the chromophoric dimethylaminobenzeneazo function with a reactive isothiocyanate group which reacts with amines. The limit of detection of its amino-acid derivatives (20—50 pmol) is reported to be lower than for dansyl derivatives, and t.l.c. systems to separate the new derivatives have been developed. Its main potential advantage over dansyl chloride is that no hydrolysis step is required before t.l.c. identification, thus permitting amides (Asn and Gln) to be distinguished from acids (Asp and Glu).

Carboxypeptidase Y has been a useful enzyme for determining *C*-terminal sequences due to its ability to release proline residues. A means of covalently attaching this enzyme to agarose beads has recently been reported;[411] the attached enzyme retains activity and the conjugate has been used for *C*-terminal sequencing studies. Its advantage is that the enzyme–agarose conjugate can be conveniently removed from degraded peptide and amino-acids when desired, but the importance of this is questionable.

A review has appeared [412] in which the properties of the main amino-acid derivatives useful in sequencing have been summarized. It is comprehensive and contains useful details concerning separation systems for the various derivatives.

Spinning Cup Sequencing.—A new coupling buffer has recently been recommended [413] for use in spinning cup sequencers. The compound, *NNN′N′*-tetrakis-(2-hydroxyethyl)ethylenediamine (THEED), is easy to prepare free of aldehyde contaminants which, if present, reduce the repetitive yield of a sequence run. Repetitive yields of 98% were reported using THEED, in comparison to 94% with quadrol under otherwise identical conditions. The problem in using THEED buffers is that impurities present are extracted by chlorobutane into the thiazolinone fraction and they subsequently interfere with identification of some PTH amino-acids by t.l.c. When identifications are made by back hydrolysis, however, these impurities do not interfere. The use of THEED is potentially important in obtaining extended *N*-terminal sequences on proteins. Nevertheless, where solvent removal of small or hydrophobic peptides is the limiting factor in sequencing, volatile tertiary amines will remain the buffers of choice.

[408] M. L. Lee and A. Safille, *J. Chromatog.*, 1976, **116**, 462.
[409] J. Y. Chang, E. H. Creaser, and K. W. Bentley, *Biochem. J.*, 1976, **153**, 607.
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[411] F. A. Liberatore, J. E. McIsaac, jun., and G. P. Royer, *F.E.B.S. Letters*, 1976, **68**, 45.
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[413] G. S. Begg and F. J. Morgan, *F.E.B.S. Letters*, 1976, **66**, 243.

For the automated degradation of small peptides, the simultaneous addition of parvalbumin to the spinning cup has been recommended[414] for use with dimethylbenzylamine as coupling buffer. Parvalbumin is blocked by an acetyl group at the *N*-terminus and so is not itself susceptible to Edman degradation but instead acts as a zwitterionic surface which reduces solvent removal of the small unblocked peptide being sequenced. The authors reported that 20 nmol of a pentapeptide was sufficient for its complete sequence determination by this method.

Reagents such as 4-sulphophenylisothiocyanate, which increase the polarity of peptides, have been valuable for sequence studies. This particular reagent covalently attaches to the *N*-termini and to lysine ε-amino-groups of peptides. Treatment with acid removes the *N*-terminal derivative, exposing the second residue, but leaves the internal lysine blocking groups attached. Recently, the *meta*-isomer, 3-sulphophenylisothiocyanate (3-SPITC), has been reported to be preferable to the original compound.[415] Its coupling efficiency is as good and the efficiency of removal of its *N*-terminal derivative by acid is faster than when 4-SPITC is used; incomplete cleavage would result in premature overlap which can limit the usefulness of sequence runs. The second advantage of 3-SPITC over 4-SPITC is claimed to be better recoveries of lysine residues when back hydrolysis of thiazolinones is used for identification.

During extended sequencer runs, background, due to acid cleavage of labile peptide bonds, sooner or later prevents positive identifications being made. It has recently been claimed[416] that non-specific cleavage can be reduced if the usual order of addition of phenylisothiocyanate (PITC) and coupling buffer is reversed. The authors suggest that the main cause of peptide bond cleavage during sequencing is the acid-catalysed N → O acyl shift, in which serine hydroxy-groups nucleophilicly attack the peptide bond involving the serine α-amino-group. Once exposed to PITC the serine α-amino-group would block and in due course cleave, thereby exposing a new section of the chain to further degradation. Delivery of coupling buffer prior to PITC allegedly causes a base-catalysed O → N acyl shift which restores the original peptide bond. New programmes for Beckman sequencers which incorporate reversed delivery regimes are presented in this report.

A device, suitable for accessory use with Beckman sequencers, for automatically converting thiazolinones into PTH amino-acids has recently been described.[417] The usefulness of automatic conversion, however, is questionable at present, since manual conversion is a short and easy operation when compared to other sequencing operations such as residue identifications or purification of starting materials.

Solid-phase Sequencing.—The attachment site of peptides coupled to aminated polystyrenes by the carbodi-imide method has recently been studied.[418] Two

[414] H. Rochat, G. Bechis, C. Kopeyan, J. Grégoire, and J. Van Rietschoten, *F.E.B.S. Letters*, 1976, **64**, 404.
[415] F. E. Dwulet and F. R. N. Gurd, *Analyt. Biochemistry*, 1976, **76**, 530.
[416] J. Thomsen, D. Bucher, K. Brunfeldt, E. Nexø, and H. Olsen, *European J. Biochem.*, 1976, **69**, 87.
[417] B. Wittmann-Liebold, H. Graffunder, and H. Kohls, *Analyt. Biochem.*, 1976, **75**, 621.
[418] C. Mech, H. Jeschkeit, and A. Schellenberger, *European J. Biochem.*, 1976, **66**, 133.

model peptides, one containing aspartic acid, the other containing glutamic acid, were both found to be attached predominantly *via* *C*-terminal carboxy (70%) rather than side-chain carboxy-groups (30%). Peptides coupled in this manner should be highly amenable to complete sequence analysis without blank cycles where acidic residues occur; the problem with this method of attachment, however, is that not all peptides couple efficiently and an excess of peptide is often necessary to ensure that sufficient is bound for sequence analysis.

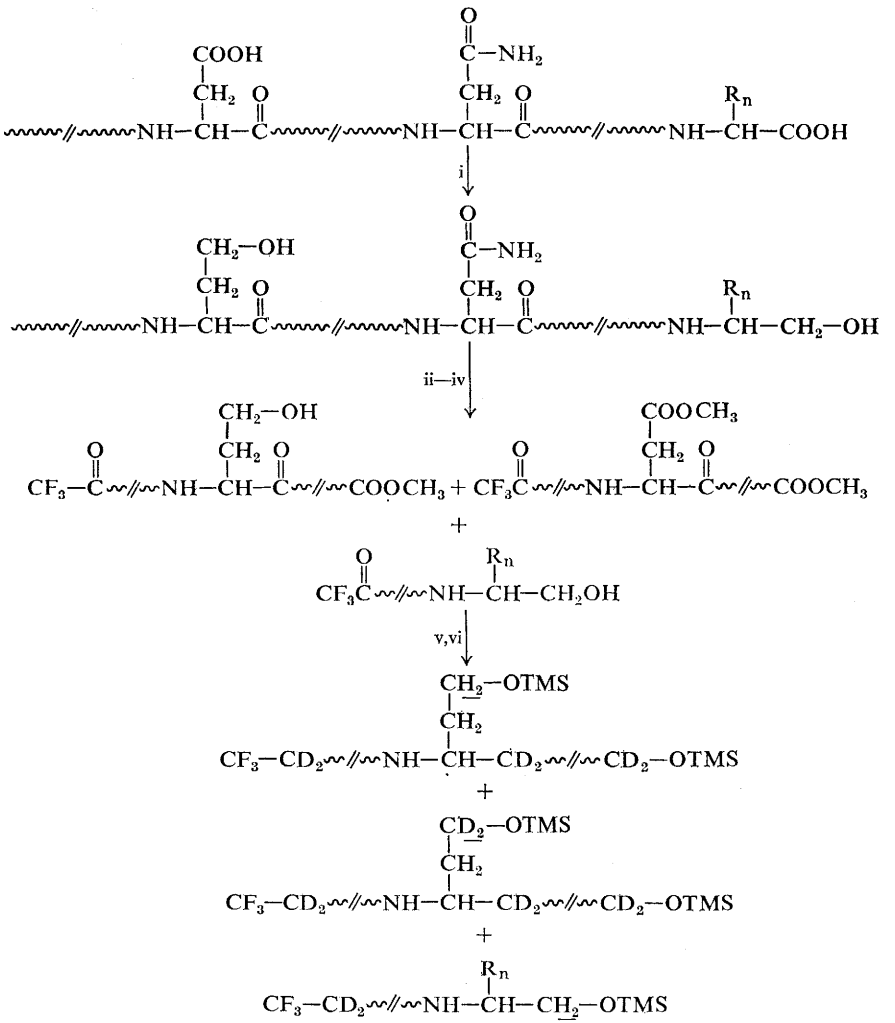
Another problem in solid-phase sequencing is that the resins commonly used for immobilization of peptides and proteins are not general enough. Polystyrene resins, for example, can be highly substituted with amino-groups for coupling but do not swell in aqueous buffers. This limits their use to peptides which are fairly soluble in organic solvents. Porous glass derivatives, on the other hand, cannot be highly substituted and covalent attachment of small peptides with only a few reactive sites is inefficient.

A resin has reportedly been developed [419] which satisfies the dual requirements of high degrees of amino-group substitution while remaining swollen in either aqueous or organic solvents. The resin, β-alanyl-hexamethylenediamine-polydimethylacrylamide, was originally developed for solid-phase synthesis of peptides but seems to be also suitable for the reverse operation, sequencing, as well as for oligonucleotide synthesis. Both insulin B chain (30 residues) and trypsin inhibitor (58 residues) were efficiently coupled to the resin by the DITC method. The latter was degraded successfully through 29 cycles by a conventional sequencing programme. Unfortunately no exact measurements of repetitive yield were carried out in this work but the qualitative data presented were very impressive. Another resin, an aminated derivative of poly(acrylic acid), has been developed [420] for similar purposes; attachment yields of polypeptides were estimated at *ca.* 80%, but no data on sequencing efficiency were made available.

Mass Spectrometry.—The sequence of a 39-residue peptide was recently deduced entirely by mass spectrometry.[421–423] The strategy used was to fragment the chain into small peptides by enzymic cleavage or partial acid hydrolysis. Peptides were then esterified, trifluoroacetylated, deuteroalkylated by reduction with $LiAlD_4$, and finally *O*-trimethylsilylated to increase their volatility. The resulting mixture was separated by g.l.c. and analysed by a mass spectrometer which produced spectra every 4.7 s. An on-line computer was used to process the data generated. The reported data were indeed adequate to deduce unambiguously the entire 39-residue sequence.

Problems encountered during mass spectrometric sequencing of peptides containing Asn or Gln, namely inefficient derivatization and deamidation, have been solved by a novel procedure (Scheme 3).[424] The protein is first treated with diborane which reduces the acid side-chains to alcohols, leaving amides unaffected. The chain is then fragmented by partial acid hydrolysis which also deamidates amides. The mixture is then esterified and trifluoroacetylated; reduction with

[419] E. Atherton, J. Bridgen, and R. C. Sheppard, *F.E.B.S. Letters*, 1976, **64**, 173.
[420] J. C. Cavadore, J. Derancourt, and A. Previero, *F.E.B.S. Letters*, 1976, **66**, 155.
[421] H. Nau and K. Biemann, *Analyt. Biochem.*, 1976, **73**, 139.
[422] H. Nau and K. Biemann, *Analyt. Biochem.*, 1976, **73**, 154.
[423] H. Nau and K. Biemann, *Analyt. Biochem.*, 1976, **73**, 175.
[424] H. Nau, *F.E.B.S. Letters*, 1976, **63**, 154.



Reagents: i, B_2H_6–THF; ii, 6N-HCl, or enzymatic digestion; iii, MeOH–HCl; iv, $(CF_3CO)_2O$; v, $LiAlD_4$; vi, Et_2NSiMe_3

Scheme 3

$LiAlD_4$ and *O*-trimethylsilylation follow. Analysis of the resultant mixture by g.l.c.–mass spectrometry allows those residues which were originally amides to be differentiated from those which were acids. Amide-derived ions are two mass units heavier than the corresponding ions derived from acids. *C*-Terminal peptides may also be distinguished from other peptides since the *C*-terminal carboxy-group was reduced by diborane rather than $LiAlD_4$ and spectral ions corresponding to *C*-terminal amino-acids will be two mass units lighter than the same internal residues.

In these applications, however, most of the sequences obtained were of di- and tri-peptides. The repeated occurrence of the same di- or tri-peptides by chance alone in larger proteins could conceivably introduce sequence ambiguities difficult to resolve due to the non-volatility of larger peptides. This, for the time being at least, may impose practical limits upon the usefulness of the technique for longer polypeptides. Moreover, it would be difficult or impossible by mass spectrometry alone to determine the number and arrangement of identical sequence repeats now known to exist in many proteins. Nevertheless, mass spectrometry has already become a useful adjunct to conventional sequencing techniques. The identification of the unusual amino-acid γ-carboxy-L-glutamic acid in prothrombin was carried out by mass spectrometry, and Morris *et al.*[425] have recently published their experimental procedure in detail.

PTH Amino-acids.—Previously reported separations of PTH amino-acids (see 1974 and 1975 Reports) by t.l.c. on polyamide plates permitted the ready identification of all amino-acid derivatives except the pairs Leu/Ile and Arg/His. The problem in separating the latter pair has now been overcome.[426, 427] Development of polyamide plates, on which the conventional two-dimensional separation has already been carried out, with a third solvent (n-butyl acetate–methanol–acetic acid, 29 : 20 : 1)[427] allows histidine and arginine phenylthiohydantoins to be adequately separated. An improved separation of methylthiohydantoin amino-acid derivatives has also appeared recently.[428a]

Several new methods of separating PTH amino-acids by high pressure liquid chromatography have appeared.[428b–432] None satisfies the optimum requirement of separating all amino-acids in a single run, but if the amount of sample for analysis is not limiting every PTH amino-acid can be unambiguously identified by judicious use of two different systems.

High-resolution field desorption mass spectrometry has also been used to identify PTH amino-acids.[433] Its advantage is that the small molecular weight differences between PTH amino-acids can easily be discriminated. About one nanomole is sufficient for identification. Its obvious disadvantage is the high cost of the hardware.

Microsequencing.—The lower limit of detection of amino acids or their PTH derivatives is not the only factor which imposes limits on the amount of protein required for sequencing. Problems in non-destructively detecting microquantities of proteins and peptides during their purification are also important. Two methods which will extend the scope and importance of microsequencing have recently appeared. Each represents a significant advance in overcoming both problems.

[425] H. R. Morris, A. Dell. T. E. Petersen, L. Sottrup-Jensen, and S. Magnusson, *Biochem. J.*, 1976, **153**, 663.
[426] T. P. Hopp, *Analyt. Biochem.*, 1976, **74**, 638.
[427] S. Bose and H. B. Brewer, *Analyt. Biochem.*, 1976, **71**, 42.
[428a] K. D. Kulbe and Y. M. Nogueira-Hattesohl, *Analyt. Biochem.*, 1976, **72**, 123.
[428b] M. R. Downing and K. G. Mann, *Analyt. Biochem.*, 1976, **74**, 298.
[429] C. L. Zimmerman, E. Appella, and J. J. Pisano, *Analyt. Biochem.*, 1976, **75**, 77.
[430] J. A. Rodkey and C. D. Bennet, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 1407.
[431] C. Bollet and M. Claude, *J. Chromatog.*, 1976, **121**, 323.
[432] G. D. Lominac and H. S. Kingdon, *Arch. Biochem. Biophys.*, 1976, **173**, 320.
[433] H. R. Schulten and B. Wittmann-Liebold, *Analyt. Biochem.*, 1976, **76**, 300.

Sequencing with ^{35}S-*PITC.* In the first approach, Bridgen[434] has developed a solid-phase procedure in which micro amounts of protein covalently attached to glass beads can be sequenced by the use of ^{35}S-PITC. The principle of his method is that an initial ^{35}S-PITC 'pulse' with reagent of high specific radioactivity ensures high levels of radioactive incorporation into the phenylthiocarbamyl (PTC) peptide during the first coupling step. The second 'cold' PITC step which employs a large molar excess of unlabelled reagent ensures that the chemical efficiency of coupling is not sacrificed. Subsequent washing and acid cleavage steps proceed in the conventional manner. The released thiazolinones are converted into PTH amino-acids, mixed with unlabelled carrier PTH amino-acids and separated by t.l.c. on polyamide plates. Identifications are made by autoradiography of the developed t.l.c. plates after overnight exposure to *X*-ray film. The general principle of the method resembles that of a previously published procedure developed for the spinning cup sequencer; however, in terms of the published amounts sequenced, the solid-phase method is more sensitive by two orders of magnitude. Bridgen reports that 70 pmol of lysozyme was adequate for the determination of 20 *N*-terminal residues.

The practical usefulness of this technique was demonstrated by the determination of the *N*-terminal sequences of mixed human histocompatibility antigens (HLA). These antigens have only recently become available in pure form owing mainly to their low abundance, which means that their preparation in chemically significant amounts entails expensive bulk culture facilities for growing vast quantities of cells. Bridgen *et al.*[435] separated a partially purified HLA preparation by SDS-polyacrylamide slab gel electrophoresis and localized the 45 000 dalton band of interest by staining guide strips with Coomassie Brilliant Blue (Figure 4). The gel strip containing this band was excised and extracted with buffer containing SDS. Extracted protein, without prior removal of detergent, was covalently coupled to DITC-activated glass (Figure 4). The glass–protein conjugate was washed and subjected to sequencing with ^{35}S-PITC. An *N*-terminal sequence through residue 16 was thereby obtained.

The factor(s) which will limit the sensitivity of this sequencing method are not clear. In the general case, however, sufficient protein must be available for its detection by stains and this amount is probably greater than is required for sequencing.

Intrinsic Labelling. Ballou *et al.*[436] have used an entirely different approach to the problem of microsequencing, that of incorporation of radioactive precursors into living cells synthesizing the protein of interest. The initial problem here was biological rather than chemical: although essential amino-acids present no difficulties in their *in vivo* incorporation into proteins, non-essentials such as alanine, glycine, and serine either do not incorporate efficiently or label other amino-acids by metabolic interconversion. One solution to the problem has been to label one or two residues at a time with essential radioactive amino-acids which do not undergo *in vivo* interconversion into other amino-acids. This

[434] J. Bridgen, *Biochemistry*, 1976, **15**, 3600.
[435] J. Bridgen, D. Snary, M. Crumpton, C. Barnstable, P. Goodfellow, and W. F. Bodmer, *Nature*, 1976, **261**, 200.
[436] B. Ballou, D. J. McKean, E. F. Freedlender, and O. Smithies, *Proc. Nat. Acad. Sci.*, 1976, **73**, 4487.

approach has been used successfully in sequence studies on mouse H-2 antigens (see Histocompatibility Antigen Section). Sequence obtained, however, contain gaps presumably occupied by residues difficult to label discretely (see Figure 15, p. 106).

The major innovation of Ballou *et al.*[436] was to develop a radioactive medium for cell culture which uniformly labelled every residue in the cellular protein of

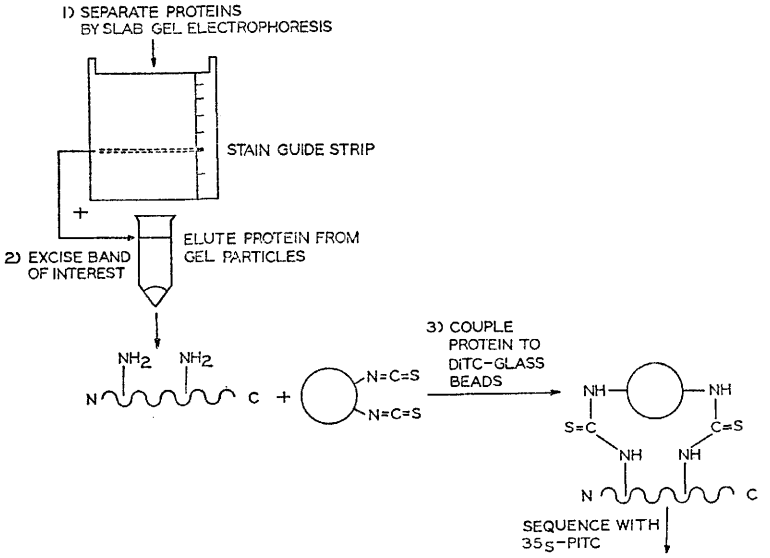


Figure 4 *High sensitivity solid-phase sequencing*

interest. Here, too, the subject of the sequence investigation was human HLA. Human lymphoblastoid cells were incubated in media containing ^{14}C-amino-acids and ^{14}C-pyruvate (which labels alanine, the acidic amino-acids, and their amides). Cells, thus labelled, were lysed by treatment with non-ionic detergent. A solid-phase adsorbent, consisting of antibodies to human β_2-microglobulin coupled to agarose beads, was used to perform a single-step purification of HLA which, in its native state, is non-covalently bound to β_2-microglobulin. Separation of HLA from β_2-microglobulin was accomplished by SDS-gel electrophoresis, and the 45 000 dalton HLA chain was subjected to automatic sequencing. Assignments were made by amino-acid analysis of the hydrolysed thiazolinone extracts of each cycle and by t.l.c. of PTH amino-acids. The *N*-terminal 27 residues of HLA were deduced by this method.

It is impossible on the basis of published work to compare the relative sensitivities of the two microsequencing strategies. Neither Bridgen *et al.* nor Ballou *et al.* attempted to measure the amount of protein they subjected to sequencing. However, in the ^{35}S-PITC application tens of litres of cell culture were used to prepare the material sequenced whereas the intrinsic labelling technique required only 40 ml of cell culture. It is therefore probable that in this case the intrinsic labelling method is more sensitive. The other major advantage of intrinsic

labelling is that isolation of suitable peptide fragments for further sequence studies should be greatly facilitated owing to their ease of detection by non-destructive radioactive techniques.

Sequencing with [^{3}H]*Dansyl Chloride.* The use of [^{3}H]dansyl chloride for sequencing short peptides has allowed the sequence of ameletin, a learning-induced peptide from rat brain, to be determined.[437a] Its amino-acid composition was determined by ^{3}H-dansylation of a hydrolysate followed by measurement of radioactivity in dansyl amino-acids separated by t.l.c. Sequence determination was accomplished by subjecting the peptide to carboxypeptidase and amino-peptidase digestion; time samples from each digest were analysed by ^{3}H-dansylation and the peptide sequence was deduced by the rate at which different residues were released. The exact amount of ameletin required for the entire operation was not stated but was in the range 10—100 pmol.

C-Terminal Sequencing.—The chemical problems associated with sequential degradation of polypeptides from the *C*-terminus have so far seriously limited its use in protein sequence analysis. A recent method [437b] in which polypeptides are coupled to solid supports by *N*-terminal amino-groups and sequenced by the ammonium thiocyanate method of Stark may extend the usefulness of *C*-terminal sequencing. Synthetic polymers are used to attach small peptides and larger polypeptides are attached to hydroxysuccinimide–glass beads; six *C*-terminal residues were successfully identified after *C*-terminal-degradation both of a model peptide and of ribonuclease, each coupled to solid supports. The authors point out the possibility of using commercially available solid-phase sequencers for *C*-terminal sequencing.

6 Primary Structures

Table 2 presents a summary of the amino-acid sequence studies reported during 1976.

Enzymes.—*Selenocysteine.* A new amino-acid has recently been discovered in *Clostridium sticklandii* glycine reductase.[438] It is selenocysteine and the sulphur atom of only one specific cysteine residue of many in the protein is substituted by selenium.

Table 2 *Amino-acid sequences reported during* 1976

Protein	*Source*	*Comments*	*Ref.*
Enzymes			
Adenylate kinase	Human	Complete sequence, 195 residues. Sequenced mainly by automatic spinning cup and solid-phase techniques	439
Alcohol dehydrogenase	*Drosophilia melanogaster*	121 residues reported, about 50% of complete sequence	440

[437] (*a*) S. R. Burzynski, *Analyt. Biochem.*, 1976, **70**, 359; (*b*) M. Rangarajan and A. Dabre, *Biochem. J.*, 1976, **157**, 307.
[438] J. E. Cone, R. M. Del Rio, J. N. Davis, and T. C. Stadtman, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2659.
[439] I. Von Zabern, B. Wittmann-Liebold, R. Untucht-Grau, R. H. Schrimer, and E. F. Pai, *European J. Biochem.*, 1976, **68**, 281.
[440] M. F. Schwartz and H. Jörnvall, *European J. Biochem.*, 1976, **68**, 159.

Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Alkaline phosphatase	Human	*N*-Terminal sequences of liver (3 residues) and placental (4 residues) isozymes	441
Anthranilate-5-phosphoribosyl transferase	*Serratia marcescens*	*N*-Terminal 38 residues	442
Anthranilate synthetase	*Pseudomonas putida*	*N*-Terminal 45 residues	443
Aspartate amino-transferase	Porcine heart	*C*-Terminal 10 residues of mitochondrial isozyme	444
ATPase (Na^+, K^+-activated)	*Anas platyrhynchos*	*N*-Terminal 13 residues of 94 000 dalton chain	445
ATPase (Ca^{2+} transporting)	Rabbit sarcoplasmic reticulum	25 residues of phosphorylated active centre peptide	446
Bromelain	Pineapple stem	Partial sequences of 3 CnBr peptides, 118 residues	447
Carbonic anhydrase	Human	Complete sequence, 259 residues: 3 discrepancies with earlier report	448
Chorismate mutase/ prephenate dehydrogenase	*E. coli*	Sequence of cysteine-containing peptides, 47 residues in all. Isolation of 4 unique cysteine peptides establishes that a single chain carries both enzyme activities	449
Cyclic nucleotide phosphodiesterase, Ca^{2+}-dependent regulatory subunit	Bovine brain	Partial sequence (57 residues) from tryptic peptides: evidence of sequence homology to Ca^{2+}-binding component of troponin (TN-C)	450
Citrate lyase	*Klebsiella pneumoniae*	*N*-Terminal sequences on 3 non-identical subunits (10 residues from each)	451
Elastase	Human leucocytes	*N*-Terminal 7 residues	452
Peroxidase	Horseradish	Complete sequence, 308 residues	453

441 K. S. Badger and H. H. Sussman, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2201.
442 M. Largen, S. E. Mills, J. Rowe, and C. Yanofsky, *European J. Biochem.*, 1976, **67**, 31.
443 Y. Goto, H. Zalkin, P. S. Keim, and R. L. Heinrikson, *J. Biol. Chem.*, 1976, **251**, 941.
444 D. Barra, F. Bossa, S. Doonan, H. M. A. Fahmy, F. Martini, and G. J. Hughes, *European J. Biochem.*, 1976, **64**, 519.
445 B. E. Hopkins, H. Wagner, jun., and T. W. Smith, *J. Biol. Chem.*, 1976, **251**, 4365.
446 G. Allen and N. M. Green, *F.E.B.S. Letters*, 1976, **63**, 188.
447 K. Goto, T. Murachi, and N. Takahashi, *F.E.B.S. Letters*, 1976, **62**, 93.
448 L. E. Henderson, D. Henriksson, and P. O. Nyman, *J. Biol. Chem.*, 1976, **251**, 5457.
449 M. J. H. Gething and B. E. Davidson, *European J. Biochem.*, 1976, **71**, 327.
450 D. M. Watterson, W. G. Harrelson, P. M. Keller, F. Sharief, and T. C. Vanaman, *J. Biol. Chem.*, 1976, **251**, 4501.
451 M. Singh, P. A. Srere, D. G. Klapper, and J. D. Capra, *J. Biol. Chem.*, 1976, **251**, 2911.
452 R. J. Baugh and J. Travis, *Biochemistry*, 1976, **15**, 836.
453 K. G. Welinder, *F.E.B.S. Letters*, 1976, **72**, 19.

Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
2-Keto-3-deoxy-6-phosphogluconic acid aldolase	*Pseudomonas putida*	^{14}C pyruvate reductively bound to active site with cyanoborohydride. Radioactive peptide sequenced, 50 residues	454
Phosphofructokinase	Rabbit muscle	104 residues sequenced in cysteine-containing peptides. No evidence for internal sequence repetition found	455
Phosphoglycerate mutase	*Saccharomyces cerivisiae*	Sequence of two histidine-containing peptides, 11 and 13 residues	456
Protease	*Streptococcus*	Complete amino-acid sequence, 253 residues. Some similarities to papain	457–459
Protocatechuate 3,4-dioxygenase	*Pseudomonas aeruginosa*	*N*-Terminal 14 residues of two non-identical subunits	460
Ribonuclease (extracellular)	*Streptomyces erythreus*	Complete sequence, 102 residues	461
Ribonuclease (pancreatic)		Complete sequences of RNases from *Myocastor coypus* (128 residues), *Chinchilla brevicaudata* (124 residues), and *Damaliscus korrigum* (124 residues)	472 473
D-Serine dehydratase	*E. coli*	*N*-Terminal sequence, 23 residues. Active centre phosphopeptide, 14 residues	462
Serine transhydroxymethylase	Rabbit liver	Active centre, pyridoxyl phosphate binding peptide, 9 residues	463
Sucrase–isomaltase complex	Rabbit intestine	Sucrose-active site labelled with ^{3}H-conduvitol-B-epoxide. 13 residue peptic peptide sequence determined	464

[454] D. D. Tsay and W. A. Wood, *Arch. Biochem. Biophys.*, 1976, **177**, 144.
[455] I. D. Walker, J. I. Harris, M. J. Runswick, and P. Hudson, *European J. Biochem.*, 1976, **68**, 255.
[456] L. A. Fothergill and G. I. Hodgson, *Biochem. J.*, 1976, **153**, 145.
[457] A. A. Kortt, J. R. Wysocki, and T.-Y. Liu, *J. Biol. Chem.*, 1976, **251**, 1941.
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[459] J. Y. Tai, A. A. Kortt, T.-Y. Liu, and S. D. Elliott, *J. Biol. Chem.*, 1976, **251**, 1955.
[460] R. Yoshida, K. Hori, M. Fujiwara, Y. Saeki, H. Kagamiyama, and M. Nozaki, *Biochemistry*, 1976, **15**, 4048.
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[462] E. Schiltz and K. D. Schnackerz, *European J. Biochem.*, 1976, **71**, 109.
[463] F. Bossa, D. Barra, F. Martini, L. V. Schirch, and P. Fasella, *European J. Biochem.*, 1976, **70**, 397.
[464] H. Quaroni and G. Semenza, *J. Biol. Chem.*, 1976, **251**, 3250.

Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Thymidilate synthetase	*Lactobacillus casei*	Active-centre peptide, 9 residues	465, 466
tRNA synthetases			
Methionyl	*Bacillus stearothermophilus*	*N*-Terminal sequence; 54 residues	467
Tryptophanyl	*E. coli*	9 residues in cysteine-containing peptides	468
Tryptophanyl	Human	23 residues in 2 cysteine-containing peptides: homology with *E. coli* found	469
Trypsinogen	Human	Activation peptide sequence of trypsinogen 2, 8 residues	470
UDP galactosyltransferase	Bovine	Active-centre sequence, 3 residues. Lysine identified as UDP-binding residue	471
Antibodies			
IgA	Human	Complete sequences of heavy (α1) and light (λ) chains, 472 and 213 residues, respectively. Disulphide bonds and carbohydrates assigned	481
Anti-*p*-azobenzoate L-chain	Rabbit	*N*-Terminal sequence; 102 residues	482
Anti-*Micrococcus lysodiekticus* H-chain	Rabbit	Residues 10—49 determined	483

[465] R. L. Bellisario, G. F. Maley, J. H. Galivan, and F. Maley, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1848.
[466] A. L. Pogolotti, jun., K. M. Ivanetich, H. Sommer, and D. V. Santi, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 972.
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[478] J. C. Jaton, *Biochem. J.*, 1976, **157**, 449.
[479] C. Sledge, D. S. Fair, B. Black, R. G. Krueger, and L. Hood, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 923.
[480] J. E. Hopper, C. Noyes, R. Heinrikson, and J. W. Kessel, *J. Immunol.*, 1976, **116**, 743.
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[482] B. Friedenson, J. Hora, and L. S. Wang, *Biochemistry*, 1976, **15**, 4876.
[483] M. Van Hoegaerden and A. D. Strosberg, *F.E.B.S. Letters*, 1976, **66**, 35.

Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
HCD protein, CHI	Human	*N*-Terminal 60 residues	484
J-Chain	Human	*N*-Terminal 17 residues. Disulphide bridge to H-chain located at residue 15 in J-chain	485
Anti-streptococcal antibodies	Mouse	*N*-terminal 30 residues on 3H chains; *N*-terminal 11 residues on 2 L chains	486
Phosphorylcholine binding H chains	Mouse	*N*-Terminal 116 residues on H chains from M167 and TEPC 15 myelomas	487
Anti-streptococcal L chain	Rabbit	*N*-Terminal sequence, 109 residues. Contains sequence differences from other b4 allotypes	488
L-chain	Rabbit	C_κ region, 103 residues, of b9 allotype sequenced. 33% divergence between b9 and b4 allotypes	489
HCD protein, 2UC	Human	Heavy chain disease protein. Complete sequence, 212 residues. Contains entire Fc region	490
HCD protein, YOK	Human	*N*-Terminal 22 residues; *C*-terminal 17 residues. Belongs to λ_1 subclass	491
H-chain	Guinea-pig	115 residues from $C\gamma 3$ domain	492
Secretory component	Bovine	*N*-Terminal 13 residues; identical with human and dog counterparts	493
Hormones			
α-Factor	*Saccharomyces cerevisiae*	Complete sequence, 13 residues	498, 499
Adipicokinetic hormone	*Locusta*	Complete sequence, 10 residues; homologous with pigment concentrating hormone from shrimp	500

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Follicle-stimulating hormone	Human	Complete sequence of β-subunit, 118 residues	501
Nerve growth factor	*Naja naja* venom	106/116 residues sequenced; 60% homologous with mouse NGF	502
Neurophysins	Bovine	Corrections made to previous neurophysin II sequence. 9 errors claimed	503
	Bovine	Foetal MSEL neurophysin identical with adult counterpart	504
	Porcine	Complete sequence, 95 residues. Only 2 differences between porcine and ovine or bovine	505, 506
Relaxin, A-chain	Porcine	Complete sequence, 22 residues	507
Somatostatin	Porcine	Complete sequence, 14 residues	508
Thyrotropin	Porcine	Complete sequences of α chain (96 residues) and β chain (112 residues)	509
Vespulakinin	*Vespula maculifrons* venom	Complete sequence, 17 residues: consists of normal bradykinin sequence preceded by octapeptide	510
Muscle proteins			
Myosin light chain	Rabbit muscle	Complete DTNB light chain sequence, 148 residues	513
Myosin light chain	Rabbit and cat muscle	Cysteine peptides of two light chains from both species sequenced	514
Troponin C	Chicken skeletal muscle	Complete sequence, 162 residues, most conserved part is Ca^{2+}-binding sequence	515

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Troponin C	Bovine heart	Complete sequence, 161 residues	516
Troponin I	Rabbit heart	Complete sequence, 206 residues; contains 26 residues more than skeletal muscle form	517
Parvalbumin	*Esox lucius*	Complete sequence, 107 residues	518
Parvalbumin	Rabbit muscle	Complete sequence, 109 residues; identical with previous reported sequence	519
Histones			
H1	*Parechinus Angulosies*	Partial sequence, 73 residues sequenced	520
F2A2	Rat chloro-leukaemia	Complete sequence, 129 residues. 3 genetic variants differing at positions 16 and 99	521
H2B	*Salmo trutta* testes	Complete sequence, 123 residues	522
H4	*Psammechinus miliaris*	Complete sequence, 102 residues	523
H5		*N*-Terminal sequences of red cell histone from *Gallus gallus domesticus* (16), *Carinia moscheta* (16), *Anser anser* (16), *Columbia livia* (16), and *Coturinx japonica* (19)	524
H5	*Gallus gallus domesticus*	Partial sequence, 41 residues; known sequence extends to residue 111	525

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Blood-clotting proteins			
Fibrinogen α-chain	Human	*C*-Terminal sequence, 27 residues, very little similarity to *C*-terminus of β and γ chains	535
Fibrinopeptides	*Petromyzon marinus*	A-peptide 6 residues; B-peptide 36 residues	536
Prothrombrin	Rat	*N*-Terminal sequence, 9 residues	537
Factor X-activating enzyme	*Vipera russelli*	*N*-Terminal sequence, 3 residues from H chain	538
Globins			
Haemoglobin:			
γ-chain	Finn-Marino lamb	Complete sequence, 145 residues	542
α- and β-chains	*Salmo irideus*	Partial sequence; 70% of both chains	543
Abnormal haemoglobin:			
Providence	Human	β82, Lys → Asn	544
Caribbean	Human	β91, Leu → Arg	545
Survesness	Human	α141, Arg → His	546
Chapel Hill	Human	α74, Asp → Gly	547
Tübingen	Human	β106, Leu → Glu	548
Altdorf	Human	β135, Ala → Pro	549
Strasbourg	Human	β20, Val → Asp	550
Henri Mondor	Human	β26, Glu → Val	551

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Dakar	Human	α-Chain with internal insertion of Glu-Phe-Thr sequence. Identical to haemoglobin Brady	552
Fannin–Lubbock	Human	β119, Gly → Asp	553
Wayne	Human	α-Chain with frameshift in *C*-terminal. Defect due to single base deletion in DNA	554
	Human		
Myoglobin	*Otocyon megalotis*	Complete sequence, 153 residues; same as dog sequence	555
	Tursiops truncatus	Complete sequence, 152 residues	556
	Eschrichtius gibbosus	Complete sequence, 153 residues	557
Viral proteins			
Gene F protein	φX174	Partial sequence; *ca.* 45% of residues accounted for	559
Gene G protein	φX174	Complete sequence, 175 residues; determined by protein and DNA sequencing	560
P23 and IP III	T4	Sequences corresponding to sites of proteolytic cleavage of precursors → mature forms. Specific Glu-Ala bonds cleaved in both cases	561
IP II	T4	Partial sequence, 106 residues	562
Capsid polypeptides	Mengo virus	*N*-Terminal sequences of α, β, and γ polypeptides, 10 residues in each case	563
Structural proteins II and IV	PM2	*N*-Terminal sequences, 39 sequences (II) and 27 residues (IV)	564

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Coat protein	Alfalfa mosaic virus (Strain 425)	Complete sequence, 220 residues	565
Coat protein	Alfalfa mosaic virus (Strain S)	Complete sequence, 217 residues; 11 substitutions and 3 deletions when compared to Strain 425 counterpart	566
Toxins			
Toxin B	*Naja naja* venom	Complete sequence, 71 residues	567
Non-curarimimetic toxins	*Naja nivea* venom	Complete sequences of toxins v″1, v″2, and v″3, all 60 residues	568
Cardiotoxin-analogue II	*Naja naja atra* venom	Complete sequence, 60 residues	569
Cardiotoxin-analogue IV	*Naja naja atra* venom	Complete sequence, 60 residues	570
Cardiotoxin	*Naja naja atra*	Complete sequence, 60 residues. 2 differences from previous sequence	571
Neurotoxins	*Aipysurus laevis* venom	Complete sequences of 3 toxins, a, b, and c: all 60 residues, sequences differ only at positions 25 and 28	572
Neurotoxins	*Dentroaspis viridis*	Complete sequences of 2 toxins, 72 and 73 residues. One longer than the other by extra *C*-terminal Gly	573
Neurotoxic phospholipase A	*Notechis scutatus scutatus*	Complete sequence, 119 residues	574
Toxins CM-8, CM-11, and CM13-a	*Naja haje annulifera*	Complete sequences, 60, 60, and 61 residues	575
Toxin II	*Anemonia sulcata*	Complete sequence, 47 residues	576
Taipoxin	*Oxyuranus scutellatus scutellatus*	*N*-Terminal sequences of α, β, and γ chains; 11, 11, and 19 residues, respectively	577

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Choleratoxin	*Vibrio cholerae*	Complete sequence, 103 residues	578
Diphtheria toxin, Fragment A		Complete sequence, 193 residues. 3 forms due to partial proteolysis at *C*-terminus	579
Electron-transport proteins			
Cytochrome *b5*	Horse	*N*-Terminal sequence, 90 residues	580
Cytochrome *c*	*Eisenia foetida*	Complete sequence, 108 residues	581
Cytochrome *c*	*Asteria rubens L.*	Complete sequence, 103 residues	582
Cytochrome *c*	*Tetrahymena pyriformis*	Complete sequence, 109 residues	583
Cytochrome *c2*	*Rhodomicrobium vannielii*	Complete sequence, 104 residues	584
	Rhodopseudomonas viridis	Complete sequence, 108 residues	584
Cytochrome *c*550	*Paracoccus denitrificans*	Complete sequence of tryptic peptides, overlaps provided by electron-density map	585
Cytochrome *c*555	*Chlorobium limicola*	Complete sequence, 86 residues	586
	Chloropseudomonas ethylica	Complete sequence, 99 residues	586
Ferredoxin	*Bacillus stearo-thermophillus*	Complete sequence, 81 residues	587
Ferredoxin	*Porphyra umbilicalis*	*N*-Terminal sequence, 40 residues	588
Ferredoxin	*Spirulina platensis*	Complete sequence, 98 residues. Identical with previous sequence except Asp-9 reported as Asn	589
Plastocyanin	*Stellaria media* L.	*N*-Terminal sequence, 40 residues	590

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Rubredoxin	*Desulphovibrio gigas*	Complete sequence, 52 residues	591
High potential iron–sulphur protein	*Rhodopseudomonas gelatinosa*	Complete sequence, 74 amino-acids	592
Plastocyanin	*Plectonema boryanum*	*N*-Terminal sequence, 38 residues	593
Cytochrome *f*	*Anabaena variabilis*	Partial sequence, 61 residues	593
	Plectonema boryanum	Partial sequence, 85 residues	593
	Synechococcus 6312	Partial sequence, 37 residues	593
Structural proteins			
Collagen (Type III)	Bovine foetal	*N*-Terminal sequence, 23 residues	597
Collagen (Type II)	Bovine	*N*-Terminal sequence, 162 residues	598
Collagen	Human	Sequences *C*-terminal to collagenase cleavage sites of α1 (II) and α1 (III) chains, 18 residues in each case	599
α-Crystallin		Complete sequences of *Macropus rufus* and *Didephis marsupialis*, 173 residues in both cases	600
Elastin	Bovine	*N*-Terminal sequence of a purified hydrophobic peptide, 17 residues: contained 3 tandem pentapeptide repeats	601
Ribosomal proteins			
S8	*E. coli*	Complete sequence, 109 residues	602
S13	*E. coli*	Complete sequence, 117 residues	603
S15	*E. coli*	Complete sequence, 87 residues	604

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
S20	*E. coli*	Complete sequence, 86 residues	605
L5	*E. coli*	Complete sequence, 178 residues	606
L10	*E. coli*	Complete sequence, 164 residues	607
L16	*E. coli*	Complete sequence, 136 residues	608
L33	*E. coli*	Complete sequence, 54 residues	609
L34	*E. coli*	Complete sequence, 46 residues	610

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Miscellaneous			
Apolipoprotein A-II	*Macaca mulatta*	Complete sequence, 77 residues	639
Apolipoprotein A-I	*Erythrocebus patas*	*N*-Terminal sequence, 20 residues	640
Apolipoprotein A-I	Canine	*N*-Terminal sequence, 34 residues; *C*-terminal, 3 residues	641
α-1-Antitrypsin	Human	*N*-Terminal sequence, 7 residues	642
Amyloid protein	Human	Complete sequence, 64 residues; shorter by 12 residues than previous sequence, probably owing to proteolysis	643
Amyloid protein	Mouse	*N*-Terminal sequence, 28 residues; 75% homologous to human and monkey counterparts	644
Alamethicin		N.m.r. study identified acetylmethylalanine as blocked *N*-terminal residue. Intra-chain cross-link identified as γ-glutamyl phenylalaninol	645
β_2-Microglobulin	Mouse	*N*-Terminal sequence, 40 residues	646
β_2-Microglobulin co-isolated protein	Rabbit urine	*N*-Terminal sequence, 26 residues; no homology with β_2 microglobulin	647
Casein	Bovine	Partial sequence, 108 residues; different casein variants had same sequence – suggest differences due to partial phosphorylation	648

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Caseinomacropeptides		Casein peptides released by chymosin from pig and human milk. Pig peptide completely sequenced, 71 residues; 59/65 residues from human peptide sequenced	649
Cholera enterotoxin		*N*-Terminal sequences of A1, A2, and B chains, 20 residues in all cases	650
Chymotrypsin inhibitor	Potato	*N*-Terminal sequence 18 residues	651
Chymotrypsin inhibitor	*Cicer Arietinum* L.	*N*-Terminal sequence, 25 residues	652
Flagellin	*Bacillus subtilis*	Complete sequence, 168 residues	653—655
Haptoglobin β chain		Partial sequence, 139 residues	656
Kininogen	Bovine	Sequence (69 residues) of fragment 1, a glycopeptide released by kallikrein	657
α-Lactalbumin	*Bubalus arnee*	*N*-Terminal sequence, 17 residues	658
Lactotransferrin	Human	*N*-Terminal sequence, 24 residues; homologous to serum transferrin and ovotransferrin	659
Lectin	*Pisum sativum*	*N*-Terminal sequences of α and β chains, 38 and 26 residues, respectively: α chain homologous to concanavalin A	660
Metallothionein 1B	Equine kidney	Complete sequence, 61 residues. The 21 cysteine residues arranged in Cys-Cys, Cys-X-Cys, or Cys-X-X-Cys sequences	661

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Table 2 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Monellin	*Dioscoreophyllum cominsii*	Complete sequence, 44 residues; determined by mass spectrometry	662
Myohemerythrin	*Themiste pyroides*	Complete sequence, 118 residues	663
Serum albumin	Human	Sequence of 90 residue fragment, agrees with previously published sequence	664
Serum albumin	Human	Acetyl salicylate binding peptide sequenced, 8 residues	666
T.C. (complex-forming) glycoprotein	Human urine	*N*-Terminal sequence, 25 residues	667
Thyrocalcitonin	Rat	Complete sequence, 32 residues	668
Transferrin	Sheep	*N*-Terminal sequence, 8 residues	669
Trypsin-plasmin inhibitor	Leech	Complete sequence, 46 residues	670

Antibodies.—*Sequence and Binding Specificity.* Much effort has been directed toward defining the nature of specific antibody combining sites. One approach has been to compare the sequence of different antibodies with binding specificity for the same antigen. Both myeloma proteins, which happen fortuitously to have suitable hapten-binding properties, and antibodies deliberately raised in animals hyperimmunized with specific antigens, have been studied.

The variable region sequences of both heavy and light chains of FR, a human IgM myeloma antibody, with phosphorylcholine binding activity have recently been reported.[474, 475] The FR light chains are less similar to mouse phosphorylcholine binding myeloma light chains than they are to human light chains of unrelated specificity.[474] In particular, the first light chain hypervariable region in mouse and human phosphorylcholine-binding antibodies contained no obvious sequence similarities which correlated with their shared binding specificity. Similarly the analysis of light chains from rabbit antibodies elicited in response to specific antigens has revealed no obvious correlation between sequence and binding specificity. Comparison of the *N*-terminal 20 residues of 22 rabbit light chains revealed that although some were indeed identical in sequence they were not necessarily of the same antigen-binding specificity.[476] Light chain sequences of different antibodies produced in a single rabbit in response to pneumococcal vaccine have recently been reported.[477] The *N*-terminal

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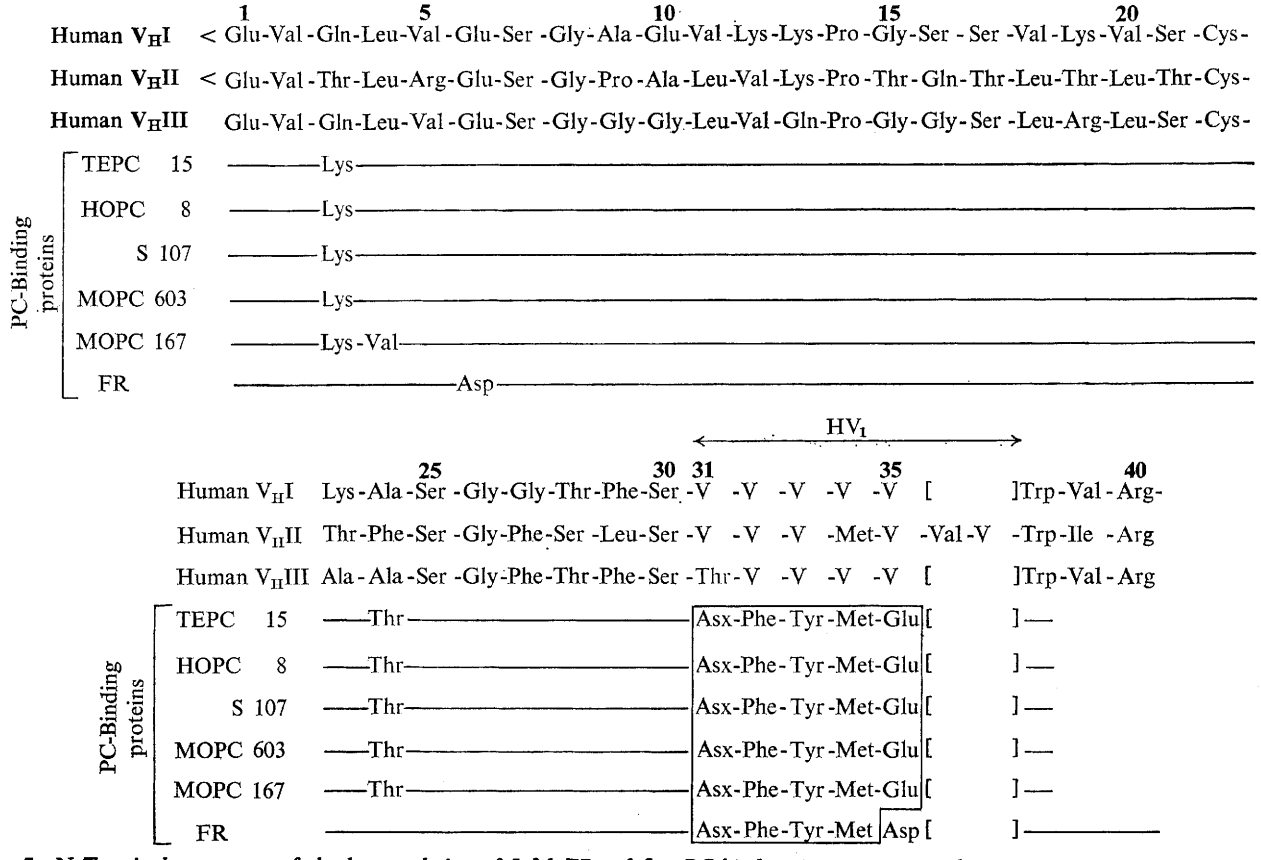

Figure 5 N-*Terminal sequences of the heavy chains of IgM FR, of five PC-binding IgA mouse myeloma proteins, and of the human* V_H *subgroups I, II, and III. Pyrrolidone carboxylic acid is symbolized by* <Glu. *A solid line represents identity with the* V_H *III subgroup. Square brackets indicate deletions.* V *indicates variable residue positions.* HV_1 *marks the extent of the first hypervariable region. Identical resin residues within hypervariable region are framed*

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40 residues of light chains from four different antibodies exhibited many sequence differences both in framework and hypervariable regions. In contrast, very extensive sequence homology has been reported [475] between the heavy chain of human FR myeloma protein and mouse phosphorylcholine-binding counterparts (Figure 5). Four out of five residues constituting the first hypervariable region of mouse and human heavy chains were conserved. This region in mouse phosphorylcholine-binding antibodies is known to interact directly with hapten, whereas the role of light chains in this interaction seems to be less important. The similarities between FR and mouse heavy chains further emphasize the importance of the first heavy chain hypervariable region in antigen binding and support the contention that specific variable region genes may be genetically stable and evolutionarily conservative. However, sequence studies on rabbit heavy chains from anti-pneumococcal antibodies do not support a simple correlation between variable region sequence and binding specificity.[478] The heavy chains from three independently isolated anti-pneumococcal antibodies exhibited a much lower degree of homology in hypervariable regions than that found between mouse and human myeloma phosphorylcholine-binding heavy chains.

Biclonal Antibodies. In certain human malignancies a single type of lymphocyte can undergo neoplastic proliferation; in most cases the clonal descendants of such cells produce antibodies containing a single homogeneous light chain and a single heavy chain. In rare diseases more complicated situations arise. Fudenberg *et al.* (*Ann. New York Acad. Sci.*, 1971, **19**, 501) reported the simultaneous occurrence of two antibodies, both from the same neoplastic cells, in a patient with Waldenström's macroglobulinaemia. The light chains of both antibodies were shown to be identical and the heavy chains, although of different classes, were shown to be identical throughout their respective variable regions. Another example of this type has more recently been reported by Sledge *et al.*;[479] the *N*-terminal sequences of the light chains of both antibodies were identical through residue 40. The heavy chains, although of γ and μ classes, respectively, were also identical through 45 *N*-terminal residues. The association of different constant regions with the same variable region in biclonal antibodies was originally interpreted in favour of different genes for constant and variable regions, fusion of which is required to generate a complete heavy chain or light chain gene. Sledge *et al.*[479] have proposed that tumours in which two antibodies are produced by the same cells arise by neoplastic transformation of normal lymphocytes in which two identical variable region genes are fused to different constant region genes. The alternative model, in which a single variable region gene is translocated from one constant region gene to another during lymphocyte development, is less attractive to the authors since it cannot so readily explain the sustained synthesis of both types of antibody by the same neoplastic clone. A still more complicated situation has been shown to occur in another patient with Waldenström's macroglobulinaemia. Here, two entirely different antibodies were shown to be produced by neoplastic cells.[480] The *N*-terminal sequences of γ and μ chains from this tumour were shown to be of different variable region subgroups and differed extensively in framework regions. The light chains also seemed to differ since the N-blockage of one light chain suggested

that it was a λ chain and the *N*-terminal sequence of the other indicated a κ chain. The unexpected feature was that both antibodies possessed very similar idiotypes and this is often a good indicator of subgroup identity.

Hormones.—*β-Lipotropin.* Recent sequence studies [494] have revealed that β-lipotropin, a 91-residue polypeptide from pituitary glands, is the precursor of

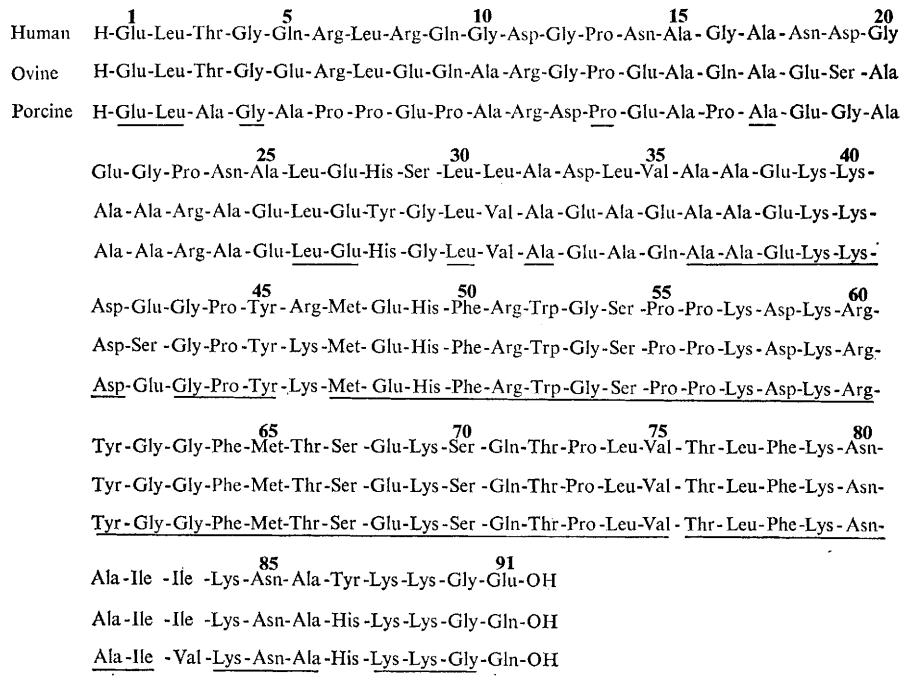

Figure 6 *Comparison of the amino-acid sequence of β_h-LPH with that of ovine and porcine hormone*
(Reproduced by permission from *Nature*, 1976, **260**, 623)

a number of peptide hormones. The sequence of human β-lipotropin has recently been determined and shows high sequence conservation when compared to porcine and ovine counterparts (Figure 6). Porcine met-enkephalin and β-endorphin were previously shown to correspond to residues 61—65 and 61—91, respectively, of β-lipotropin, and more recently two other hormones, α- and γ-endorphins, have also been shown to fit into the β-lipotropin sequence. α-Endorphin is identical in sequence with residues 61—76 and γ-endorphin with 61—77.

Insulin-like Polypeptides. Human serum contains two polypeptides of mol. wt. 6000 daltons with insulin-like activity (NSILA I and II). They are less efficient in their insulin-like activity than insulin but considerably more potent than insulin in their growth-promoting properties on cultured cells. Their *N*-terminal sequences have been determined [496, 497] (Figure 7), and NSILA I can be seen to

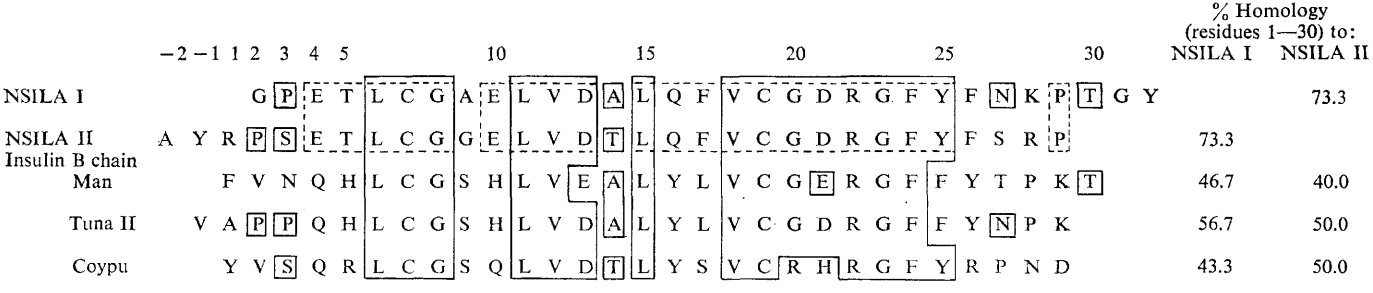

Figure 7 *Alignments of amino-acid sequences of NSILA I and II and insulin B chains from tuna fish, man, and coypu. One-letter symbols for amino-acid residues are as recommended. The numbering of residues of NSILA was chosen to correspond to conventional numbering of insulin B chains. Broken lines indicate residues identical in NSILA I and II. Solid lines indicate residues identical in NSILA and insulin B chains* (Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4379)

differ from NSILA II by only a few amino-acid interchanges and by the absence of three *N*-terminal residues present in NSILA II. Both are *ca.* 50% homologous to insulin B chains from various species, and it will be interesting to see if their *C*-terminal sequences are homologous to insulin A chain as would be expected from their similarities to proinsulin in molecular weight and *N*-terminal sequence.

Muscle Proteins.—*Regulatory Myosin Light Chains.* The effect of calcium ions in muscle contraction is mediated by two independent mechanisms. In

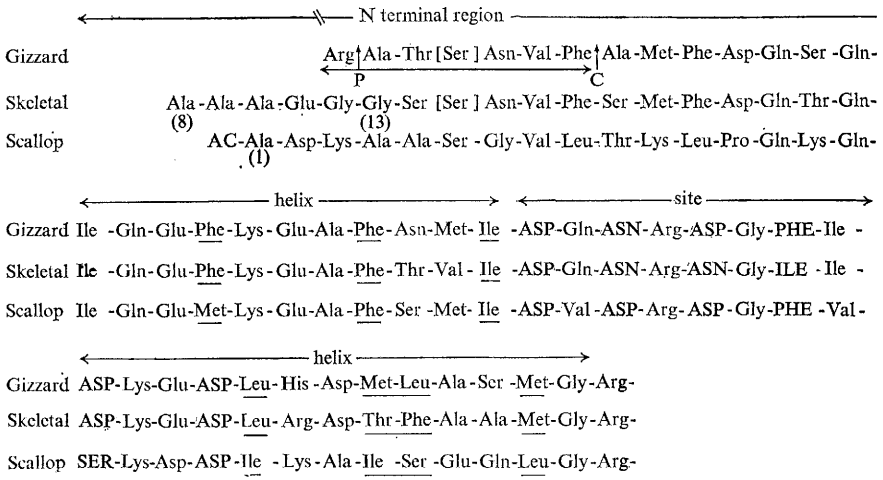

Figure 8 *Primary sequence of the gizzard 'regulatory' light chain containing the phosphoserine residue and calcium binding region, compared with the same region of the sequence of the rabbit and scallop regulatory light chains. With the exception of the N-terminal region, the sequences have been drawn out to correspond to the main structural features of carp parvalbumin. In the calcium binding site, the positions of the residues which, by analogy with parvalbumin, are believed to be involved in co-ordinating calcium are indicated by capital letters. In the 'helical' regions which surround each calcium binding site, the residues underlined correspond to the positions of hydrophobic amino-acids which are buried and form the core of parvalbumin. Hydrophobic residues are present in each of the light chain sequences in the required positions, which would further suggest that the three-dimensional structure of the light chains in this region is related to parvalbumin*

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vertebrate striated muscle calcium regulation is actin linked and occurs *via* calcium binding by the troponin complex. In molluscan muscle and vertebrate smooth muscle, calcium regulation is myosin linked. It has been demonstrated that calcium regulation of scallop muscle is mediated by a specific class of myosin light chains, the regulatory light chains. Selective removal of this chain from scallop myosin renders its ATPase activity insensitive to variations in calcium concentrations. Reconstitution of calcium regulation can be accomplished with myosin light chains either from scallop or from chick gizzard smooth muscle, but not with light chains from rabbit skeletal muscle. The

affinity with which each of the light chains binds calcium ions parallels its ability to restore calcium regulation to desensitized molluscan muscle.

Sequence studies have recently been carried out on the regulatory light chain of chick gizzard muscle (Figure 8).[511] When compared to corresponding regions of mollusc and rabbit skeletal muscle, the gizzard chain exhibited a high degree of sequence homology. The site thought to be responsible for calcium binding in all three cases was particularly well conserved. The rabbit skeletal chain,

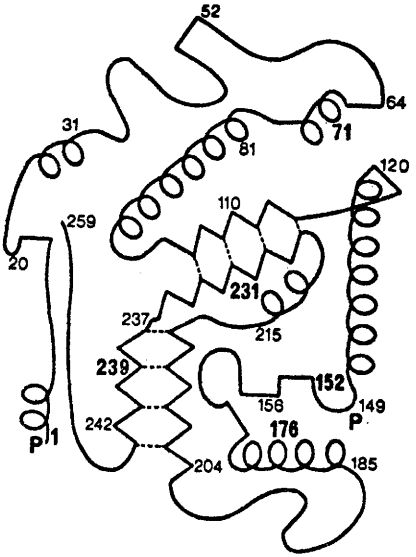

Figure 9 *Schematic representation of the secondary structure of troponin-T as predicted by the method of Chou and Fasman. The first residues of each cyanogen bromide fragment are shown in bold numbers*
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1904)

however, contained only three negatively charged residues as compared to four in the gizzard and scallop cases. These residues are believed to act as ligands which stabilize the binding of calcium. The authors suggest that the lack of one such ligand in the skeletal muscle chain may be sufficient to account for its reduced avidity for calcium ions relative to gizzard and scallop chains and for its inability to restore the calcium-binding properties to densensitized scallop myosin.

Troponin T. The complete amino-acid sequence of troponin T, the tropomyosin-binding component of troponin, has recently been reported.[512] Its secondary structure has been predicted using the Chou and Fasman method (Figure 9). A CNBr peptide of troponin T was previously known to bind to tropomyosin and the region corresponding to this peptide in the complete sequence (residues 71—151) has two interesting features; it contains two prominent α-helical sections one or both of which could interact with the two-stranded coiled-coil of tropomyosin. These α-helices contain many charged residues, as would be expected for segments located on the surface of the molecule.

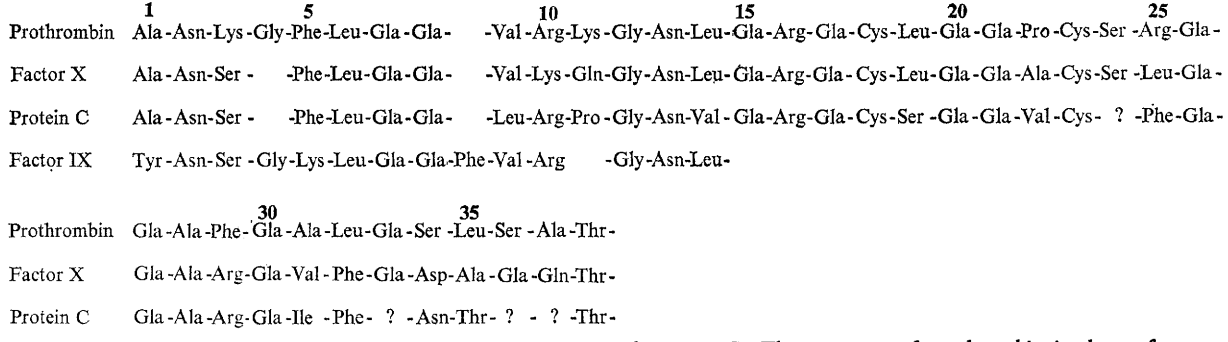

Figure 10 *γ-Carboxyglutamic acid residues (Gla) in factors IX, X, and protein C. The sequence of prothrombin is shown for comparison. The numbering is that of prothrombin*
(Reproduced by permission from *J. Biol. Chem.*, 1976, **251**, 355)

Blood-clotting Proteins.—*Factors VII and IX.* Sequence studies on the components of the blood-clotting cascade have continued to reveal striking homology among its different components and between blood-clotting enzymes and the serine proteases. Factor VII (53 000 daltons) has been shown [526] to be activated by factor Xa to a two-chain molecule, factor VIIa, consisting of heavy (29 500 daltons) and light (23 500 daltons) chains. Intact factor VII and VIIa light chain were shown to have the same *N*-terminal sequence, thereby establishing the order of the two fragments within the intact factor VII chain. The *N*-terminal sequence of heavy chain indicated that the site at which factor Xa cleaved factor VII was

↓
-Arg-Ile-Val-Gly-Gly-

Homology in sequence and activation mechanism between factor VII, prothrombin, and factor IX was strongly indicated by these studies. *N*-Terminal sequence studies on both bovine and human factor IX have established their similarities to the corresponding prothrombin and factor X sequences.[527]

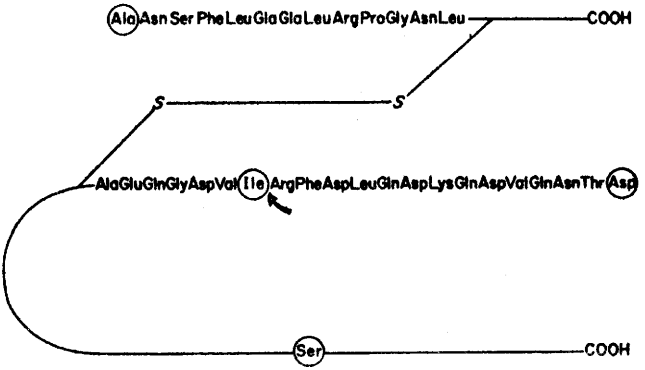

Figure 11 *Partial structure of bovine protein C. Arrow indicates the point of cleavage in the heavy chain of protein C by either RVV-X or trypsin*
(Reproduced by permission from *Biochemistry*, 1976, **15**, 4893)

Protein C. A new protein has been isolated from bovine plasma.[528] Its origin and function are not yet known, but its amino-acid sequence strongly suggests a function related to that of the vitamin K-dependent blood-clotting enzymes.[529, 530] Protein C is a two-chain molecule with 44 000 and 21 000 dalton subunits. The *N*-terminal sequence of the smaller subunit is clearly homologous with those of key blood-clotting proteins (Figure 10). Nine residues of γ-carboxyglutamic acid were shown to occur in protein C light chain at the same positions as in factor X and prothrombin, suggesting the involvement of vitamin K in its postsynthetic carboxylation. Protein C was shown to be a protease zymogen by its activation by either trypsin or a snake venom activator of factor X. The *N*-terminal sequences of intact and activated heavy chains (Figure 11) indicated that scission of this chain had occurred at an Arg-Ile bond during activation. This bond and the sequence following resemble the corresponding activation sites in trypsinogen, chymotrypsinogen, and prothrombin. Despite its sequence

homology to prothrombin, when tested for its ability to promote blood clotting, protein C was completely ineffective.

Plasminogen. Recently determined partial amino-acid sequences have revealed extensive similarities between plasminogen and prothrombin.[531, 532] Prothrombin contains two different disulphide-linked segments called 'kringles' (after Scandinavian cakes of the same shape) in the 'pro' part of its sequence; recently plasminogen was shown to have five such kringles, each of which has *ca.* 80

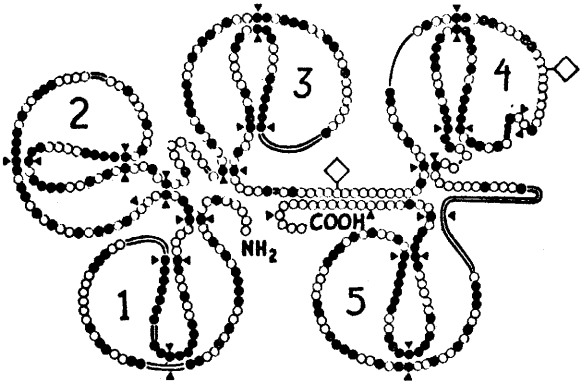

Figure 12 *Partial sequence data of residues* 76—*approx.* 587 *of heavy chain of plasmin arranged as five kringle regions (marked* 1—5) *with 'connecting strands'. The kringles represent the sequences from Cys-*1 *to Cys-*6. *Circles: Sequenced residues. Filled circles: Identities with corresponding residue in A- and/or S-kringle of prothrombin. Double lines: Stretches of known composition, not sequenced. Single line: Stretch not yet found. Squares: Attached carbohydrate. Filled triangles point at Cys-residues*
(Reproduced by permission from *F.E.B.S. Letters*, 1976, **61**, 20)

residues, interspersed with non-kringle regions of variable length.[531] Sequence homology between plasminogen and prothrombin kringles (Figure 12) was estimated at 55%, and a gene quintiplication during the evolution of plasminogen was suggested to account for the internal sequence repetition within this molecule. Although disulphide bonds were not assigned in the plasminogen sequence, Claeys *et al.*,[531] have surmised that the secondary and tertiary structures of the kringles in both prothrombin and plasminogen would be similar and have presented a model for the secondary structure of the latter (Figure 12).

Bone Protein. The complete sequence of a bone protein containing γ-carboxyglutamic acid residues has recently been determined (Figure 13).[533] It bears no similarity to any known blood-clotting component and the amino-acid sequences around its three γ-carboxyglutamic acid residues are unlike the carboxylated sites in blood-clotting proteins. Its function is unknown, although its properties of binding to crystalline but not to amorphous calcium phosphate suggest that it may play a role in demineralization of tissues.[534]

Haemoglobins.—Differences between an embryonic globin chain and adult mouse β-type chains have been located by quantitative sequence analysis of polypeptide mixtures.[539] Globin samples from mouse embryos containing

mixtures of the α-, β-, and embryonic γ-chains were sequenced: the sequences of α- and β-chains were already known and two of the three residues at each position could therefore be accounted for. The third residue was assigned to the γ-chain. Where a residue, common to γ- and either α- or β-chains occurred, quantitative analysis revealed higher amounts of this residue than accountable from one chain alone. A 39-residue *N*-terminal sequence of mouse γ-chain was deduced by this method. Mixture analysis of this type has also been applied to purified mouse β-chains after CNBr digestion.[540] Knowledge of the *N*-terminal sequence

```
                1                   5                  10                  15
 1 Tyr-Leu-Asp-His-Trp-Leu-Gly-Ala-Hyp-Ala-Pro-Tyr-Pro-Asp-Pro-

16 Leu-Gla-Pro-Lys-Arg-Gla-Val-Cys-Gla-Leu-Asn-Pro-Asp-Cys-Asp-

31 Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-

46 Tyr-Gly-Pro-Val-
```

Figure 13 *Primary structure of the γ-carboxyglutamic acid-containing protein from calf bone*
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1447)

derived from a sequencer run on intact chain allowed the additional sequence, originating from the single site of CNBr cleavage, to be determined. Mouse β-globin genes are duplicated in some strains and single in others. The amino-acid sequences of different β-chains have led to proposals accounting for their evolution and diversification within related mouse species.[540, 541]

Viral Proteins.—A remarkable and unexpected example of genetic economy has recently been uncovered by protein and DNA sequence studies on φX174.[558] It was previously known that the sites of two genes, E and D, seemed to overlap in the genetic map of φX174. Moreover, the sum of the molecular weights of proteins encoded by the phage was greater than the coding capacity of phage DNA. Protein sequence studies on the gene D product have now established its correspondence to the phage DNA sequence. DNA sequence studies on an amber mutant of the E gene have shown that the single base change responsible occurred within the gene D coding sequence but, because of the degeneracy of the genetic code, caused no alteration in gene D protein. D and E proteins must therefore be encoded by the same segment of DNA. The reading frame of the amber mutation permitted the location of putative initiators and terminators of the E gene, and the probable sequence of the gene E protein was deduced from the DNA sequence. It was concluded that gene E is entirely included in gene D, is transcribed from the same strand as D, but is translated in a different reading frame to D.

Structural Proteins.—*Keratins.* In vertebrates, epidermal structures such as skin, hair, and feathers contain cystine-rich proteins collectively called 'keratins'. Mammalian hair keratins have been extensively studied and consist of at least three major classes, namely, the high sulphur proteins, the low sulphur proteins,

and the high glycine–tyrosine proteins. The low sulphur, or cystine-poor, keratins constitute the microfibrils, the prominent structural elements of hair, which are elongated tubular structures, rich in α-helix. The high sulphur proteins are believed to act as the molecular cement in which the microfibrils are fixed. Previous studies on feather keratins had established that microfibrils and matrix elements also were present; however, in contrast to the situation in mammalian hair, no evidence had been found for multiple classes of avian keratin subunits. Instead, different feather keratin chains, whether variants occurring in the same species or in different species, exhibit remarkable constancy in molecular weight (*ca.* 11 000) and cystine content.[594] Previous sequence studies on purified feather keratin chains from emu and gull, and recent studies on chick down feather keratin,[595] have shown that most or all feather keratin chains contain eight half-cystine residues which are concentrated at the *N*-terminal and *C*-terminal segments. The central sequence in the three species is devoid of cystine but contains a preponderance of neutral and hydrophobic residues ideally suited to form β-pleated sheets, known to exist in feather microfibrils. The cystine-rich *N*-terminal and *C*-terminal ends of feather keratin chains probably constitute the matrix which maintains the stability and parallel orientation of the microfibrils which arise from the hydrophobic central segment. The occurrence of both crystalline and matrix elements on the same polypeptide chains of feather keratin suggests that, despite some ultrastructural similarities between feather and hair, the evolution of the two tissues has been markedly different. In support of this contention, comparisons between feather and hair keratin sequences have revealed no significant homology.

Sequence analysis of its tryptic peptides has indicated that down feather keratin is not a single protein but a rather large family of closely related proteins.[595] Multiple amino-acid substitutions were found to occur between different electrophoretic variants isolated from this tissue and the chemical heterogeneity observed by a combination of sequencing and gel electrophoresis was compatible with no fewer than 19 genetically different proteins.

Nucleic acid hybridization experiments using labelled cDNA, complementary to feather keratin mRNA, have shown that 25—35 different keratin genes are transcribed in down feather tissue. This estimate is in good agreement with that based on the number of electrophoretic variants and independently supports the notion that many homologous feather keratin genes are present in the chick genome.

Limited sequence data on chick scale keratin chains have revealed some interesting relationships between tissue-specific forms of keratin.[596] The *N*-terminal sequences and *C*-terminal sequences of three electrophoretically homogeneous scale fractions were shown to be highly homologous with the corresponding regions of feather counterparts (Figure 14); half-cystine residues are particularly well conserved, suggesting, by analogy to the feather situation, that the two ends of scale keratin chains are also the matrix-forming parts. However, a major segment of scale keratin chains bears no relationship to any known feather keratin sequence. It is largely composed of tandemly repeated tripeptide units of the type Gly-Gly-X, where X represents Phe, Tyr, or Leu (Figure 14). The identity of the third residue in the repeats differed at a few

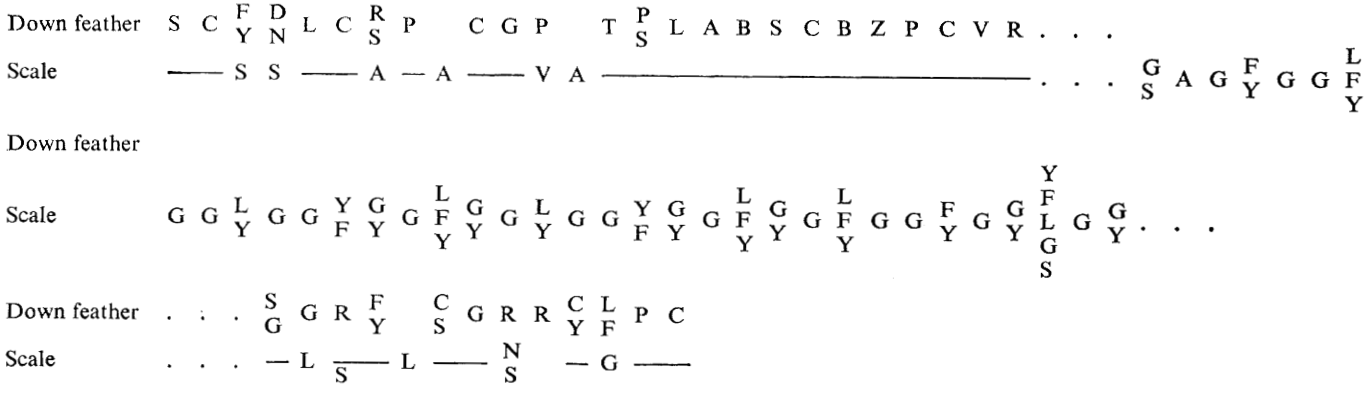

Figure 14 *Homology between scale and down feather keratins. The two sequences have been aligned to maximize homology. Unbroken lines indicate identity between the two sequences. Dotted lines indicate regions of unknown sequence. Blanks indicate deletions*

positions between different scale fractions, but, in contrast, the glycine doublets were particularly well conserved. Moreover, within a single scale fraction, two or more residue alternatives occurred at some 'X' positions, indicating sequence heterogeneity even within a single electrophoretic variant. Quantitative analysis of the sequence heterogeneity indicated that three genetically distinct proteins occurred in all three scale fractions, or at least nine in total scale tissue.

On the basis of the extensive similarity between *N*-terminal and *C*-terminal segments of scale and feather keratins, it has been suggested that significant homology might exist between the DNA sequences which encode them. This homology has been invoked to explain the high gene frequency estimates obtained when feather keratin cDNA was used to detect complementary sequences in total chick DNA. By this technique a value of 100—240 keratin genes was estimated, in contrast to the 25—35 genes actually expressed in down feather tissue. Some of the keratin genes detected by feather cDNA but silent in feather tissue may be scale (and possibly other tissue-specific keratin) genes which possess sufficient homology to form stable duplexes with feather cDNA.

Histocompatibility Antigens.—The major histocompatibility complex (MHC) in both man and mouse is a chromosomal segment whose products are intimately associated with such diverse immunological processes as transplant rejection, ability to respond to specific antigens, and complement activities. These products are, at present, the subject of intense investigation at the cellular, genetic, and structural levels.

Alloantisera, *i.e.* antibodies elicited in one individual by tissue transplants from another individual, react predominantly with antigens termed SD (serologically defined) antigens. The individually specific behaviour of human alloantibodies and the strain specificity of alloantibodies in inbred mouse strains makes it clear that the genetic loci which encode SD antigens are immensely polymorphic. In both mouse and man, however, only two genes per haploid complement encode the major SD antigens. The genes, moreover, are closely linked.

Chemical studies have been begun with a view to determining how the products of the genetic loci and their alleles differ and to define the antigenic determinants of different SD antigens. The use of alloantibodies or of antibodies to β_2 microglobulin has permitted the purification of a number of mouse (H-2) and human (HLA) antigens of differing immunological specificity. In both species, the antigenic chain, a 45 000 dalton glycoprotein, has been shown to be non-covalently associated with β_2 microglobulin, which is an 11 000 dalton protein first isolated from urine.

Specific H-2 antigens have been isolated in a number of laboratories from mouse spleen cells, radioactively labelled in culture.[611–615] The approach used to sequence these proteins has been first to label cells with one essential amino-acid at a time, then to purify the H-2 antigen with specific alloantibody and to sequence automatically the radioactive immunoprecipitate. The radioactivity in the thiazolinone fraction from each cycle is determined and residue assignments are made without the necessity of further analytical steps to identify PTH amino-acids. Results thus obtained are summarized in Figure 15. Several salient points are clear from these sequences.

Mouse H-2 sequences

	1			5					10				15			20					25	
K^b	M	P	H	L	R	Y	F	V		A	V	R	P	L			R	Y	M			Y
K^k	M	P	H	L	R	Y	F	H		A	V	I	P	L	K	P	F	A	M			Y
K^d	M		H		R	Y	F															
D^b		P			R	Y				A	V	R	P	L		P	R	Y				
D^d	M	P	H	L	R	Y	F	V		A	V		P			P		Y				

Human HLA sequences

	1			5					10				15			20					25	
A2	G	S		S	M	R	Y	F	F	T	S	V	S	R	P	G		G	E		F	I	A	V	
B(7 + 12)	G	S		S	M	R/V	Y	F	Y	T	S	V	S	R	P	G		G	E		F	I	A	V	
B(7 + 14)	G	S	S	S	M	R	Y	F	Y	T	S	V	S	R	P	G		G	E	S	B	F			
A1 + B8	G	S	H	S	M	R	Y	F	F/D	T	S/A	M/V	S	R	P	G	R	G	E	P	P/R	F	I	V/S	Y
A(1 + 2), B(8 + 13)		S		S	M	R	Y	F/Y	F/Y	T/S	A/S	V	A/S	R	P	G									

Figure 15 N-*Terminal sequences of human and mouse histocompatibility antigens. Blanks indicate residues as yet unassigned*

(i) The products of different loci (*e.g.* K^b and D^b) and of different alleles at the same locus (K^k and K^d) have different amino-acid sequences. These sequence data therefore strongly support the argument that the serological differences between specific H-2 antigens are determined by amino-acid interchanges rather than secondary modifications (such as glycosylation) of a sequence common to all antigens.

(ii) All H-2 sequences in Figure 15 are nonetheless similar, indicating that mouse SD antigens arose during evolution as a consequence of duplication and mutational alteration of a single ancestral gene.

(iii) Gene duplications are not uncommon events in evolution; β and δ chains of haemoglobin are examples of this. However, of more than one hundred alleles identified at the β chain locus, all are far more similar to other β alleles than to any known δ chain. This is not the case for mouse SD antigens. Proteins specified by different loci seem to be no less similar than those of different alleles at the same locus.

The human HLA sequences (Figure 15) were obtained by three different approaches. Terhorst *et al.*[616] used massive quantities of cells to obtain sufficient material to sequence antigens A2 and B(7 + 12) conventionally. Appella *et al.*[617] used a similar approach for antigens A2 and B(7 + 14). Bridgen *et al.*[435] used modest quantities of cells to obtain mixed antigens A(1 + 2) and B(8 + 13). Sequences were obtained by the ^{35}S-PITC procedure (see Microsequencing section, p. 74). Ballou *et al.*[436] used small quantities of highly radioactive cells to obtain the A1, B8 antigen mixture for sequencing (p. 75). The lack of specific alloantisera prevented as good a resolution of human antigens as was possible with mouse counterparts. However, several differences between specific antigens can be deduced by examination of the data in Figure 15. For example, residue 9 in A2 is phenylalanine whereas in B7 and B12 it is tyrosine; residue 6 in B7 and B14 is arginine whereas it is valine in B12.

Comparison of H-2 and HLA sequences reveals a highly significant degree of homology between SD antigens of the two species (at least 30%) and suggests that their biological function(s) in mouse and man is the same.

Biosynthetic Precursor Proteins.—Proteins which are destined for secretion are invariably synthesized as precursors which contain extra sequences at the *N*-terminal end of the mature sequence; the 1975 Report (pp. 139—143) summarizes techniques now commonly used to identify and purify precursors. Typically, mRNA from a suitable tissue is translated in a cell-free system, supplied with radioactive amino-acids, and precursor proteins are isolated by immunoprecipitation with antibodies directed against the mature form. In addition to the examples cited below, putative precursors for placental lactogen,[618] prolactin,[619] and growth hormone [620] have recently been isolated.

Preproparathyroid Hormone. The exact length of the *N*-terminal precursor portion of preproparathyroid hormone has been determined [621] (Figure 16). The 'prepro' hormone contains an *N*-terminal appendage of 26 residues preceding the prohormone sequence. The extra sequence is very hydrophobic and it has been proposed that this property is important in directing ribosomes synthesizing such nascent chains to membrane sites, namely those located on the rough endoplasmic reticulum. Shortly after its discharge through the membrane the

preprohormone is cleaved to the prohormone. That the preprohormone is indeed a genuine translation product of parathyroid cells rather than an artefact of cell-free synthesis has been demonstrated [622] by its isolation from parathyroid tissue.

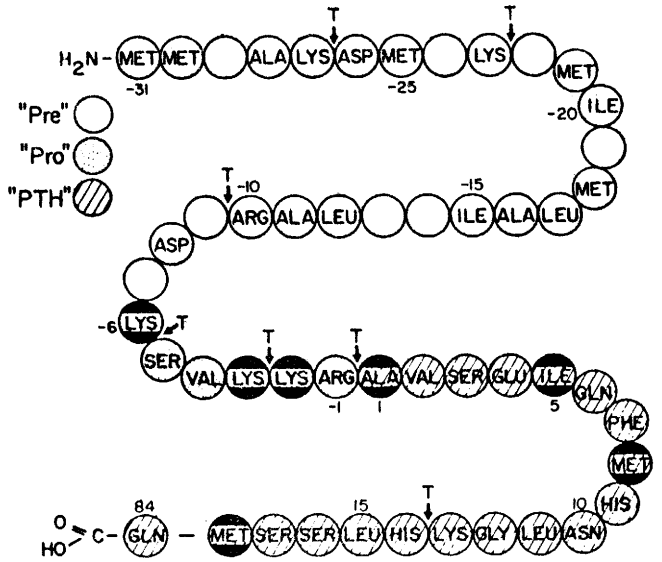

Figure 16 *Partial amino-acid sequence of Pre-ProPTH. The numbers refer to the polypeptide numbering system, beginning with* −31 *at the amino terminus of Pre-ProPTH. The sequence beginning at the amino terminus of ProPTH (lysine,* −6*) is taken from previous analyses of the structures of ProPTH and PTH. The shaded residues in this region represent overlapping residues detected in the present analysis of Pre-ProPTH*
(Reproduced by permission from *Biochemistry*, 1976, **15**, 18)

Preproinsulin. The isolation and characterization of preproinsulin have recently been accomplished [623] by translation of mRNA, isolated from rat islets of Langerhans, in a wheatgerm cell-free system. The precursor contains 23 additional amino-acids located at the *N*-terminal end of the proinsulin sequence.

Immunoglobulin Precursors. Schechter and colleagues have assembled an impressive amount of data on mouse immunoglobulin light chain precursors.[624–628] Light chain mRNA's from different myelomas were translated in wheatgerm cell-free systems and precursor proteins were sequenced. The data (Figure 17) show that the precursor sequences of light chains from the same subgroup are

	1			5				10				15				20	
M321	M		T		T	L	L	L	W	V	L	L	L	W	V	P	S T
M63	M				L	L	L			L	L	L			P	S	
M41	M	M		A	P	A		I	F		F	L	L	L	L	F	P	T	C
M104E	M	A				L		L		L	L	A	L			A			

Figure 17 N-*Terminal sequences of light chain precursors (from refs.* 624—628). *Blanks indicate unknown residues. Solid lines indicate mature sequences*

identical (*e.g.* M321 and M63). On the other hand, the precursor sequences from different subgroups (M321 and M41) and of different subclasses (M104E and M321) differ. In common with precursors of other types of protein, those of light chains are hydrophobic which suggests an interaction with membrane components. Schechter and Burstein [627] suggest that an ancillary function of the *N*-terminal appendages may be to anchor antibody molecules, with precursor sequences still attached, to the outer membrane of B cells where they could act as specific antigen receptors.

Penicillinase Precursor. There are both extracellular and membrane-bound forms of *Bacillus licheniformis* penicillinase. Both are encoded by the same gene, and sequence studies on the two forms have pinpointed the difference between them. The extracellular form of the enzyme is unblocked and has the *N*-terminal sequence Lys-Thr-Glu-Met-. The membrane-bound form is blocked. Both forms have the same *C*-terminal sequence, -Met-Asn-Gly-Lys.[629, 630]

The membrane-bound form of the enzyme has been shown to contain an additional *N*-terminal sequence preceding the normal sequence of the extracellular form.[631] The appendage is 25 residues in length and consists largely of repeating sequence units which have diverged from the ancestral sequence, Asx-Glx-Ser-Gly. The *N*-terminal residue is phosphatidylserine, which accounts for the apparent *N*-terminal blockage. The authors suggest that the membrane-bound form of penicillinase is a precursor of the extracellular form, but their data cannot eliminate the formal possibility of a peptide addition mechanism whereby the initial form of penicillinase is either secreted or enzymically extended to produce the membrane-bound form. Even if the membrane form is the precursor of the extracellular form, it must itself be derived from an even larger precursor. Kelly and Brammar (*J. Mol. Biol.*, 1973, **80**, 135) isolated a penicillinase frameshift mutant and compared the *C*-terminal sequence of the mutant to that of wild type. As can be seen in Figure 18, the first possible terminator codon in the DNA sequence of the non-frameshift form deduced from the two out-of-phase amino-acid sequences occurs four codons distal to the *C*-terminal residue of mature penicillinase. The initial product of translation of penicillinase mRNA must therefore be extended by at least three residues at the *C*-terminus of the mature form.

Miscellaneous.—*Disease Antigens. Viral antigens.* Influenza virus has the unfortunate ability to mutate key antigens from time to time so as to escape immunity mechanisms which can recognize and eliminate pre-existing forms of the virus. Current interest centres around which antigen differs in different viral strains. Viral haemagluttinin (HA) consists of two chains, HA1 and HA2, which are generated by post-synthetic cleavage of a precursor containing both. Immunological studies and peptide-mapping experiments had previously suggested that HA1 rather than HA2 contained the major sites of variability. Recent sequence [632] studies have lent strong support to this idea. The *N*-terminal sequences of HA1 and HA2 have shown that four of the first 10 positions differ between HA1 chains of two different strains of flu virus whereas the *N*-terminal sequences of HA2 from the two strains differed at only one position in 24.

Trypanosome antigens. Trypanosomes such as *Trypanosoma brucei* evade destruction by altering surface antigens more rapidly than their hosts can mount

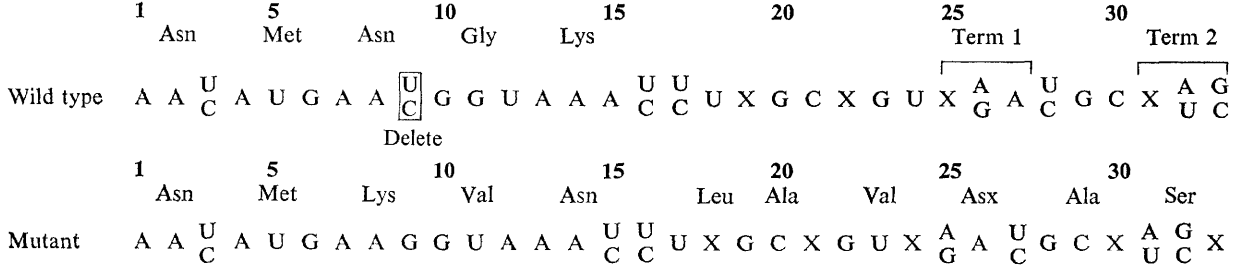

Figure 18 *C-Terminal amino-acid sequences (upper) and corresponding mRNA sequences (lower) of wild type and frameshift forms of penicillinase.* X *positions in the sequences can be occupied by any of the four nucleotides without altering the amino-acid sequence*

an effective immunological response. *T. brucei* surface antigens are all glycoproteins of mol. wt. 65 000 daltons and the phenotypic variation between different antigens has recently been investigated.[633] *N*-Terminal sequences of antigens from four trypanosome clones, isolated from a single infected rabbit, were determined (Figure 19). In each case a single sequence was present but the

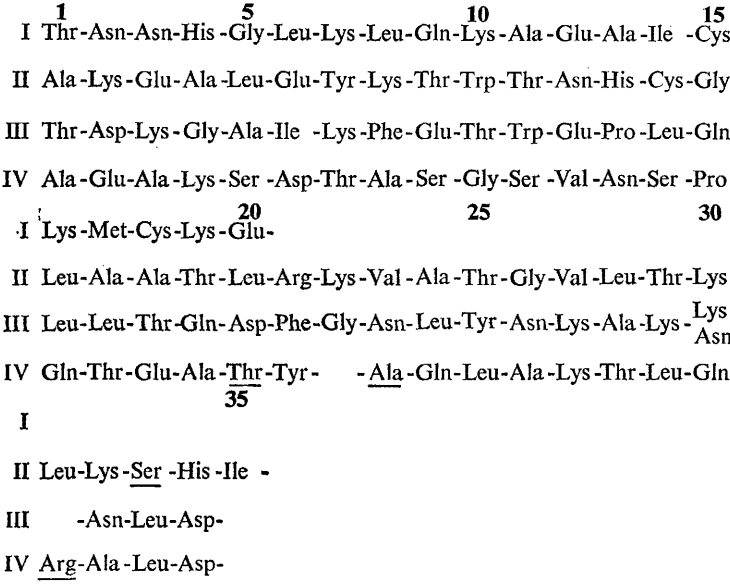

Figure 19 *Amino-terminal sequences of variant specific surface antigens from four successive trypanosome variants*

sequences were markedly different; little or no homology was apparent between different antigens, indicating that their phenotypic differences were determined by different structural genes rather than by secondary modifications of a single shared sequence.

Non-histone Proteins. Chromatin proteins can be fractionated into two general classes, histones and non-histones. The histones are the predominant, if not the sole protein constituent of the nucleosomes; few if any non-histones have yet been assigned a functional role. Sequence studies on two purified non-histone proteins have recently established that they are related to histones.

Nuclear protein A24 from calf thymus has been shown by peptide mapping studies to include the entire H2a histone sequence plus additional sequences.[634, 635] It is not a precursor of histone H2a in the normal sense since its single *C*-terminal sequence is identical with that of H2a and it contains the normal acetylated *N*-terminal peptide of H2a. Yet A24 has also an unblocked *N*-terminus which makes it amenable to sequencing, and the *N*-terminal 37 residues have recently been determined.[635] The sequence bears no relation to that of any known histone. Neither disulphide nor γ-glutamyl cross-links can explain the paradoxical

situation of two *N*-termini (H2a is blocked) and one *C*-terminus unless both proteins have the same *C*-terminus. Possibly the *C*-terminus of the non-histone chain is covalently attached to a specific side-chain residue in F2A.

Another non-histone from calf thymus, HMG-17, has been sequenced through 39 *N*-terminal residues.[636] The sequence (Figure 20) is not significantly homologous to mammalian histones but closely resembles trout histone T.

```
                1                   5                  10                  15
HMG-17  Pro-Lys-Arg-Lys-Ala-Glu-Gly-Asp-Ala-Lys-Gly-Asp-Lys-Ala-Lys-Val-Lys-
                1                   5
Histone-T Pro-Lys-Arg-Lys-Ser-Ala-Thr-Lys-Gly-
                   20                  25                  30                  35
HMG-17  Asp-Glu-Pro-Gln-Arg-Arg-Ser-Ala-Arg-Leu-Ser-Ala-Lys-Pro-Ala-Pro-Pro-Lys-Pro-
                   10                  15                  20                  25
Histone-T Asp-Glu-Pro-Ala-Arg-Arg-Ser-Ala-Arg-Leu-Ser-Gly-Arg-Pro-Val-Pro-Lys-Pro-Ala-
```

Figure 20 *Sequence homology between HMG-17 and histone-T*
(Reproduced by permission from *Biochem. Biophys. Res. Comm.*, 1976, **73**, 77)

Staphylococcus aureus A Protein. The ability of a protein A, a cell-wall constituent of *Staphylococcus aureus*, specifically to bind antibodies has been a useful tool in identifying antibodies on cell surfaces and in mixtures of other proteins. Protein A binds to the Fc portion of antibodies and is multivalent, having two or more binding sites per subunit. Peptide fragments of protein A which bind to an Fc immunoadsorbent have recently been purified and sequenced (Figure 21).[637] The interesting feature is that three different peptides bind. They are remarkably alike in sequence and their molar yields establish that all occur on the same chain. This provides an explanation for the multiple Fc binding sites of protein A. Whatever the function of protein A and whether its *in vivo* role is to bind antibody or not, the repeated sequences have been well conserved (*ca.* 80%) during evolution.

Purple Membrane Protein. Halobacterium halobium is a purple photosynthetic bacterium first isolated from a highly saline environment. Its purple colour is due to a single retinal-containing protein (bacteriorhodopsin or purple membrane protein) which is the major cell membrane polypeptide. Membrane vesicles, whose sole protein constituent is purple membrane protein, can establish a pH gradient between external and extracellular environments by converting light energy into chemical energy. Structural studies (see Vol. 8, p. 161) have revealed that native purple membrane protein subunits consist of seven α-helices all essentially perpendicular to the place of the membrane. Recent chemical studies have established, beyond reasonable doubt, the exact subunit molecular weight and have localized the peptide to which the retinal chromophore is covalently attached.[638] Preliminary molecular weight studies gave serious discrepancies in the results depending upon the technique used; values obtained ranged from 20 000 to 28 000 daltons. The problem was resolved by determining the exact number of lysine residues per subunit by peptide mapping thermolytic digests of the ^{14}C-succinylated derivative. By this technique six lysine containing peptides were identified, all of unique sequence; one contained two lysine residues. Using the value of seven lysines per chain and an accurate knowledge

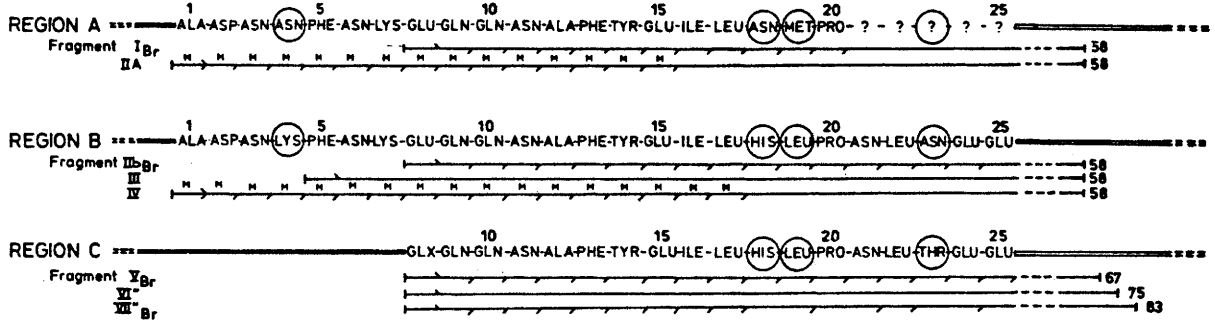

Figure 21 *Assignment of the various Fc-binding fragments to different regions within protein A according to amino-acid compositions and N-terminal sequences*
(Reproduced by permission from *F.E.B.S. Letters*, 1976, **67**, 62)

of the amino-acid composition of purple membrane protein, an exact molecular weight value of 25 380 daltons was determined.

To determine which of the lysine-containing peptides was attached to retinal in intact purple membrane protein, a procedure involving borohydride reduction and irradiation was used to attach the chromophore irreversibly to the protein. A thermolytic peptide map of reduced, ^{14}C-succinylated protein exhibited one major radioactive peptide absent in the non-reduced digest. Sequence analysis of this peptide established that it contained two adjacent lysine residues, the first being succinylated, the second containing the reduced retinal group. The sequence of the retinal peptide is

Val-Ser-Asp-Pro-Asp-Lys(suc)-Lys(ret)

It contains only two hydrophobic residues and five polar or charged residues and would be expected to occur on the surface of the membrane, possibly bridging two α-helices.

The detail in which the three-dimensional structure of purple membrane protein is known should facilitate the building of a molecular model when the amino-acid sequence has been determined.

PART IB: Chemical Modification *by G. Winter and A. Dell*

A comprehensive list of proteins modified is given in the Table.

Table *Chemical modification of proteins*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Acetylcholinesterase	Bovine brain, eel	[^{3}H]Di-isopropyl fluorophosphate	Ser	Active-site titrations	1
Acetylcholinesterase	*Torpedo californica*	Dimethyl suberimidate/DTT	Lys	Cross-links between head and tail units	2
Acetylcholinesterase	*Torpedo marmorata*	1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl-(methyl or ethoxy)-fluorophosphate	Ser	Spin label	3, 4
Acetylcholine receptor	*Torpedo californica*	4-Azido-2-nitrobenzyltrimethyl-ammonium fluoroborate			5
Acetylcholine receptor	*Torpedo ocellata*	*p*-(*N*-Maleimido)benzyl-(^{3}H-trimethyl)ammonium iodide	Cys	Affinity label	6
α_1-Acid glycoprotein	Human plasma	Trinitrobenzenesulphonic acid, arylisocyanates,	Lys	One Lys protected by progesterone	7
		Tetranitromethane	Tyr	One in active site	
		Various	Trp	Decreased steroid binding	
α_1-Acid glycoprotein	Human plasma	Various		Topography study	8
Acid phosphatase	Bovine brain	DTNB	Cys	One highly reactive at low pH	9
Actin	Chicken fibroblasts	Iodo-[^{14}C]- and -[^{3}H]acetic acid	Cys	6.8% of protein of fibroblasts estimated to be actin	10
Actin	Rabbit muscle	*N*-Iodo-[^{3}H]acetyl-*N'*-(1-sulpho-5-naphthyl)ethylene diamine	Cys	Cys-10 reactivity increased on binding myosin	11

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Actin	Rabbit muscle	5-Amino-1*H*-[^{14}C]tetrazole	Tyr-53	Inhibits polymerization; labelled tryptic peptide isolated	12
Actin(F)	Rabbit skeletal muscle	*N*-(3-Pyrene)maleimide	Cys-373	Fluorescence study	13
Actin(F)	Rabbit muscle	*N*-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)maleimide	Cys	Interaction of actin with spectrin studied	14
Adenine nucleotide carrier	Rat heart mitochondria	[^{3}H]N_4-azido-2-nitrophenyl-aminobutyryl-atractyloside/ photoirradiation		Photoaffinity label: ATR binding protein has mol. wt. 30 000	15
S-Adenosylmethionine decarboxylase	Yeast, rat liver	1,1′-(Methylethanediylidene-dinitrilo)-*bis*(3-aminoguanidine)		Irreversible inhibition	16
Adenylate kinase	Porcine muscle	Tetranitromethane	Tyr-153	Tyr-153 involved in ATP binding	17
5′-Adenylic acid aminohydrolase	Rat skeletal muscle	Periodate oxidized GTP	Lys	Inactivates enzyme: made irreversible by $NaBH_4$ reduction (Schiff base)	18
ADP glucose pyro-phosphorylase	*E. coli*	Pyridoxal phosphate/$NaBH_4$	Lys	Lys at allosteric activator binding site	19
β-Adrenergic receptor	Turkey erythrocytes	*N*-[2-Hydroxy-3-(1-naphthoxy)-propyl]-*N*′-bromoacetyl-ethylenediamine		Affinity label	20
ACTH	Ovine	Substituted *o*-nitrophenyl-sulphenyl chlorides	Trp, Met	Effect on activity studied	21
ACTH	Synthetic	N^{ε}-(+)-biotinyllysine amide	*C*-Terminus	Affinity chromatography on avidin-Sepharose	22
Agglutinin	Wheatgerm	*N*-Bromosuccinimide	Trp	2/3 Trp modified	23
Albumin	Bovine serum	Iodo[^{14}C]acetamide	Cys	Isoelectric focussing with 'aged' and 'oxidized' BSA	24
Albumin	Bovine serum	Ribose-5-phosphate, adenosine diphosphoribose	Lys	Made irreversible by $NaBH_4$ reduction (Schiff base)	25
Albumin	Bovine serum	*N*-(*N*-Dinitrophenylaminoalkyl)-maleimides	Cys	Accessibility to anti-DNP antibody examined	26

Albumin	Bovine serum	Fluorescein isothiocyanate	Lys-220	Destroys catalytic activity	27
Albumin	Bovine serum	Fluorescein isothiocyanate	Lys ?	No loss of activity	28
Albumin	Bovine serum	Methyl *p*-benzoylphenyl-acetimidate hydrochloride	Lys	Photochemical study suggests photoreductive coupling of benzophenone chromophores to polypeptide backbone	29
Albumin	Human serum	1-(*p*-Benzenediazonium)-(ethylenedinitrilo)-tetra-acetic acid	His, Lys	Powerful chelating agent; used ^{111}In as radioactive tracer	30
Albumin	Human serum	Difluorodinitrobenzene/ bradykinin, glucagon	Lys	Efficient coupling of peptides to albumin	31
Albumin	Human serum	Product of *N*-ethyl-5-phenyl-isoxazolium-3′-sulphonate with bilirubin		Affinity label for bilirubin binding site	32
Albumin	Human serum	[1-^{14}C]Acetylsalicylic acid	Lys-199		33

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Alcohol dehydrogenase	Horse liver	Iodoacetate	Cys	Kinetic study	34
Alcohol dehydrogenase	Horse liver	Diazonium-1*H*-tetrazole	Cys-174	Structure of product studied	35
Alcohol dehydrogenase	Horse liver	α-Bromo-β-(5-imidazolyl)-propionic acid	Cys-46	Specifically labelled	36
Aldehyde dehydrogenase	Ovine liver	Disulphiram analogues	Cys	Mitochondrial enzymes less susceptible to inhibition than cytoplasmic	37
Aldolase	Rabbit muscle	3,4,5,6-Tetrahydrophthalic anhydride, pH 8	Lys	Reversible; remove at pH 6	38
Aldolase	Rabbit muscle	Phenylglyoxal	Arg	One Arg/subunit	39
		Butane-2,3-dione	Arg	Some reaction with Lys	
Amine oxidase	*Aspergillus niger*	3-Methyl-2-benzothiazolinone hydrazone		Modification of an essential carbonyl group	40
D-Amino acid oxidase	Porcine kidney	*N*-Chloro-D-leucine		Chlorinates active site	41
Aminoacylase	Porcine kidney	Various thiol reagents	Cys	4 Cys freely available	42
Aminopeptidase	*Clostridium histolyticum*	Dimethyl suberimidate	Lys	Hexameric structure	43
cAMP binding proteins	Lamb testis	[^{3}H]Guanosine-3′,5′-monophosphate/photoirradiation		Cross-linking of nucleotide to protein	44
AMP deaminase	Chicken and rabbit skeletal muscle	[^{14}C]Iodoacetic acid	Cys	Tryptic digest suggests α_4 structure	45
α-Amylase	Porcine pancreas	[^{14}C]Bromoacetate	Cys	S—S/SH exchange studied	46
α-Amylase	Porcine pancreas	2,4-Dinitrofluorobenzene	Lys	Unique Lys labelled at pH 7.9	47
α-Amylase protein inhibitor	Wheat kernel	Iodoacetic acid	Cys	Loss of inhibitory activity	48
Apolipoprotein A-I and II	Human plasma	Dimethyl suberimidate	Lys	Cross-linking	50
Apolipoprotein A-II	Human serum	Iodoacetic acid	Cys		51
Arylsulphatase B	Human liver	*p*-Chloromercuribenzoate, *N*-ethylmaleimide	Cys	No loss of activity	52
		N-acetylimidazole	Tyr	No loss of activity	
		Iodoacetate	His, Cys	His in active site	
Asparaginase	*E. coli*	2-Hydroxy-5-nitrobenzyl bromide	Trp		53
		Thiol reagents	Cys		

Asparaginase	*E. coli*	5-Diazo-4-oxo-L-norvaline		DMSO facilitates labelling; binds to active site	54
Asparaginase	*E. coli*	2,4,6-Trinitrobenzene sulphonic acid	Lys	Effect on subunit interactions	55
Asparagusate dehydrogenase	Asparagus mitochondria	Various thiol reagents	Cys	Activity inhibited	56
Aspartate amino-transferase	Porcine heart	$KN^{14}CO$	Lys	pK of Lys-258 = 7.98	57
Aspartate amino-transferase	Porcine heart	*trans*-5′-Deoxy-5′-carboxy-methylenepyridoxal	Lys	Affinity label	58
Aspartate amino-transferase	Porcine heart	Chloropyruvate		Cys-390 *not* modified	59

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Aspartate amino-transferase	Porcine heart	L-2-Amino-4-methoxy-*trans*-3-butenoic acid		Irreversibly inactivates enzyme; detailed mechanism proposed	60
Aspartate amino-transferase	Porcine heart	[^{14}C]Iodoacetic acid	Cys	Single Cys modified and peptide sequenced	61
Aspartate amino-transferase	Porcine heart	L-[2-^{14}C]propargylglycine		Irreversible inactivation	62
Aspartate trans-carbamylase	*E. coli*	Pyridoxal 5′-phosphate/$NaBH_4$	Lys	Partial inactivation of enzyme	63
Aspartate trans-carbamylase	*E. coli*	Phenylglyoxal	Arg	Essential Arg	64
ATPase	Bovine heart, rat liver	2,3-Butanedione, [^{14}C]phenyl-glyoxal	Arg	Essential Arg at hydrolytic site ?	65
ATPase F1	Bovine heart	Dimethyl suberimidate, dimethyl adipimidate, methylmercapto-butyrimidate	Lys	Arrangement of subunits determined	66
		N-[^{14}C]ethylmaleimide	Cys		
ATPase	Bovine heart	4-Chloro-7-nitro-benzofurazan	Tyr	Method for estimating ATPase content of energy transducing membranes	67
ATPase F1	Bovine heart	Arylazido aminopropionyl-ATP		Photoaffinity reagent	68
ATPase	Canine kidney	(*o*-Phenanthroline)$_2$Cu^{2+}/air	Cys	Subunit structure is $\alpha_2\beta_2$	69
ATPase	*E. coli*	Dithiobis(succinimidyl propionate)	Cys	Subunit cross-linking studied	70
ATPase	Rabbit muscle	1-(2,4-Dinitrophenylamino)hexane, 6-(*N*-maleimido)hexane, *N*-ethyl-(2,3-$^{14}C_2$)maleimide, *NN′*-bis(2,4-dinitrophenyl)-L-cystine	Cys	ATP protects Cys; antibody binding study	71
ATPase	Rabbit and sheep kidney	*N*-Ethylmaleimide	Cys		72
ATPase	Rabbit sarco-plasmic reticulum	(*o*-Phenanthroline)$_2$Cu^{2+}	Cys	ATPase in bilayer as tetramer ?	73

ATPase	Rabbit sarcoplasmic reticulum	[^{14}C]Iodoacetate	Cys		74
ATPase	Rabbit sarcoplasmic reticulum	2,3-Butanedione	Arg	Inactivation	75
ATPase	Rat brain	Dimethylaminonaphthalene-cysteine-Hg^{+}	Cys	New fluorescent probe; more reactive than *N*-ethylmaleimide	76
Bacteriorhodopsin	*Halobacterium cutirubrum*	Glutaraldehyde	Lys	No large motion of protein necessary for the photoreaction cycle	77
Bacteriorhodopsin	*H. halobium*	Sodium borohydride/light		Modifies covalent linkage between chromophore and protein	78
Bisphosphoglyceromutase/ 2,3-bisphosphoglycerate phosphatase/ phosphoglyceromutase	Human erythrocytes	Trinitrobenzenesulphonate	Lys	Evidence for one active site for the three substrates	79

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Carbonic anhydrase B	Human erythrocytes	Iodoacetate, bromoacetate, *p*-nitrophenylacetate	His	Labels active site, studying pH-dependent properties	80
Carboxypeptidase A	Bovine	Tetranitromethane	Tyr	80% of label on Tyr-248	81
Carboxypeptidase A	Bovine	*p*-Azobenzenearsonate	Tyr-248		82
Carboxypeptidase A	Human	Tetranitromethane, *N*-acetyl-imidazole	Tyr	2.3 Residues/mol, increases esterase and decreases peptidase activity	83
Carboxypeptidase A	Bovine	Diazotized *p*-arsanilic acid	Tyr-248	Forms intramolecular chelate with Zn; c.d. probe	84
Carboxypeptidase inhibitor	Potato	Various		Activity studies	85
DD-Carboxypeptidase transpeptidase	*Streptomyces* R61	[^{14}C]benzylpenicillin	Ser	Single Ser labelled: peptide sequenced	86
Catechol-*O*-methyl-transferase	Rat liver	*N*-ethyl [2,3-$^{14}C_2$]maleimide	Cys ?	Inactivation	87
Cathepsin D	Bovine spleen	Dithiophosgene, 2,2-dichloro-1,3-dithiacyclobutanone		New reagents for alkylation	88
Cholera toxin	*Vibrio cholerae*	Dimethyl suberimidate	Lys	Cross-linking of subunits studied	89
		[^{125}I]Iodide, chloramine T	Tyr	Tyr in A_1 only modified	
		[^{14}C]Iodoacetamide	Cys	Inter S—S A_1 to A_2; intra S—S in B subunits	
Cholera toxin	*Vibrio cholerae*	[^{14}C]Iodoacetate, *N*-ethyl maleimide	Cys	A chain only, no inactivation	90
Choriogonadotropin	Human	Thioredoxin/iodo[^{3}H]acetate	Cys	All S—S in subunit surface oriented	91
Chromatin nonhistone protein	Murine Krebs II ascites tumour cells	Photoirradiation		Cross-linking to DNA	92
Chymotrypsin		*N*-Acetyl-*p*-cyanato-L-phenyl-alanine ethyl ester	Ser-195, Cys-1	Ser-195 carbamylated; amino-group of Cys-1 linked *via* isoureido linkage	93
Chymotrypsin	Bovine	[*carbonyl*-^{14}C]Phenacyl bromide	Met-192	Secondary alkylation at neutral pH	94

Chymotrypsin	Bovine	Glycine methyl ester/carbodi-imide	Carboxy-groups	Specificity studied: mechanism suggested which uses water rather than Asp-102 as the proton sink	95
Citrate synthase	*Acetobacter xylinum*	*p*-Hydroxymercuribenzoate	Cys	Inhibits enzyme	96
Citrate synthase	Porcine heart	Rose Bengal immobilized on Sepharose		Photo-oxidation	97
Clathrin A	Porcine brain, bovine brain, and adrenal medulla	CNBr 2-Nitro-5-thiocyanatobenzoic acid	Met Cys	Peptide maps demonstrate Similarities between clathrins from different sources	98
Clostripain	*Clostridium histolyticum*	N^{α}-*p*-Nitrobenzyloxycarbonyl arginine chlormethyl ketone		Affinity label	99
Coat protein	Bacteriophage MS2	U.v. irradiation (λ = 254 nm)	Lys	RNA cross-links to protein: ε-*N*-(2-oxopyrimidyl-4)-lysine isolated	100

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Collagen	Bovine dentine	CNBr	Met	CNBr peptide isolated – contains site of inter-molecular cross-link	101
Collagen	Chicken	Sodium borohydride		Two principal reducible cross-links identified	102
Collagen	Chicken	Sodium [^{3}H]borohydride			103
Collagen	Foetal calf, steer skin	Potassium [^{3}H]borohydride		Reduces hydroxylysino-5-oxo-norleucine	104
Collagen (types I and II)	Porcine invertebral disc	CNBr	Met	Proportions of I and II vary gradually across annulus fibrosus	105
Collagen	Rabbit	Sodium [^{3}H]borohydride/CNBr		Isolated cross-linked CNBr peptide; tissue-dependent variability	106
Complex III	Beef heart mitochondria	Iodo[^{14}C]acetamide	Cys	All radioactivity located in a single 50 000 dalton polypeptide	107
Concanavalin A		Succinic anhydride, fluorescein isothiocyanate	Lys		108
Concanavalin A		Fluorescein isothiocyanate	Lys	Con A bound to Sephadex G50 during reaction	109
Corticosteroid binding globulin	Human	21-Diazo-21-[6,7-^{3}H]deoxy-corticosterone		Photoaffinity label of high specific activity	110
Coupling factor	Spinach chloroplasts	1-Methyl acetimidate 2-trinitro-benzenesulphonate	Lys	First reagent reacts with exposed Lys; second reagent reacts with newly exposed Lys when protein brought into its high-energy state	111
Coupling factor	Spinach chloroplasts	Various cross-linking reagents		Minimal sub-unit stoicheiometry is $\alpha_2\beta_2\gamma\delta\varepsilon_2$	112
Coupling factor	Spinach chloroplasts	Substituted maleimides	Cys	Selectively labels 2 Cys in γ sub-unit after heat activation	113

Coupling factor	Spinach chloroplasts	*o*-Iodosobenzoate/light	Cys	Reagent selective for vicinal dithiols	114
Creatine kinase	Rabbit muscle	*N*-(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide	Cys	Use of label with paramagnetic Mn^{II} to map 3D structures of enzyme–substrate complexes	115
Creatine kinase	Rabbit skeletal muscle	CNBr-activated Sepharose		Evidence for active subunits of matrix bound kinase	116
Creatine kinase	Rabbit muscle	Performic acid	Cys, Met, Trp	Some Tyr modified	117
Creatine kinase	Rabbit muscle	Potassium cyanide	Cys	Cyanylated thiols not required for activity	118
Cyclohexanone oxygenase	*Nocardia globerula* and *Acinobacter*	DTNB	Cys	Essential cysteine	119
Cyclopentanone oxygenase	*Pseudomonas*	DTNB, *p*-mercuribenzoate	Cys	Functional Cys	120

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Cytochrome *c*	Horse heart	Methylene blue	Met	Role of Met-80 described	121
Cytochrome *c*	Horse heart	Various alkylating reagents		Carboxymethyl derivatives of His, Met, Lys	122
Cytochrome *c*	Horse heart	Iodoacetate	Met		123
Cytochrome *c*	Horse heart	Ethylthiotrifluoroacetate	Lys	Selective trifluoroacetylation; ^{19}F n.m.r. study	124
Cytochrome *c*	Horse heart	Maleic anhydride, methyl acetamidinate	Lys	Haeme-linked alkaline ionization is not triggered by deprotonation of Lys	125
Cytochrome *c*		Methylene blue	Met-80	Photo-oxidation	126
Cytochrome oxidase	Yeast	DTNB/Iso-1-cytochrome *c*	Cys	Cytochrome *c* behaves as an affinity label: specifically forms a mixed disulphide with sub-unit III of oxidase	127
3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase	*E. coli*	Various		Cys and possibly Lys in catalytic site	128
Deoxyribose 5-phosphate aldolase	*Salmonella typhimurium*	Acrolein	Lys	Forms Schiff base; inactivates irreversibly *via* Michael addition of a second nucleophile	129
Dihydrofolate reductase	*Lactobacillus casei*	Dansyl chloride	Lys	Single Lys modified; protected by DNSOH	130
Dihydrofolate reductase	*Lactobacillus casei*	Phenylglyoxal	Arg	Protected by NADPH	131
Dipeptidyl peptidase IV	Porcine kidney	Di-isopropyl fluoro[^{32}P]phosphate	Ser		132
DNA polymerase	*E. coli*	[^{35}S]β-D-Ribosyl-6-methyl-thiopurine periodate oxidation product	Lys	Single Lys and Arg in or about the active site	133
		[^{14}C]phenylglyoxal	Arg		
Elastase-like proteinases	Horse leucocytes	Di-isopropyl fluorophosphate,	Ser, Lys	Inactivates	134
		Alkyl isothiocyanates	Lys	Inactivates	
Elastase	Human pancreas	Phenylmethanesulphonyl fluoride, 1-tosylamido-2-phenylethyl-chloromethyl ketone	Ser	Inactivates	135

Elongation factor Tu	*E. coli*	*p*-Nitrophenylchloroformate	Lys	Tu cross-linked to L1, L5, L7/L12, L15, L20, L30, L33	136
Elongation factor Tu	*E. coli*	Methyl-4-mercapto-butyrimidate	Lys	Tu cross-linked to L23, L28, L1, L3, L24	137
Elongation factor G	*E. coli*	*N*-Ethyl maleimide	Cys	Selective modification of a single Cys near nucleotide binding site	138
Elongation factor 1	Mouse ascites tumour cells	[^{14}C]Formaldehyde/sodium borohydride	Lys		139
Elongation factors Tu and Ts	Qβ phage	Dimethyl suberimidate	Lys	Tu:Ts cross-linked product	140
Erabutoxins a, b, c	*Laticauda semi-fasciata*	*O*-Methylisourea, acetic anhydride	Lys	All Lys guanidated; mono-acetyl derivatives isolated	141
Estradiol-17β-dehydro-genase	Human placenta	Nicotinamide-(5-bromoacetyl-4-methylimidazole)dinucleotide	Cys	Affinity label for co-enzyme binding site	142

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Estradiol-17β-dehydrogenase	Human placenta	3-Chloro[2-^{14}C]acetyl pyridine–adenine dinucleotide	Cys	Labelled peptides isolated after chymotryptic digest	143
Estrogen binding proteins	Calf uterus	Sodium thiocyanate	Cys	Studies of aggregation and dissociation of receptor	144
Factor VII	Bovine	[^{3}H]Di-isopropyl fluorophosphate	Ser	Stoicheiometric incorporation into same site of 1, 2, and 3 chain forms of protein	145
Fatty acid synthetase	Chicken liver	Phenylmethanesulphonyl fluoride	Cys	Inhibits thioesterase function	146
Fatty acid synthetase	Goose	[^{3}H]Di-isopropyl fluorophosphate	Ser	One mole of inhibitor per mol subunit	147
Fatty acid synthetase	Yeast	[1-^{14}C]Iodoacetamide	Cys	Central and peripheral thiol groups on same polypeptide chain	148
Ferredoxin	*Clostridium acid-urici*	Edman degradation, followed by coupling to Boc-amino-acid-*p*-nitrophenyl esters or Boc-dipeptide-*N*-hydroxysuccinimide esters	*N*-terminal	Semi-synthetic ferredoxin derivatives	149
Ferredoxin-NADP reductase	Spinach chloroplast	[*Me*-^{14}C]Dansyl chloride	Lys	Essential Lys, protected by NADP or NADPH	150
Apoferritin	Horse spleen	Maleic anhydride	Lys		151
		Glycine amide	Asp, Glu	Modification of carboxy-groups inactivates enzyme	
Fibrinogen	Bovine	[^{125}I]Iodide, lactoperoxidase	Tyr	2 to 5 atoms of I per mol	152
Ficin	*Ficus glabrata*	2,2′-Dipyridyl disulphide	Cys	Characterization of active centre	153
Ficin	*Ficus glabrata*	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	Cys	Reactivity probe: ficin lacks carboxy-groups conformationally equivalent to Asp-158 of papain	154
Flavoprotein lipoamide dehydrogenase	Porcine heart	Iodoacetate	Cys	Selective alkylation of one thiol from active centre S—S	155

Folch-Pi apoprotein	Bovine and human myelin	3-(2-Iodoacetamido)-2,2,5,5-tetramethylpyrrolidine-1-oxyl	Cys	Spin label: organic solvents	156
		3-Carboethoxycarboxyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl	Cys	Spin label: aqueous solvents	
Fructose bisphosphate aldolase	Rabbit muscle	*N*-Bromoacetylethanolamine phosphate, pH 8.5	Lys-146	Essential Lys	157
β-Fructosidase	Yeast	Conduritol-β-epoxide		Inactivation: carboxylate, $pK_a = 3.05$, is reactive group ?	158
Fumarase	Porcine heart	Benzyl bromide	Met	Evidence that reagent has greater specificity than previously recognized	159
α-D-Galactopyranosyl binding lectin	*Bandeiraea simplicifolia*	Various reagents		Carboxy-groups participate in carbohydrate binding	160
Galactose binding protein	*E. coli*	Dimethyl suberimidate	Lys		161

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
β-Galactosidase	*E. coli*	2,6-Anhydro-1-diazo-1-deoxy-D-*glycero*-L-*manno*-heptitol		Specific label for active site	162
Galactosyltransferase	Bovine milk	Photoirradiation	Trp	Inactivates: protected by substrates	163
Glucagon	Bovine	2-Nitrophenylsulphenyl chloride, 2,4-dinitro-1,5-phenyldisulphenyl chloride	Trp	Glucagon dimer active	164
Glutamate apodecarboxylase	*E. coli*	Bromo[2-^{14}C]pyruvate	Cys	Inactivation: 1-carboxyl-1-(DL)hydroxyethyl cysteine isolated	165
Glutamate dehydrogenase	Bovine liver	8-Azido-ADP, photoirradiation		Presence of ADP reduces incorporation	166
Glutamate dehydrogenase	Bovine liver	2,3-Butanedione	Arg	Arg involved in both regulation and catalysis	167
Glutamate dehydrogenase	Bovine liver	*p*-Chloromercuribenzoate	Cys	One Cys per subunit: disagrees with previous data	168
Glutamate dehydrogenase	Bovine liver	[^{3}H]-4-Amino-6-chloro-5-oxo-hexanoic acid	Lys-126	Negative co-operative interactions in reactivity of Lys-126	169
Glutamate dehydrogenase	Bovine liver	2,3-Butanedione, phenylglyoxal	Arg	4 Arg substituted; co-enzyme protects 2	170
Glutamate dehydrogenase	Bovine liver	*p*-Chloromercuribenzoic acid	Cys-319	Identified by replacing mercurial derivative with iodo[2-^{14}C]acetic acid	171
Glutamate dehydrogenase	*Neurospora crassa*	1,2-Cyclo[1-^{14}C]hexanedione	Arg	Peptic containing modified Arg identified	172
Glutamate dehydrogenase	Tuna liver	Pyridoxal 5′-phosphate/sodium borohydride	Lys	Essential Lys identified	173
Glutamate synthase	*E. coli* K12	L-2-Amino-4-oxo-5-chloro-pentanoic acid, iodo[^{14}C]-acetamide	Cys	Selective inactivation of Gln-dependent activity	174
Glyceraldehyde phosphate dehydrogenase	*Bacillus stearo-thermophilus*	Succinylation of apoenzyme	Thr-150 ?	Loss of activity, Thr adjacent to active site cysteine	175

Glyceraldehyde phosphate dehydrogenase	*Bacillus stearothermophilus*	Potassium [^{125}I]tri-iodide	Tyr, His	Active site Cys protected with tetrathionate	176
Glyceraldehyde phosphate dehydrogenase	Porcine liver	Pyridoxal 5′-phosphate	Lys-212	Inactivation of holoenzyme	177
Glyceraldehyde phosphate dehydrogenase	Yeast, sturgeon, rabbit muscle	2,4,6-Trinitrobenzene sulphonate	Lys	Structure/function	178
Glyceraldehyde phosphate dehydrogenase	Rabbit and porcine muscle	DTNB	Cys		179
Glyceraldehyde phosphate dehydrogenase	Rat skeletal muscle	DTNB	Cys	Nucleotide-dependent inactivation has no effect on the reactivity of the thiols	180
Glyceraldehyde phosphate dehydrogenase	Rat skeletal muscle	2,3-butanedione	Arg	2 Arg per subunit modified	181
Glyceraldehyde phosphate dehydrogenase	Spinach chloroplasts	*p*-Chloromercuribenzoate	Cys	Activation of enzyme exposes buried thiols	182

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Glycogen phosphorylase b	Rabbit muscle	*N*-Acetylimidazole	Tyr	Inactivates reversibly; protected by AMP	183
Glycophorin A	Human erythrocytes	Iodo[^{14}C]acetate	Met-8, 81	Met-81 involved in subunit interactions	184
Gonadotropin	Human chorionic	Tetranitromethane	Tyr	Subunit and receptor binding studies	185
Growth hormone	Human	DTT/bromo[2-^{14}C]acetate	Cys	Accessibility of disulphide bonds probed	186
Growth hormone	Ovine	Thrombin then DTT/iodoacetamide	Cys	Polypeptide chain dissected into 'functional' areas	187
Haemoglobin A	Human	Iodoacetamide	G11(104)-α-Cys	Co-operative oxygen binding decreased	188
Haemoglobins A and S	Human	Potassium cyanate *p*-Mercuribenzoate	α-NH_2 β-93-Cys		189
Haemoglobin S	Human	Potassium [^{14}C]cyanate	α-NH_2	Labelled for determination of the area per molecule	190
Haemoglobin S	Human (sickle)	Methyl isothiocyanate	α-NH_2	Powerful antisickling activity	191
Haemoglobin S	Human (sickle)	Methyl acetimidate	α-NH_2, Lys	Inhibits sickling	192
Haemoglobin S	Human (sickle)	Cystamine	Cys	Inhibits sickling	193
Haemoglobin	Human	[^{14}C]Glucose-6-phosphate	α-NH_2	Affinity label	194
Haemoglobin	Human	Sodium and potassium [^{14}C]cyanate	α-NH_2	Cyanate anion binds to vicinity of Val-1 prior to carbamylation	195
Haptoglobin	Human	Iodoacetamide	Cys	Subunit composition	196
Hemerythrin	*Phascolosoma gouldii*	Various reagents		Reactivity studies	197
Hemopexin	Rabbit	Bromoacetic acid, diethylpyrocarbonate	His	Decreases protein's ability to form a low-spin hemichrome complex	198
Hemopexin	Rabbit	NBS	Trp	4 Trp oxidized	199
Heparin-neutralizing protein	Human platelets	*O*-Methyl isourea	Lys	Decreases activity, heparin protects	200
		1,2-Cyclohexanedione	Arg	No effect on activity	

Histidyl-tRNA synthetase	*Salmonella typhimurium*	DTNB, *p*-hydroxymercuri-benzoate	Cys	SH groups not directly involved in binding of substrate or in catalysis	201
Histones	Calf thymus	Acetic anhydride	Lys	Effect of modification on displaceability from DNA	202, 203
Histones	Calf thymus	Acetic anhydride	Lys	Increased ability to support DNA-dependent RNA synthesis	204
Histones	Calf thymus	[^{14}C]- and [^{3}H]acetic anhydride	Lys	Unmasking of amino-groups at high pH	205
Histone H4	Calf thymus	[^{14}C]-ATP, u.v. light λ 290 nm, acetone as photosensitizer		Adenine moiety linked directly to polypeptide chain	206

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Histones	Calf thymus	Dimethyl suberimidate	Lys	Histone dimers: a stable intermediate in histone assembly ?	207
Histones	Hamster	Photoirradiation		Protein–DNA adducts	208
Histones	HeLa cells	Sodium borohydride reduction after methylation and depurination of DNA		Reduction of Schiff base; histones coupled to DNA	209
Histones	Hepatoma tissue	[^{3}H]Acetate, [^{32}P]phosphate		Natural modification immediately after synthesis	210
Histones	Mouse LA-9 cells	Photoirradiation $\lambda = 280$ nm		Histones 2A and 2B linked	211
Histones	Rat liver	Di-isopropyl fluorophosphate	Ser		212
Histone H3	Rat liver	Histone transacetylase/acetyl coenzyme A	Lys	Sites of enzymic acetylation determined	213
Histones	*Tetrahymena pyriformis*	Sodium [^{3}H]acetate	Lys	*In vivo* experiments	214
Histone F2a1	*Xenopus laevis*	Sodium [^{125}I]iodide/chloramine T	Tyr	Sensitive probe for conformation	215
Histone kinase (cAMP-dependent)	Porcine brain	[^{32}P]-ATP	His	*N*-Phosphohistidine formed	216
L-4-Hydroxymandelate oxidase	*Pseudomonas convexa*	Various thiol reagents	Cys	Inactivate	217
Immunoglobulin G1	Human	Iodoacetate	Cys		218
Immunoglobulin G	Rabbit	Bis(α-bromoacetyl-ε-2,4-dinitrophenyllysylproline) ethylene diamine	Lys, Tyr	Affinity label: dimers and trimers of IgG formed	219
Initiation factor	*Artemia salina*	*N*-Ethylmaleimide	Cys	Destroys ternary complex with GTP and initiator tRNA	220
Initiation factor (IF-E2)	Rabbit reticulocytes	[^{14}C]Formaldehyde/sodium borohydride	Lys	Labels all three proteins, no loss in biological activity	221, 222
Isocitrate dehydrogenase	Porcine heart	Iodoacetate	Cys	Protected by isocitrate	223
Isoleucine tRNA synthetase	*E. coli*	L-[^{14}C]Isoleucyl-bromoethyl ketone	Cys	Affinity label: radioactive peptide isolated	224

Keratin	Chicken feathers	DTT/iodoacetate	Cys	22 different proteins identified	225
2-Keto-3-deoxy-6-phosphogluconic aldolase	*Pseudomonas putida*	[^{14}C]Pyruvate/cyanoborohydride	Lys	Peptide containing labelled Lys sequenced	226
Kininogen (H.M.W.)	Bovine	Citraconic anhydride Chloramine T	Lys Met	Isolation of Ser-Leu-Met-Lys-Bradykinin	227
Lac repressor	*E. coli*	Dimethyl(2-hydroxy-5-nitrobenzyl)-sulphonium bromide, 2-hydroxy-5-nitrobenzyl bromide	Trp	Trp-190 and 209 have limited access to solvent	228
Lac repressor	*E. coli*	2-Mercuri-4-nitrophenol	Cys	2/3 Cys per monomer: reporter group	229
Lac repressor	*E. coli*	3-F-Tyr substitution by growing bacteria in medium containing the modified Tyr		N.m.r. studies: 4 or 5 surface Tyr; 2 buried; 1 with OH ionized or H-bonded	230

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Lac repressor	*E. coli*	2-Chloromercuri-4-nitrophenol	Cys	Reporter group: 2/3 Cys per monomer	231
Lac repressor-Galactosidase (chimera)	*E. coli*	Dimethyl suberimidate	Lys	Dimers only	232
α-Lactalbumin	Human	Diethyl pyrocarbonate	His-32	Inactivation	233
Lactate dehydrogenase	Porcine heart	*N*-Phenyl[2,3-$^{14}C_2$]maleimide	Cys	Affinity chromatography	234
Lactogen	Rabbit	[^{125}I]Iodide/lactoperoxidase	Tyr	Studying binding to various tissues	235
Lactose synthetase	Bovine milk	Dimethyl suberimidate	Lys	Galactosyl transferase and α-lactalbumin cross-linked	236
Lectin	*Lens esculenta*	Maleic anhydride *N*-acetylimidazole	Lys Lys, Tyr		237
Lectin	Pea	2-Nitrophenylsulphenyl chloride	Trp	2/10 Trp react; in heavy subunit sequenced around one Trp	238
Lipase	Porcine pancreas	Diethyl pyrocarbonate	His	Essential His	239
Lipoamide dehydrogenase	Porcine heart	Iodo[^{14}C]acetamide	Cys	Differential reactivity of 2 active-site cysteines, tryptic peptide isolated	240
Lipoxygenase	Soybean	Iodo[^{14}C]acetic acid, pH 3.5	Met	Single Met alkylated, activity lost	241
Luciferase	*Beneckea harveyi*	Diethyl pyrocarbonate	His	Essential His	242
Lutropin	Bovine	Iodoacetate	Cys	Studying accessibility of cystines of α and β subunits	243
Lutropin	Bovine	DTE/Iodoacetate	Cys		244
Lutropin	Bovine	Iodo[^{14}C]acetate	Met	Selective modification of Met	245
Lutropin	Bovine	Iodoacetic acid, pH 3.0	Met	1—2 Met per subunit involved in hormone–receptor binding ?	246
Lutropin	Ovine	1,2-Cyclohexanedione	Arg	Modification of isolated subunits does not affect recombination	247
Lutropin	Ovine	Tetranitromethane	Tyr	Derivative *other* than the 3-nitrotyrosyl identified	248

Lutropin	Ovine	Tetranitromethane	Tyr	Selective nitration affects subunit association	249
Lysozyme	Egg white	7-Chloro-4-nitrobenz-2-oxa-1,3-diazole	Tyr-23, Lys-33	Lys reacts *after* Tyr	250
Lysozyme	Egg white	β-Propiolactone	Met	Met not in antigenic sites	251
Lysozyme	Egg white	Potassium triiodide	Trp, Tyr	^{13}C-N.m.r. used; Trp-108 is *not* oxindolealanine	252
Lysozyme	Egg white	2,3-Dioxo-5-indoline-sulphonic acid	Trp-123	Delineation of antigenic reactive site	253
D-Mannitol dehydrogenase	*Absidia glauca*	Methyl mercurinitrate, *p*-hydroxymercuribenzoate	Cys	Inhibits	254
Membrane proteins	*Achyla ambisexualis*	*p*-Chloromercuribenzene sulphonate	Cys	Mimics cytochalasin A inhibition	255

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Membrane proteins	Bovine adrenal	*N*-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)iodoacetamide, *N*-(1-oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)maleimide	Cys	Spin label	256
Membrane proteins	Chicken erythrocytes	Dimethyl suberimidate	Lys	77 000 polypeptides arranged in envelope as oligomers	257
Membrane proteins	Crayfish muscle	DTNB, *p*-chloromercuribenzoic acid	Cys	2 Cys react in native protein, 6 Cys react in Ca^{2+}-free protein	258
Membrane proteins (Protein 1)	*E. coli* outer cell envelope	CNBr	Met	Analysis of CNBr fragments indicates Ia almost identical with Ib	259
Membrane proteins	*E. coli* (ATPase complex)	Dicyclohexyl[^{14}C]carbodi-imide		Only one protein specifically reacts – isolated and purified	260
Membrane protein (purple)	*Halobacterium halobium*	[^{14}C]Succinic anhydride, sodium borohydride/retinal	Lys	Tryptic succinylated peptide containing retinal binding site isolated	261
Membrane proteins	Human erythrocytes	Protoporphyrin IX/photo-irradiation		Evidence that cross-linking plays a crucial role in cell lysis	262
Membrane proteins	Human erythrocytes	*N*-Dansylaziridine, *N*-(1-anilino-naphthalene-4-)maleimide	Cys	Fluorescent label	263
Membrane proteins	Human erythrocytes	Potassium [^{3}H]borohydride		Hexosyl-lysine is major reducible component	264
Membrane proteins	Human erythrocytes	Substituted maleimides	Cys	New reagents: impermeant to membranes	265
Membrane proteins	Human erythrocytes (muscular dystrophy)	*N*-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)maleimide	Cys	Significant differences in e.s.r. spectra of normal and muscular dystrophy membranes	266
Membrane proteins	Human erythrocyte	Cytochalasin B	Cys	Does *not* bind to high affinity sites ?	267

Membrane proteins	Human erythrocytes	1,5-Diazidonaphthalene, 4,4′-diazidobiphenyl, 4,4′-dithiobisphenylazide/photoirradiation		At low pH cross-linking can occur in the membrane core	268
Membrane proteins	Human platelets	[^{14}C]Glycine ethyl ester/transglutaminase		A fourth glycoprotein identified on platelet membrane	269
Membrane proteins	Rabbit	*N*-[^{14}C]Ethyl maleimide	Cys	Specificity of ATP protection investigated by proteolytic cleavage	270
Membrane proteins	Rabbit	*N*-(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodoacetamide	Cys	Label insensitive to Mg^{2+}, sensitive to Ca^{2+}	271
Membrane proteins	Rabbit	Ethoxyformic anhydride	Lys, His	1 Lys/mol ATPase, 1 or 2 His	272
Membrane proteins	Rat	*N*-[^{14}C]- and -[^{3}H]-ethyl maleimide	Cys	Specific labelling of 1 Cys involved in phosphate transport	273
Membrane proteins	Rat synapse	DTNB	Cys	Proteins are cross-linked by cystine into polymers of >350 000 dalton	274

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Meromysin	Rabbit muscle	*N*-Ethyl maleimide, *p*-phenylenedimaleimide	Cys	Affinity chromatography	275
Messenger ribonucleoprotein (myosin heavy chain)	Chicken embryo	Iodoacetate	Cys	Subunit structure	276
Metallothionein 1B	Horse	4-Vinylpyridine, iodoacetamide	Cys	Sequence studies	277
Mitochondrial pyruvate transporter	Rat liver	α-Cyano-4-hydroxycinnamate	Cys	Specific, reversible	278
Monellin	*Dioscoreophyllum comminsii*	Various thiol reagents CNBr	Met Cys	Abolish sweetness	279
Monoamine oxidase	Bovine liver	*p*-Mercuribenzoate, methyl mercuric iodide, DTNB	Cys	2 essential Cys	280
Myoglobin	Sperm whale	Methyl acetimidate hydrochloride	Lys	Sequence study, tryptic cleavage at Arg only	281
Myoglobin	Sperm whale	[^{13}C]Methyl iodide	Met	^{13}C enrichment for n.m.r.	282
Myosin	Bovine, chicken, rabbit	2,4,6-Trinitrobenzene sulphonate	Lys	Affects binding affinity, improves resolving power of ATP columns	283
Myosin	Chicken, human, rabbit	2,4,6-Trinitrobenzene sulphonate	Lys	Effect on ATPase activity in different tissues studied	284
		N-Ethyl maleimide	Cys	Effect on ATPase activity	
Myosin	Rabbit	6,6′-Dithiobis(inosinyl-5′-yl imidodiphosphate), adenyl-5′-yl imidodiphosphate	Cys	Specific modification of unique light chain Cys	285
Myosin	Rabbit	*N*-Iodoacetyl-*N*-(5-sulpho-1-naphthyl)ethylenediamine	Cys	Fluorescent probe, 2 fast-reacting thiols on heavy chain	286
Myosin	Rabbit	*N*-Ethylmaleimide, *NN′-p*-phenylenedimaleimide	Cys		287
Myosin	Rabbit	*N*-(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodoacetamide	Cys	Reduction of nitroxyl by ascorbate used to study states of subfragment 1	288
Myosin	Rabbit, porcine	*N*-[^{14}C]ethylmaleimide	Cys	Effect on Ca^{2+} and K^+ ATPase activity noted	289

Myosin	Rabbit	Various di-imido esters	Lys	Heavy-chain dimers, extensive cross-linking between globular heads	290
Myosin	Rabbit	*N*-Ethylmaleimide	Cys	Inhibition of relaxation	291
Myosin subfragment I	Rabbit	*N*-Iodo[^{3}H]acetyl-*N'*-(1-sulpho-5-naphthyl)ethylenediamine	Cys	Reactivity of SH_1 decreases on formation of (S1)-F-actin complex	292
Myosin	Various sources	2,4,6-Trinitrobenzene sulphonate *N*-Ethylmaleimide	Lys Cys	Effect on ATPase activities	293
NADH-ubiquinone reductase	Bovine heart	[^{125}I]Iodide/lactoperoxidase	Tyr, His	75 000 and 53 000 peptides labelled	294
Nerve growth factor	Cobra venom	Iodo[^{14}C]acetate	Cys		295
Neurophysins I and II	Bovine	*S*-Adenosyl-L-[*methyl*-^{3}H]-methionine/protein carboxy-methylase	Carboxyls	Kinetic studies of ligand binding	296

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Neurotoxins I, II, and III	*Androctonus australis*	DTT/methyl iodide	Cys	One S—S bridge	297
		Formic acid/dry HCl	Trp	Trp not in active site	
		Glycine ethyl ester	Carboxyls	Suppresses toxicity	
		Various amino reagents	Lys		
Nucleotide pyrophosphatase	Mouse lymphocytes	[^{125}I]Iodide/lactoperoxidase	Tyr	Enzyme located on outer side of membrane	298
Nucleotide pyrophosphatase	Rat liver	[^{125}I]Iodide/lactoperoxidase	Tyr	Differential labelling of enzyme in plasma membranes compared with endoplasmic reticulum	299
Nucleotidyltransferase	Yeast	'Bromoacetamido'-tRNA^{Phe}		1:1 covalent complex	314
Ovalbumin	Egg white	Succinic anhydride	Lys		300
Ovomucoid	Egg white	CNBr	Met	Active fragments produced	301
2-Oxoacid dehydrogenase	*E. coli*	*N*-Ethylmaleimide, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide	Cys	No reaction in absence of substrates, only transacetylase component affected	302
Papain	Papaya latex	Chloromethyl ketone derivatives	Cys-25	*X*-ray crystallography of derivatives	303
Papain	Papaya latex	Methylene blue/photoirradiation	Cys	Active-site Cys oxidized	304
Papain	Papaya latex	1-Ethyl-3-(3-dimethylaminopropyl)-carbodi-imide/ethyl glycinate		Modifies Cys-25, 6/14 CO_2H, 9/19 Tyr, *no* His	305
Papain	Papaya latex	Methyl 4-dimethylamino-3-nitro-(α-benzamido)cinnamate	Cys-25	Resonance Raman studies	306
Papain	Papaya latex	Iodoacetamide, methyl iodide	Cys		307
Papain	Payaya latex	Ninhydrin	Arg	Proposed as a new modification	308
Papain	Papaya latex	3-Bromo-1,1,1-trifluoropropanone	Cys-25	^{19}F n.m.r. study shows co-operative ionization of Asp-158 and His-159	309
Papain	Papaya latex	*SS*-(2,2,2-trifluoro-1,1′-dideuteroethyl)*O*-methyl dithioperoxycarbonate	Cys-25	^{19}F n.m.r.: rates of proton transfer for Asp-158 and His-159 calculated	310
Papain	Papaya latex	Trifluoromethylmercuric trifluoroacetate	Cys-25	^{19}F n.m.r. probe	311

Pepsin	Porcine	Acetylimidazole	Tyr	5/16 Tyr react	312
Phenylalanyl tRNA synthetase	*E. coli*	'Azido'-tRNA^{Phe}/ultraviolet irradiation		Affinity labelling: 0.3 mol reagent per mol enzyme	313
Phosphate transport	Spinach chloroplasts	*p*-(Diazonium)benzene [^{35}S]sulphonate	His, Tyr	29 000 protein labelled	315
Phosphodiesterase (alkaline)	Mouse lymphocytes	[^{125}I]Iodide/lactoperoxidase	Tyr	Enzyme located on outer side of membrane	316
Phosphoenolpyruvate carboxykinase	Rat liver	Acetaldehyde	Cys ?	Inactivation	317
Phosphofructokinase	Rabbit muscle	Iodo[^{14}C]acetate	Cys	No repeat units within subunit	318
Phosphofructokinase	Rabbit muscle	Pyridoxal phosphate/sodium borohydride	Lys	Modified at citrate binding site	319
Phosphofructokinase	Yeast	Dimethyl suberimidate	Lys	Dimers, trimers, and tetramers	320

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
6-Phosphogluconate dehydrogenase	*Candida utilis*	Periodate oxidized NADP/ sodium borohydride	Lys		321
3-Phosphoglycerate kinase		Iodine	Tyr	Unique Tyr, protected by 3-phosphoglycerate	322
Phosphoglycerate kinase	Yeast	2,3-Butanedione	Arg	Essential Arg	323
Phosphoglycerate kinase	Yeast	Tetranitromethane	Tyr	Sulphate ion protects critical Tyr	324
Phosphoglycerate kinase	Yeast	Tetranitromethane	Tyr	4 buried Tyr; selective nitration of 1 Tyr close to MgATP site	325
Phosphoglycerate mutase	Chicken muscle	Glycerate-1,3- and -2,3-diphosphate	His	Enzyme intermediates ?	326
Phosphoglycerate mutase	Rabbit muscle	2,3-Butanedione	Arg	0.13 mM – 1 Arg/subunit 0.5 mM – 3 Arg/subunit	327
Phosphoglycerate mutase	Rabbit muscle	*N*-Bromoacetylethanolamine phosphate	Cys	Inactivates; tryptic peptide containing modified Cys isolated	328
Phosphoglycerate mutase	Yeast	Trinitrobenzene sulphonate	Lys	1 essential Lys per subunit	329
Phospholipase A_2	*Bitis gabonica* venom	*N*-Bromosuccinimide, 2-nitro-phenylsulphenyl chloride	Trp	Trp-28 essential for activity	330
Phosphorylase a and b	Rabbit muscle	*N*-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)iodoacetamide	Cys	E.s.r. spectra analysed with various ligands bound	331
Phosphorylase b	Rabbit muscle	DTNB	Cys	Essential Cys	332
Phosphorylase b	Rabbit muscle	Potassium ferrate	Tyr, Cys	Phosphate analogue: protection against inactivation by nucleotides	333
Phosphorylase b	Rabbit muscle	*N*-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)iodoacetamide	Cys	Heterotropic interactions of ligands studied by e.s.r.	334
Phosphorylase b	Rabbit muscle	*N*-(1-Oxyl-2,2,5,5-tetramethyl-pyrrolidin-3-yl)iodoacetamide, *N*-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidin-3-yl)acetamido-iodoacetamide	Cys	Spin label studies	335
Pinguinain	Pineapple juice	DTNB, *N*-ethylmaleimide	Cys	Enzymatic activity decreased	336

Plastocyanin	*Scenedesmus*	DTNB	Cys	Thiols of different activity	337
Potato protein water	Potato powder	Trinitrobenzene sulphonate	Lys	Determination of protein	338
Procollagen Type III	Bovine foetal	Iodoacetate	Cys	Determination of S—S links	339
Prolactin	Ovine pituitary	Hydrogen peroxide, iodoacetate	Met		340
Proline reductase	*Clostridium sticklandii*	DTNB	Cys	20 thiols	341
Prostaglandin cyclo-oxygenase	Ram seminal vesicles	*N*-Ethylmaleimide	Cys	Disulphide at active site ?	342
Protamine sulphate		1-Ethyl-3-(3-dimethylamino-propyl)carbodi-imide		Dimers, tetramers *etc.*; not trimers, pentamers *etc.*	343
Protease (chromatin bound)	Various sources	Phenylmethanesulphonyl fluoride, di-isopropyl fluorophosphate	Ser	Inactivation	344
		Carbobenzoxyphenylalanine chloromethyl ketone		Inactivation	

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Protease inhibitor (double headed)	Black-eyed pea	Dansyl chloride	Lys	Several residues inaccessible due to subunit interactions ?	345
Protein S-100	Bovine, horse brain	DTNB	Cys	Effects of K^+ and Ca^{2+} on reaction studied	346
Proteoglycan	Porcine cartilage	Various reagents		Effect on hyaluronate binding	347
Purple phosphatase	Porcine uterus	Various thiol reagents	Cys	1 essential Cys	348
Pyrimidine nucleoside monophosphate kinase	Rat liver	Iodoacetate	Cys	Activation of enzyme involves cleavage of S—S bonds	349
Pyruvate carboxylase	Sheep liver	2′,3′-Dialdehyde derivative of ATP/borohydride	Lys	Affinity label	350
Pyruvate dehydrogenase	*E. coli*	*NN′*-Bis-(2-carboximidoethyl) tartarimide dimethyl ester, *NN′*-bis-(2-carboximidomethyl)-tartarimide dimethyl ester	Lys	Periodate-cleavable bis(imido-esters)	351
Pyruvate dehydrogenase	*E. coli*	*N*-Ethyl(2,3-$^{14}C_2$)maleimide, bis(*N*-maleimidomethyl)ether		Lipoate acetyltransferase bears two functionally active lipoic acid residues	352
Pyruvate kinase	Human erythrocytes	*N*-Ethylmaleimide	Cys	Conformation studies	353
Pyruvate kinase	Rabbit muscle	5-Chloro-4-oxo-[3,5-$^3H_2$]-pentanoic acid	Cys	Inactivates enzyme: reaction controlled by a more basic group at the active site ?	354
Quinolinate phospho-ribosyltransferase	Porcine liver	Various reagents			355
Relaxin	Human ovary	*N*-Bromosuccinimide	Trp	One essential Trp	356
Rhodanese	Bovine liver	*N*-Bromosuccinimide *N*-Acetylimidazole	Trp Tyr	Re-examination of aromatic amino-acid content	357
Rhodanese	Bovine liver	DTNB	Cys	2/4 Cys do not react after denaturation	358
Rhodopsin	Bovine retina	[^{3}H]Azidonaphthalene/photo-irradiation		Hydrophobic probe	359
Rhodopsin	Bovine retina	*N*-Bromosuccinnimide	Trp	1 Trp more reactive in bleached compared with unbleached	360

Riboflavin binding protein	Egg white	*N*-Bromosuccinnimide	Trp	1 to 2 essential Trp accessible	361
Ribonuclease A	Bovine pancreas	[^{3}H]- and [^{14}C]-acetic anhydride	Lys	Double labelling	362
Ribonuclease A	Bovine pancreas	Dimethyl suberimidate and related compounds	Lys	Activity of cross-linked products toward d.s. RNA studied	363
Ribonuclease A	Bovine pancreas	Fluorescein isothiocyanate	α-NH_2	pK of fluorescein used as index of conformation	364
Ribonuclease A	Bovine pancreas	Cytidine and uridine 2′(3′),5′-diphosphate/photoirradiation		Peptide linked to nucleotide: identified as Asn-67 to Arg-85	365
Ribonuclease A	Bovine pancreas	1-Guanyl-3,5-dimethylpyrazole, pyridoxal 5′-phosphate/sodium borohydride	Lys		366
Ribonuclease A	Bovine pancreas	Tetranitromethane followed by sodium hydrosulphite	Tyr	Fluorescence study using the aminotyrosine residues	367

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ribonucleases H_1 and H_2	Yeast	*N*-Ethylmaleimide	Cys	H_1 inactivated; H_2 unaffected	368
Ribonucleoside diphosphate reductase	*E. coli*	2′-Deoxy-2′-chloro-ribonucleoside diphosphate, 2′-deoxy-2′-azido-ribonucleoside diphosphate	Cys	Cys oxidation: active site formed by both B1 and B2 subunits	369
Ribosomes	*E. coli*	*p*-Chloromercuri[^{14}C]benzoate	Cys	Gel electrophoresis modified to ensure stability	370
Ribosomes	*E. coli*	U.v. irradiation		Covalent cross-links of RNA with unique ribosomal proteins	371
Ribosomes	*E. coli*	*p*-Nitrophenylchloroformate	Lys	EFTu cross-linked to L1, L5, L7/L12, L15, L20, L30, L33	372
Ribosomes	*E. coli*	Monoiodoamphenicol		Labels S6, L16, L24	373
Ribosomes	*E. coli*	*p*-Azido-*N*-tBocPhe-[^{35}S]Met-tRNA^{met}/photoirradiation		Label mostly on S18	374
Ribosomes	*E. coli*	[^{131}I]Iodide/lactoperoxidase	Tyr	Iodination of 5 ribosomal proteins altered following 30S-IF3 interaction	375
Ribosomes	*E. coli*	U.v. irradiation of 16S RNA-S7 complex		Photocross-links: regions cross-linked identified after tryptic digest	376
Ribosomes	*E. coli*	5-Bromoacetamido uridine 5′-phosphate bonded to 3′-end of A-U-G		Cross-linking mainly to S1	377
Ribosomes	*E. coli*	U.v. irradiation		Ribosome-dependent photo-induced chain breakage of the 16S rRNA	378
Ribosomes	*E. coli*	5′-4-(Bromo[2-^{14}C]acetamido)-phenylphospho-uridylyl-(3′-5′)-guanylyl-(3′-5′)adenosine		Predominant cross-linking of UGA analogue to S4 and S18	379
Ribosomes	*E. coli*	Methyl-4-mercaptobutyrimidate	Lys	21 protein pairs identified	380
Ribosomes	*E. coli*	3,3′-Dithiobispropionimidate	Lys	S4–S5–S8, S2–S3, S4–S5, and S5–S8 cross-linked	381
Ribosomes	*E. coli*	G-U-U-5-iodo[^{3}H]acetylamino-uridine		S18 and S1 are constituents of 30S ribosomal decoding site; affinity label	382

Ribosomes	*E. coli*	U.v. light on 30S subunits		16S RNA–S7 covalent complex isolated; site of cross-linking to 16S RNA identified	383
Ribosomes	*E. coli*	[^{125}I]Iodide/lactoperoxidase	Tyr	Surface topography of 30S subunits studied	384
Ribosomes	*E. coli*	Dimethyl adipimidate	Lys	L7/L12–L10 and L7/L12–L11 cross-links	385
Ribosomes	*E. coli*	Methyl-4-mercaptobutyrimidate	Lys	L23, L28, L1, L3, L24 cross-links to EFTu	386
Ribosomes (L7/L12)	*E. coli*	Dimethyl suberimidate		L7 and L12 exist in solution as dimers	387
Ribosomes (L7/L12)	*E. coli*	Dimethyl-3,3′-dithiobis-propionimidate	Lys	Cross-links between L7/L12 and IF2	388
Ribosomes (L27)	*E. coli*	*p*-Nitrophenylcarbamyl-tRNA^{met}	Lys	Affinity label	389
Ribosomes	Rabbit reticulocyte	*N*-(3-Pyrene)maleimide	Cys	Fluorescent probe	390

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ribosomes	Rat liver	2-Methoxy-5-nitrotropone	Lys	L21 and/or L26 in the peptidyl transferase centre ?	391
Ribosomes	*E. coli*	(2-Nitro-4-azidobenzoyl)-hydrazone of periodate-oxidized GTP, guanosine 5′-(α,β-methylene)-triphosphate plus the γ-(4-azido-benzyl) amide of GTP; each reaction is followed by photo-irradiation		Photoaffinity reagents	392
Ribulose-1,5-bisphosphate carboxylase	Spinach leaves	Glycidate(2,3-epoxypropionate), iodoacetamide	Cys	Oxygenase activity inhibited	393
RNase A		Ethyl trifluorothioacetate	Lys	S-peptide analogues, recognition of S-protein	394
RNA polymerase	*E. coli*	5-Formyl-1-(α-D-ribofuranosyl)-uracil 5′-triphosphate/sodium borohydride	Lys	Inhibits *via* Schiff base	395
RNA polymerase	*E. coli*	*N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulphonate DNSCl	Cys	Unique Cys Surface labelling	396
RNA polymerase	*E. coli*	[^{14}C]phenylglyoxal	Arg	Essential Arg, corrected to 10 Arg in second reference	397, 398
RNA polymerase I	Rat liver	[γ-^{32}P]-ATP and N^6,$O^{2'}$-dibutyryl-cyclic 3′,5′-AMP	Ser, Thr	^{32}P on enzyme subunits SA1, SA3, SA5, and SA6	399
RNA replicase	Phage Qβ	Dimethyl suberimidate	Lys	Quaternary structure changes with ionic strength and addition of Qβ RNA	400
Selenoprotein from glycine reductase	*Clostridium sticklandii*	Various alkylating agents, selenocystine	Se	Selenium behaves analogously to Cys	401
D-Serine dehydratase	*E. coli*	Butanedione, [^{14}C]phenylglyoxal	Arg	Essential Arg at pyridoxal binding site	402
D-Serine dehydratase	*E. coli*	Pyridoxal phosphate/sodium borohydride	Lys	Tryptic peptide containing bound pyridoxal phosphate isolated	403

D-Serine transhydroxymethylase	*E. coli*	Pyridoxal phosphate/sodium borohydride	Lys	Pyridoxal phosphate binding site sequenced	404
Subtilisin BPN'		L-Phenylalanyl-L-alanyl-L-lysine chloromethyl ketone	His-64, Ser-221	Mechanism for halogenomethyl ketone inactivation of serine proteases suggested	405
Subtilisin inhibitor	*Streptomyces albogriseolous*	Various reagents		Met essential for activity	406
Succinate dehydrogenase	Bovine heart	*N*-Ethylmaleimide	Cys	Site-directed inhibitor ?	407
Succinate dehydrogenase	Bovine heart	*N*-[^{14}C]Ethylmaleimide and 3-(maleimidomethyl)-2,2,5,5-tetramethylpiperidinoxyl	Cys	E.s.r. study: two types of thiol group	408
Succinic thiokinase	*E. coli*	Permanganate	Cys	Selective oxidation	409
Sucrase–isomaltase complex	Rabbit intestine	[^{3}H]conduritol-β-epoxide	Asp	Label bound to β-carboxyl	410
Taipoxin	*Oxuryanus s. scutellatus*	Iodo[^{14}C]acetamide	Cys	Sensitive detection of subunits	411

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Thrombin	Bovine	1: Di-isopropylfluorophosphate; 2: [^{125}I]iodide/chloramine T Glutaraldehyde	1: Ser 2: Tyr	[^{125}I]DIP-thrombin used for platelet binding studies Cross-linking of labelled thrombin to platelet	412
Thrombin	Human	Various reagents		Active site Ser and His	413
Thrombin	Rabbit	[^{3}H]Di-isopropylfluorophosphate	Ser	DIP-thrombin binds to Sepharose	414
Thymidylate synthetase	*Lactobacillus casei*	5′-Fluoro-2′-deoxyuridylate-[^{14}C]-5,10-methylenetetrahydrofolate/Raney nickel		Carbon–sulphur bonds cleaved	415
Thymidylate synthetase	*Lactobacillus casei*	Iodo[^{14}C]acetic acid, 5-fluoro-[2-^{14}C]deoxyuridine 5′-phosphate	Cys	Labelled CNBr peptide isolated and partly sequenced	416
Thymidylate synthetase	*Lactobacillus casei*	5-Fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate	Cys	N.m.r. evidence for covalent binding	417
Thymidylate synthetase	*Lactobacillus casei*	5-Fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate	Cys	Labelled peptide isolated	418
Thyroglobulin	Human (goitre)	[^{125}I]Iodide		25–30 I atoms incorporated/mol	419
Thyroglobulin	Human and rat	DTT/iodoacetamide	Cys	Tetrameric structure	420
α-Toxin	*Staphylococcus aureus*	[^{125}I]Iodide/lactoperoxidase	Tyr	Unique Tyr: enables resolution of biological and cell binding activities	421, 422
Troponin	Rabbit	*N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, *S*-mercuric-*N*-dansyl cysteine	Cys	Modified protein has same Ca^{2+} binding properties as unlabelled	423
Trypsin	Bovine	Sodium borohydride followed by iodoacetamide, iodoacetic acid, or ethyleneimine	Cys-179, Cys-203	Enzyme specificity changes	424
Trypsin–chymotrypsin inhibitor	Chick pea	CNBr	Met	Biological activities dissected; one CNBr peptide inhibits trypsin, the other chymotrypsin	425
Trypsin inhibitor	Bovine pancreas	Formaldehyde/borohydride	Lys	Intramolecular interaction of Lys-41 with Tyr-10	426

Trypsin inhibitor	Bovine pancreas	Iodoacetamide after DTT	Cystine-14—38	N.m.r. studies	427
Trypsin inhibitor	Soybean	*O*-Methylisourea Cyanate	Lys α-NH_2	Protein further modified by conversion of Ile-64 into Ala	428
Trypsin inhibitor (Bowman–Birk)	Soybean	*N*-Acetylimidazole	Tyr	Origins of the u.v. c.d. bands identified	429
Trypsin-like proteases		*p*-Nitrophenyl ester of *p*-amidinophenylmethane sulphonic acid	Ser	Utilizing differences in leaving group to produce a selective affinity label for the different proteases	430
Trypsinogen	Bovine	Sodium borohydride/iodoacetate	Cys	Refolding of immobilized *S*-(carboxymethyl)$_2$-trypsinogen	431
Trypsinogen	Bovine	Maleic anhydride	Lys	Mutual exclusion in tryptic digestion: half of molecules cleave at each Arg	432

[412] D. M. Tollefsen and P. W. Majerus, *Biochemistry*, 1976, **15**, 2144.
[413] K. H. Mrymel, R. L. Lundblad, and K. G. Mann, *Biochemistry*, 1976, **15**, 1767.
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[420] R. Pitt-Rivers, *Biochem. J.*, 1976, **157**, 767.
[421] P. Cassidy and S. Harshman, *Biochemistry*, 1976, **15**, 2342.
[422] P. Cassidy and S. Harshman, *Biochemistry*, 1976, 2348.
[423] J. D. Potter, J. C. Seidel, P. Leavis, S. S. Lehrer, and J. Gergely, *J. Biol. Chem.*, 1976, **251**, 7551.
[424] R. J. Knights and A. Light, *J. Biol. Chem.*, 1976, **251**, 222.
[425] P. Smirnoff, S. Khalef, Y. Birk, and S. W. Applebaum, *Biochem. J.*, 1976, **157**, 745.
[426] L. R. Brown, A. de Marco, G. Wagner, and K. Wuthrich, *European J. Biochem.*, 1976, **62**, 103.
[427] G. H. Snyder, R. Rowan, and B. D. Sykes, *Biochemistry*, **15**, 2275.
[428] D. Kowalski, and M. Laskowski jun., *Biochemistry*, 1976, **15**, 1300.
[429] E. Kay, *J. Biol. Chem.*, 1976, **251**, 3411.
[430] S.-C. Wong and E. Shaw, *Arch. Biochem. Biophys.*, 1976, **176**, 113.
[431] A. Light and N. K. Sinha, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 1188.
[432] T. A. Grooms, and J. D. Ogle, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 876.

Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Tryptophanyl tRNA synthetase	*E. coli*	Various thiol reagents	Cys	'Required' Cys located in tryptic peptide	433
Tryptophanyl tRNA synthetase	*E. coli*	DTNB, (^{14}C)iodoacetic acid	Cys	Inactivates enzyme	434
Tubilin	Bovine brain	Diethyl pyrocarbonate, photo-oxidation	His	Self assembly inhibited	435
Urease	*Canavalia ensiformis*	2,2′-Dipyridyl disulphide	Cys	3 classes of thiol groups	436
Urease	Jack bean	β-1-[3,3-Dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyrene)]-propionic anhydride		Photochromic reagent	437
Uridine diphospho-glucose dehydrogenase	Bovine liver	Iodoacetic acid, iodoacetamide	Cys	Half-of-the-sites reactivity	438
Urocanase	*Pseudomonas putida*	4-Bromocrotonate		Irreversibly inactivates	439
Urokinase	Human	Acetyl-Gly-Lys-CH_2Cl, Nle-Gly-Lys-CH_2Cl	His	Affinity labels, Nle identified in heavy chain	440
β-D-Xylosidase	*Bacillus pumilus*	*p*-Azidophenyl xylopyranoside		Pseudo-photoaffinity label	441

[433] G. V. Kuehl, M. L. Lee, and K. H. Muench, *J. Biol. Chem.*, 1976, **251**, 3254.
[434] M. S. Lipscomb, M. L. Lee, and K. H. Muench, *J. Biol. Chem.*, 1976, **251**, 3261.
[435] Y. C. Lee, L. L. Houston, and R. H. Himes, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 50.
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[438] J. S. Franzen, R. Ishman, and D. S. Feingold, *Biochemistry*, 1976, **15**, 5665.
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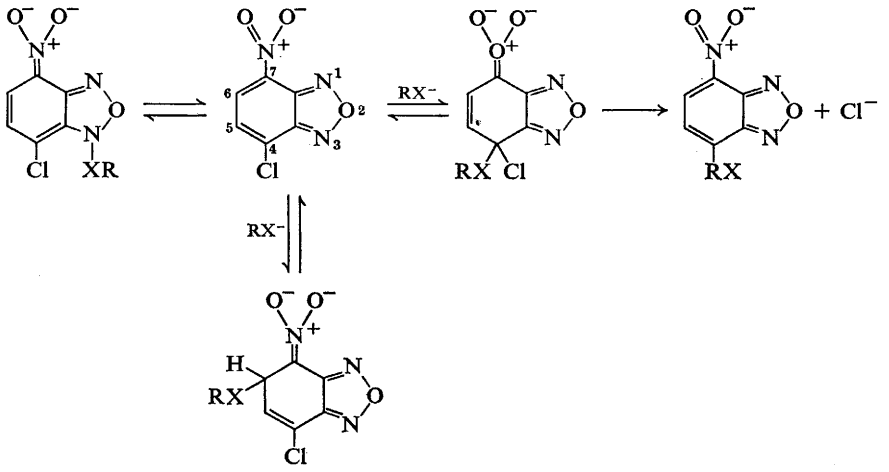
[440] E. B. Ong, A. J. Johnson, and G. Schoellmann, *Biochem. Biophys. Acta*, 1976, **429**, 252.
[441] E. Saman, M. Claeyssens, H. Kersters-Hilderson, and C. De Bruyne, *F.E.B.S. Letters*, 1976, **63**, 211.

1 Reinvestigation of Known Reagents

Several modification procedures have recently come under scrutiny.

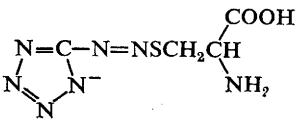
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl).—This reagent, commonly used for the fluorescent labelling of proteins, is attacked by nucleophiles which displace the chlorine atom at position C-4. The reaction probably occurs rapidly through a Meisenheimer intermediate at position C-4,[442] although this may well be preceded by reversible reaction at other electrophilic centres, such as N-3 or C-6 (Scheme 1).[442a] Adduct formation by a *second* protein nucleophilic group



Scheme 1

at one of these other centres *before* the alkylation at C-4 might well dictate the site in the protein alkylated. Alternatively, adduct formation by the second nucleophile *after* alkylation at C-4 would be expected to change the spectrum of the fluorophore from that of a simple 4-substituted nitrobenzofurazan.

Diazonium 1*H*-tetrazole.—Normally assumed to react with lysine, histidine, and tyrosine residues, this reagent also appears to react selectively with Cys-174 of alcohol dehydrogenase to give *S*-(5-tetrazoleazo)cysteine (1) or with 2-mercapto-

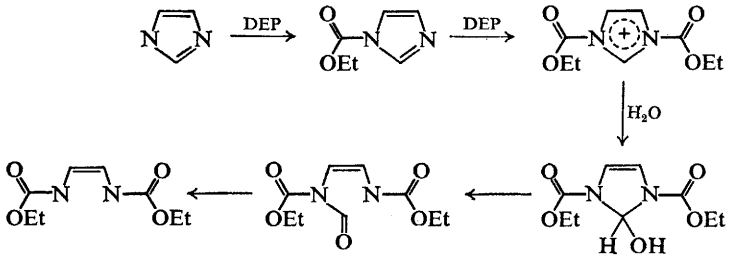


(1)

ethanol to give 5-tetrazoleazo-2-thioethanol. The azothiol can be destroyed by irradiation at 320 nm or by exposure to extremes of pH.[443]

[442] M. Shipton, T. Stuchbury, and K. Brocklehurst, *Biochem. J.*, 1976, **159**, 235.
[442a] B. S. Baines, G. Allen, and K. Brocklehurst, *Biochem. J.*, 1977, **163**, 189.
[443] D. C. Sogin and B. V. Plapp, *Biochemistry*, 1976, **15**, 1087.

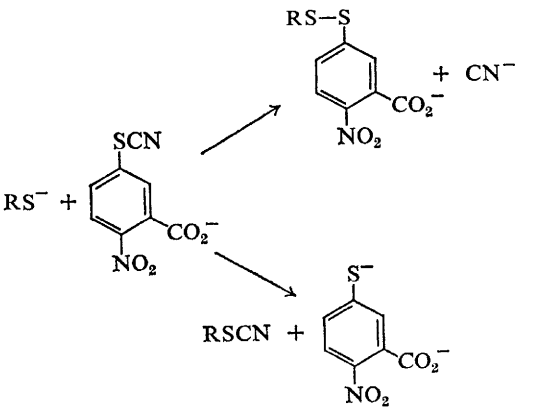
Diethyl Pyrocarbonate.—The *N*-carbethoxylation of histidine residues by excess diethyl pyrocarbonate can apparently proceed to cleavage of the imidazole ring. The isolation of *cis-NN'*-dicarbethoxy-*N*-formyl-1,2-diaminoethene and *cis-NN'*dicarbethoxy-1,2-diaminoethene as products from the addition of excess diethyl pyrocarbonate to imidazole suggests that a Bamberger cleavage of the imidazole ring has occurred, probably by attack of water on C-2 of a *NN'*-dicarbethoxy-imidazolium ion (Scheme 2). Hydroxylamine quantitatively



Scheme 2

removes the *N*-carbethoxy-group from histidine (regenerating histidine), but it cannot reverse the Bamberger cleavage. Hence the irreversible loss of histidine residues after hydroxylamine treatment of diethyl pyrocarbonate modified protein would determine the extent of imidazole cleavage.[444]

2-Nitro-5-thiocyanatobenzoic Acid.—The cyanylation of cysteinyl residues by 2-nitro-5-thiocyanatobenzoic acid has proved useful not only for delineating the role of cysteine in proteins, but also as a way of cleaving the polypeptide chain at cysteine. It appears, however, that the reaction of 2-nitro-5-thiocyanato-benzoic acid with cysteine can also produce the mixed disulphide (Scheme 3). The precise ratio of the two products depends on the protein concerned. This

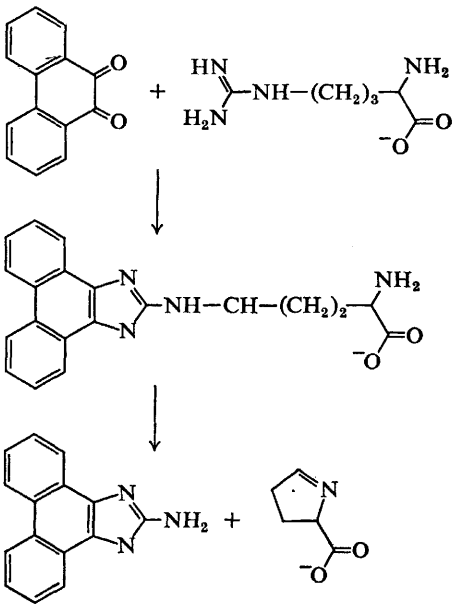


Scheme 3

[444] M. J. Loosemore and R. F. Pratt, *F.E.B.S. Letters*, 1976, **72**, 155.

stresses the importance of quantitating the yield of the *S*-cyano-derivative (by measuring incorporation of ^{14}C cyanide from the radioactively labelled reagent) and the yield of the mixed disulphide (by spectrophotometry). The formation of the *S*-cyano-derivative can be favoured by addition of excess cyanide to the reaction mixture.[445]

Phenanthraquinone.—The reaction of arginine with phenanthraquinone to give a highly fluorescent product is commonly used for specific identification of arginine-containing peptides. The products have now been identified as 2-amino-1*H*-phenanthro[9,10-*d*]imidazole (fluorescent) and Δ^1-pyrroline-5-carboxylic acid, and the reaction occurs through a Schiff base intermediate (Scheme 4).[446]



Scheme 4

Succinic Anhydride.—Apo-glyceraldehyde phosphate dehydrogenase is rapidly inhibited by small amounts of succinic anhydride, and it was possible to narrow down the critical residue to one of two threonines adjacent to the active-site cysteine residue. It proved impossible to identify the precise threonine labelled, as the Edman degradation persistently blocked on approaching the critical area. It was suggested that O → N migration of the succinyl group blocked the newly exposed amino-group of *O*-succinylthreonine.[447]

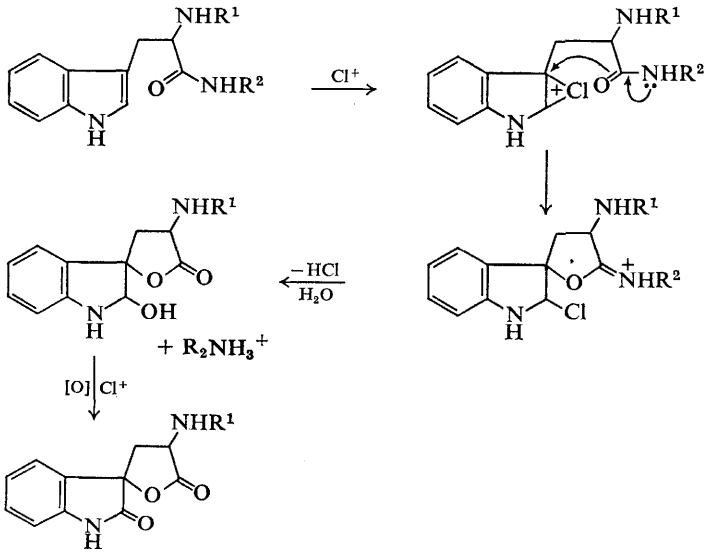
Tetranitromethane.—Tetranitromethane is customarily assumed to react with tyrosyl residues to generate the 3-nitro-tyrosyl derivative; however, after the reaction of lutropin with tetranitromethane and proteolytic digestion, peptides

[445] N. C. Price, *Biochem. J.*, 1976, **159**, 177.
[446] H. A. Itano, K. Hirota, I. Kawasaki, and S. Yamada, *Analyt. Biochem.*, 1976, **76**, 134.
[447] G. Allen and J. I. Harris, *European J. Biochem.*, 1976, **62**, 601.

could be isolated which contained some other tyrosine derivative. The derivative did not react with fluorescamine or ninhydrin in the usual way, but did appear to undergo a normal Edman degradation. The authors suggest that the derivative may therefore be a secondary amino-compound, analogous to proline.[448]

2 New Reagents and Techniques

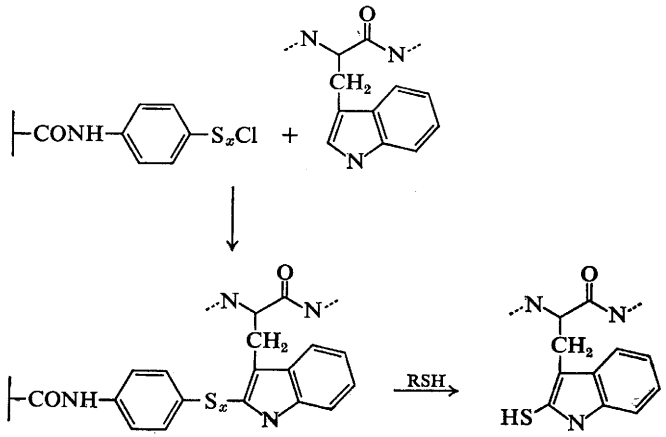
Several new reagents have been used for the modification of tryptophan: *N*-chlorosuccinimide has been suggested in preference to *N*-bromosuccinimide for the selective cleavage of tryptophanyl bonds. Two equivalents of chlorine are consumed during the cleavage and the indole ring is oxidized to an oxindole derivative (Scheme 5). Although the yields of cleavage of tryptophanyl bonds



Scheme 5

(35—45%) are lower than those with *N*-bromosuccinimide, only tryptophanyl bonds are split and the only other side-chains oxidized are methionine and cysteine.[449] It remains to be seen whether the reagent has any significant advantages over BNPS-Skatole. 2,3-Dioxo-5-indoline sulphonic acid (reported 1975) has now been seen in action against lysozyme, where it selectively modifies one of the more exposed tryptophanyl residues (Trp-123).[450] Novel reagents, polymeric analogues of aryl sulphenyl halides, have been developed for the reversible binding of tryptophanyl residues. Tryptophan-containing peptides, present in peptide mixtures, were selectively immobilized on the polymers, then stripped off by simple thiols to emerge as 2-thiotryptophan peptides (Scheme 6).[451]

[448] B. D. Burleigh, W. K. Liu, and D. N. Ward, *J. Biol. Chem.*, 1976, **251**, 308.
[449] Y. Schechter, A. Patchornik, and Y. Burstein, *Biochemistry*, 1976, **15**, 5071.
[450] M. Z. Atassi and W. Zablocki, *J. Biol. Chem.*, 1976, **251**, 1653.
[451] M. Rubinstein, Y. Schechter, and A. Patchornik, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 1257.



Scheme 6

Tryptophan, and peptides containing *N*-terminal tryptophan, react with fluorescamine to form derivatives which have a high fluorescence in strong acid. This has been used to measure tryptophan in the alkaline hydrolysates of proteins.[452]

N-[^{14}C]Acetoxybenzotriazole has been proposed as a new reagent for the acetylation of proteins: the acetyl group is preferentially transferred to nucleophilic centres such as alcohols or amines, rather than to water.[453]

Dimethyl sulphate has been proposed for selectively converting cysteinyl residues into *S*-methylcysteine, thereby introducing a bond sensitive to cyanogen bromide. *S*-Methylcysteine is relatively stable to acid hydrolysis and can be quantitated directly from the amino-acid analysis.[454] However, with slightly more vigorous conditions, other workers have successfully methylated tobacco mosaic virus coat protein on the phenol groups of Tyr-139 and Tyr-72, and at Lys-68 as well as at Cys-27.[455] Furthermore, the cyanogen bromide cleavage of *S*-methylcysteine is not straightforward and involves two competing mechanisms of cleavage, one of which may give rise to an *N*-terminally blocked peptide.[456]

Benzyl bromide has been used for the selective alkylation of methionine to *S*-benzylmethionine.[457] The extent of alkylation can be determined by performic acid oxidation followed by acid hydrolysis and amino-acid analysis. Methionine sulphonium salts, but not methionine, resist performic acid oxidation; the protected *S*-benzylmethionine residues are then converted into free methionine by acid hydrolysis. Hence the methionine in the amino-acid analysis reflects the methionine originally alkylated. It is interesting that the *S*-benzylmethionine should break down quantitatively to methionine on acid hydrolysis: *prima facie* sulphonium salts would be expected to cleave to alcohol and thioether in three

[452] H. Nakamura, and J. J. Pisano, *Arch. Biochem. Biophys.*, 1976, **172**, 98.
[453] M. Reboud-Ravaux and C. Ghelis, *European J. Biochem.*, 1976, **65**, 25.
[454] J. Eyem, J. Sjodahl, and J. Sjoquist, *Analyt. Biochem.*, 1976, **74**, 359.
[455] H. J. Staab and F. A. Anderer, *Biochim. Biophys. Acta*, 1976, **427**, 453.
[456] E. Gross and J. L. Morell, *Biochem. Biophys. Res. Comm.*, 1974, **59**, 1145.
[457] G. A. Rogers, N. Shaltiel, and P. D. Boyer, *J. Biol. Chem.*, 1976, **251**, 5711.

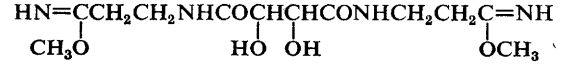
different ways. Presumably the greater stability of the intermediate benzylic carbonium ion restricts the other modes of cleavage.

3 Chemical Cross-linking

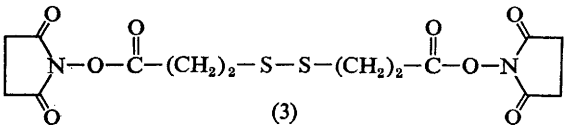
Cross-linking with bifunctional reagents has been used to determine the symmetry of oligomeric proteins and in particular to distinguish between heterologous and isologous tetramers.[458] The main assumption is that cross-linking across one symmetry axis does not influence that across another. By quantitating the monomer, dimer, trimer, and tetramer species on an SDS-polyacrylamide gel, the ratio (k_p/k_q) of the rate constants of cross-linking along two of the two-fold symmetry axes (P and Q) can be calculated. Since $k_p \neq k_q$ for an isologous tetramer such as a dimer of dimers, whereas $k_p = k_q$ for a heterologous tetramer, the equality or non-equality of k_p and k_q is decisive in distinguishing between heterologous and isologous associations. When applied to aldolase and lactate dehydrogenase, both isologous tetramers, the ratio of k_p to k_q was found to decrease with increasing chain length of the bifunctional reagent. Thus the shorter the bifunctional reagent (provided it can make cross-links at all) the greater the chance of discriminating between different symmetries.

Dithiophosgene (2,2,4,4-tetrachloro-1,3-dithiacyclobutane) and its derivative 2,2-dichloro-1,3-dithiacyclobutanone will react with a variety of amines to form the corresponding disubstituted thiuram compounds.[459] They have been shown to react at least monofunctionally with cathepsin D,[460] and it is therefore legitimate to speculate that the reagents could form cross-links between proteins.

The synthesis of three cleavable cross-linking reagents has been described: periodate cleavable bis(imido-esters) have been synthesized by using carbodiimide to couple tartaric acid and 2-aminopropionitrile. The resultant *NN'*-bis-(2-cyanoethyl)tartaramide is converted into *NN'*-bis-(2-carboximidoethyl)tartarimide dimethyl ester (2) by methanol–HCl.[461] Such bis(imido-esters)



(2)



(3)

with a periodate-cleavable *vic*-glycol bond offer alternative warheads to the periodate-cleavable bis(azides) already described.[462] An improved synthesis of cleavable disulphide bifunctional imido-esters, such as dimethyl 3,3-dithiobispropionimidate, uses ω-olefinic nitriles and ammonium polysulphide to

[458] J. Hajdu, F. Bartha, and P. Friedrich, *European J. Biochem.*, 1976, **68**, 373.
[459] J. Wortmann and G. Gartow, *Z. anorg. Chem.*, 1970, **377**, 79.
[460] E. T. Rakitzis and T. B. Malliopoulou, *Biochem. J.*, 1976, **153**, 737.
[461] J. R. Coggins, E. A. Hooper, and R. N. Perham, *Biochemistry*, 1976, **15**, 2527.
[462] L. C. Lutter, F. Ortanderl, and H. Fasold, *F.E.B.S. Letters*, 1974, **48**, 288.

produce the dithiobisnitrile. These are converted into the dithiobisimido-ester by methanol–HCl.[463] The synthesis of ^{35}S-labelled dithiobis(succinimidyl-propionate) (3), another cleavable disulphide cross-linker, has been described. 3,3′-[^{35}S]dithiopropionic acid, obtained by the alkylation of sodium [^{35}S]-disulphide with propiolactone, was coupled to *N*-hydroxysuccinimide using carbodi-imide. This reagent has great advantages over the bifunctional imido-esters, in its mild reaction conditions (pH 7.0 rather than 8.5) and its long solution half-life.[464]

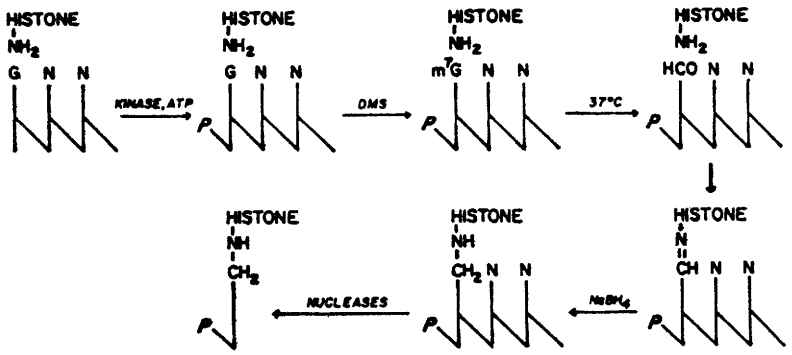


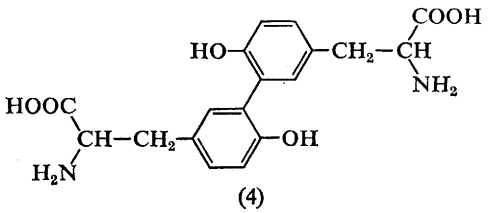
Figure 1 *Scheme for labelling the 5′-termini of nucleosome DNA with* ^{32}P *and cross-linking the labelled phosphate to a neighbouring histone lysyl residue. DMS is dimethyl sulphate*
(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1976, 73, 4400)

A method of chemically cross-linking protein and DNA has been used to show that histones H3 and H4 occur with equal frequency as the nearest protein neighbours to the 5′-end of DNA in nucleosomes. The DNA is labelled at its 5′-terminus with ^{32}P using [γ-^{32}P]ATP and polynucleotide kinase. Purines at the 5′-termini are methylated with dimethyl sulphate and then depurinated; the aldehyde so created reacts with a nearby histone lysyl residue to form a Schiff base. Reduction by sodium borohydride fixes the cross-link, and the non-cross-linked DNA is digested with DNase I and venom phosphodiesterase. The histone is thus labelled with ^{32}P and can be identified by autoradiography (Figure 1).[465]

4 Enzymic Cross-linking

Enzymic oxidation of proteins with peroxidase and hydrogen peroxide at basic pH can lead to an oxidative coupling of adjacent tyrosyl residues. The dityrosine cross-links (4) can be identified by their fluorescence, by thin-layer chromatography, or by amino-acid analysis.[466]

[463] H. Peretz and D. Elson, *European J. Biochem.*, 1976, 63, 77.
[464] A. J. Lomant and G. Fairbanks, *J. Mol. Biol.*, 1976, 104, 243.
[465] R. T. Simpson, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, 73, 4400.
[466] R. Aeschbach, R. Amado, and H. Neukom, *Biochim. Biophys. Acta*, 1976, 439, 292.



(4)

5 Photocross-linking

The photocross-linking of simple nucleotides and nucleic acids to proteins has been extensively investigated, and several possible side-reactions noted, *viz.*, photoinduced breakdown of polypeptide chain [467] and ribonucleic acid,[468] and protein–protein cross-linking.[469] In the irradiation of chromatin, the extent of protein–protein cross-linking depends on the wavelengths used. At $\lambda = 280$ nm the main product is the cross-linked dimer of histones 2A and 2B, although at $\lambda = 254$ nm DNA–histone cross-linking predominates.[469] DNA is generally less photoreactive than RNA, but can be photosensitized by incorporation of 5-bromo-2′-deoxyuridine; using this strategy the cross-linking of non-histone chromosomal proteins to DNA could be increased dramatically.[470]

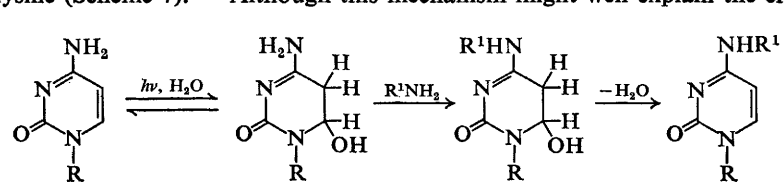
Ribosomal RNA has been cross-linked to several ribosomal proteins. The extent of cross-linking (at a given dose of irradiation) for each protein component of the 50S ribosome was determined by quantitating the amount of each (non-cross-linked) component remaining on a two-dimensional polyacrylamide gel after irradiation. The method assumes that no protein–protein cross-linking occurs and that components cross-linked to RNA, due to the polyanionic nature of nucleic acid, do not migrate from the positively charged origin of the second gel dimension at pH 4.6. It was found that one group (L2, L4, L5, L7, L10, L12, L16—19, L21, L22, L28, and L29) cross-linked at a relatively low dose and of these a sub-group (L2, L4, L5, L16, L21, and L29) cross-linked with single hit kinetics. It was suggested that this sub-group not only binds ribosomal RNA, but that the reactive groups of both the protein and nucleic acid are in close proximity.[471] A more direct way of identifying cross-linked ribosomal components involved labelling the nucleic acid with ^{32}P, photocross-linking, and cutting off the excess nucleic acid with RNases A and T_1. The autoradiography of the two-dimensional polyacrylamide gel showed that L2 and possibly L4 and S8 were now labelled with ^{32}P. One difficulty with this strategy lay in rigorously identifying the ^{32}P-labelled proteins since their electrophoretic mobilities had changed owing to the attached RNA fragments.[472]

Similarly those precise parts of a polypeptide chain which are photocross-linked have been identified both by direct and indirect means. In the indirect approach, the *absence* of certain peptides from the tryptic fingerprints of

[467] J. E. Celis, M. Fink, and K. Kaltoft, *Nucleic Acid Res.*, 1976, **3**, 1065.
[468] L. Gorelic, *Biochemistry*, 1976, **15**, 5474.
[469] H. G. Martinson, M. D. Shetlar, and B. J. McCarthy, *Biochemistry*, 1976, **15**, 2002.
[470] J. N. Lapeyre and I. Bekhor, *J. Mol. Biol.*, 1976, **104**, 25.
[471] L. Gorelic, *Biochim. Biophys. Acta*, 1976, **454**, 185.
[472] O. G. Baca and J. W. Bodley, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 1091.

photocross-linked S7—16S RNA was used to identify four peptides which must have photocross-linked.[473] In the direct approach, a single ^{14}C-labelled tryptic peptide corresponding to residues Asn-67 to Arg-85 could be isolated from the photocross-linked [^{14}C]cytidine 2′(3′),5′-diphosphate– or uridine 2′(3′),5′-diphosphate–RNase A complex. It is known from the crystallographic model of RNase A that part of this peptide lies close to the enzyme's binding site for pyrimidines. During the photocross-linking, or work-up of the peptide, the phosphate moieties of the nucleotide were lost, suggesting that use of ^{32}P-labelled nucleotide might prove hazardous. The authors used wavelengths >300 nm but with acetone as a photosensitizer in preference to direct irradiation at 254 nm, and thus obtained fewer protein–protein cross-linked aggregates. They reason that at 254 nm *both* nucleic acid and enzyme are excited, while at 300 nm acetone absorbs most of the radiation and transfers it mainly to the nucleic acid.[474]

The structure of a photocross-link between nucleic acid and protein has been described for the first time: after photocross-linking bacteriophage MS2 RNA to coat protein at $\lambda = 254$ nm, ε-*N*-(2-oxopyrimidyl-4)-lysine could be isolated from the acid hydrolysates of the complex. A mechanism for the photocross-linking is suggested in which the cytosine nucleus is activated by photohydration, and the exocyclic amino-group is substituted by the ε-amino-group of lysine (Scheme 7).[475] Although this mechanism might well explain the cross-



Scheme 7

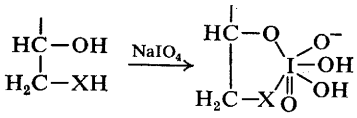
linking of ribosomal RNA to lysine clusters of protein S4 (see Vol. 8), it clearly cannot apply to the cross-linking of nucleotide to a lysine-deficient part of RNase A (see above).

6 Affinity Labelling

Halogenomethyl ketones are often used as the alkylating warhead on affinity labels. To identify the residue modified (lysine, histidine, or cysteine), performic acid oxidation can be used and the modified residue identified as its carboxymethyl derivative. However, the precise yield of the carboxymethyl derivative will depend on the competition between two oxidative cleavage pathways at either C—C bond adjacent to the carbonyl group. After the performic acid oxidation of 5-chloro-4-oxo[3,5-^{3}H]pentanoate labelled pyruvate kinase, both [^{3}H]succinic acid and [^{3}H]carboxymethylcysteine were identified,[476] with the latter in 30% yield.

[473] B. Ehresmann, J. Reinbolt, C. Backendorf, D. Tritsch, and J. P. Ebel, *F.E.B.S. Letters*, 1976, **67**, 316.
[474] J. Sperling and A. Havron, *Biochemistry*, 1976, **15**, 1489.
[475] E. I. Budowsky, N. A. Simukova, M. F. Turchinsky, I. V. Boni, and Y. M. Skoblov, *Nucleic Acids Res.*, 1976, **3**, 261.
[476] R. A. Chalkley and D. P. Bloxham, *Biochem. J.*, 1976, **159**, 213.

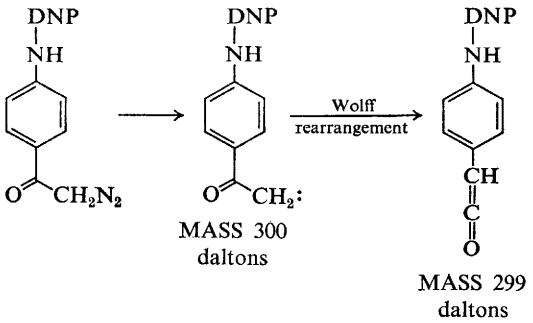
A way of distinguishing between the [^{14}C]halogenomethyl ketone alkylation of ε-aminolysine and cysteinyl residues has been suggested: the ketone group of the alkylated derivative is first reduced with sodium borohydride to produce a secondary alcohol; subsequent periodate oxidation will only occur if a cyclic periodate ester can be formed (Scheme 8): this is possible when X = O, S, NH_2,



Scheme 8

or *N*-alkyl, but not when X = *S*-alkyl. Thus only the lysyl derivative will release [^{14}C]formaldehyde on oxidation. The labelled formaldehyde (with carrier formaldehyde added) can be detected as its dimedone complex.[476]

Affinity-labelled residues have also been identified by mass spectrometry: rabbit anti-2,4-dinitrophenyl-*p*-aminobenzoylglutamate antibody was labelled with 2,4-dinitrophenyl-*p*-aminobenzoyldiazoketone and proteolytically digested. The partially purified labelled peptides were degraded by the Edman reaction using methyl isothiocyanate in place of phenyl isothiocyanate. The mass spectra of the methylthiohydantoin amino-acids were obtained by thermally inducing the fragmentation of an aliquot of the thiourea peptide in the ion source. The main fraction of the thiourea peptide was then cleaved with trifluoroacetic acid, and for the next step coupled again with methyl isothiocyanate. At the fifth step of the degradation, the mass spectra were compatible with substituted glycine and histidine methylthiohydantoins. Moreover, the precise mass of the attached label indicated that DNP-*p*-aminobenzoyl diazoketone reacts with the protein chain *via* the ketocarbene rather than the keten (arising from Wolff rearrangement of the ketocarbene) (Scheme 9).[477]



Scheme 9

Affinity labelling has been used to probe the active sites of estradiol-17β-dehydrogenase[478] and ribonucleotide reductase.[479] The dehydrogenase was

[477] J. Lindeman and R. E. Lovins, *Analyt. Biochem.*, 1976, **75**, 682.
[478] M. Pons, J.-C. Nicolas, A.-M. Boussioux, B. Descomps, and A. Crastes de Paulet, *European J. Biochem.*, 1976, **68**, 385.
[479] L. Thelander, B. Larsson, J. Hobbs, and F. Eckstein, *J. Biol. Chem.*, 1976, **251**, 1398.

labelled with estradiol-17β which carried alkylating groups, halogeno[2-^{14}C]-acetates or -acetamides, in nine different positions on the steroid nucleus. The ^{14}C-alkylated residues were identified as one histidine and two cysteines after chymotryptic digestion and separation of the peptides. For successful digestion it was found necessary to detach the body of the steroid from its warhead by

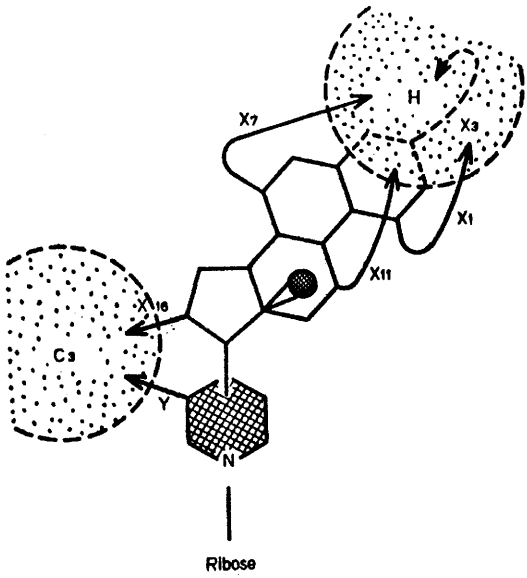


Figure 2 *Relative orientation of the substrate, nicotinamide, histidine residue, and cysteine in the evolutive ternary complex.* X_1, X_3, X_7, X_{11}, *and* X_{16} *refer to the positions of the alkylating group in the affinity label.* Y = *chloroacetyl group in 3-chloroacetylpyridine adenine dinucleotide.* H *is the reactive histidine residue and* C_3 *one of the reactive cysteines*
(Reproduced by permission from *European J. Biochem.*, 1976, **68**, 385)

mild alkaline hydrolysis, a strategy applicable to the acid derivatives but not to the alkali-resistant amides. The histidine residue was clearly located in the vicinity of ring A of the steroid, and one cysteinyl residue was located at the junction of ring D of the steroid with the nicotinamide moiety of the coenzyme (Figure 2). Ribonucleotide reductase consists of two subunits, B1 and B2, and was shown to have a composite active site by probing with the affinity labels 2′-deoxy-2′-chlororibonucleoside diphosphate and 2′-deoxy-2′-azidoribonucleoside diphosphate. The former inactivates exclusively subunit B1 (but only in the presence of B2) whereas the latter inactivates exclusively B2 (but only in the presence of B1). The active site must therefore be formed from both subunits.

A detailed mechanism for the affinity labelling of subtilisin has been inspired by the crystallographic model of the L-Phe-L-Ala-L-Lys-chloromethyl ketone–subtilisin complex. This shows two covalent bonds between inhibitor and

[480] T. L. Poulos, R. A. Alden, S. T. Freer, J. J. Birktoft, and J. Kraut, *J. Biol. Chem.*, 1976, **251**, 1097.

enzyme: the first between the ketone carbonyl carbon atom of the inhibitor and the hydroxy-group of the catalytic Ser-221 and the second between the methylene carbon of the inhibitor and the imidazole nitrogen of the catalytic His-64. The enzyme's catalytic apparatus is presumed to form first a hemiketal with the inhibitor which retaliates with a rate-limiting alkylation of the catalytic histidine.[480]

7 Competitive Labelling

The amino-groups in chromatin have been competitively labelled with acetic anhydride and their reactivities noted as a function of pH: at pH 7—10 the majority of amino-groups in all five histones are buried, whereas at higher pH they are exposed.[481] An attempt to identify the regions of the tyrosyl tRNA synthetase which interact with its cognate $tRNA^{Tyr}$, has been made by competitively labelling the free enzyme and also the enzyme–tRNA complex with acetic anhydride. After chymotryptic digestion of the acetylated protein, at least three peptides could be isolated in which the lysine residues had decreased reactivities in the complex.[482] One of these peptides showed a dramatic seven-fold decrease in reactivity, while the others showed a two-fold decrease. It is not clear whether this reflects an upward shift of the pK_a of ε-aminolysine concomitant on binding the tRNA or a genuine physical shielding of these groups by tRNA. The technique of reductive methylation, in contrast to acetylation by acetic anhydride, has been used for the competitive labelling of elongation factor EF-Tu. Free EF-Tu and complexes of EF-Tu with GDP, GTP or GTP and $Phe\text{-}tRNA^{Phe}$ were labelled with trace amounts of formaldehyde and sodium[^{3}H]borohydride. Amino-groups were thus methylated according to their reactivities. After completing the methylation with non-radioactive reagents the protein was mixed with fully ^{14}C-methylated EF-Tu and proteolytically digested. One substantial difference in (^{3}H/^{14}C) ratio for the same peptide isolated from the different complexes was noted,[483]

PART II: *X*-Ray Studies *by H. Muirhead*

1 Introduction

The techniques of protein crystallography are well established and can be used to study the structures of complicated macromolecular assemblies as well as those of pure proteins. In the past year advances have been made in several areas. Improved techniques for data collection and refinement have led to some structure determinations of such quality that protein conformation can be determined with considerable accuracy. For example, *X*-ray diffraction can be used to determine the position of every atom in an enzyme. The analysis of an electron-density map is the last stage in a protein structure determination. Given a suitable preliminary model methods have been described for refining the map, and Greer has described a method for the automatic interpretation of

[481] B. L. Malchy and H. Kaplan, *Biochem. J.* 1976, **159**, 173.
[482] H. R. Bosshard and G. L. E. Koch, Abstracts Tenth International Congress of Biochemistry, July 25, 1976.
[483] B. Kraal and B. S. Hartley, Abstracts Tenth International Congress of Biochemistry, July 25, 1976.

the map to give such a model. In this way it should be possible to analyse an electron-density map without building a physical model. Early structure determinations demonstrated the great variability of protein structure. As the number of structure determinations has increased, similar patterns of folding have been observed in proteins that may or may not have related functions and may or may not have homologous amino-acid sequences. The origin of these related 'domains' or 'super secondary structures' is a subject of considerable debate. Once the structure of an enzyme has been determined it is possible to locate a substrate or competitive inhibitor bound in the active site and effectors bound in regulatory sites, and to define the conformational changes that accompany binding. Although this gives a static picture it can be used to explain the results of experiments in solution and can lead to an understanding of the catalytic activity of the enzyme. However, it is difficult to study the binding of true substrates or productive intermediates since the time taken to complete an *X*-ray experiment is much greater than that taken to complete an enzymic reaction. At room temperature the only enzyme–substrate complexes which can be studied are the Michaelis complexes of enzymes with favourable reactions and equilibrium constants. Petsko has shown that it is possible to study true enzyme–substrate complexes in a stable form between -50 and -70 °C. This means that the binding of true substrates can be studied for an increased number of enzymes.

When studying larger, more complicated structures a major limitation has been the intensity of the beam available from a conventional *X*-ray tube. For this reason there has been considerable interest in the possibility of using synchrotron radiation for diffraction studies, and molecular biologists have recently had the opportunity to work with *X*-rays from electron synchrotrons and storage rings.

Crystallographic studies of amino-acids and peptides have given information about hydrogen bonding and the conformation of amino-acid residues and the preferred orientations of their side-chains. Complexes of amino-acids with nucleic acids, carbohydrates, and metals can give useful information about the possible interactions of proteins with such substances. Structures which have been determined are listed in Table 1.

Preliminary *X*-ray data have been published for many proteins and these are listed in Table 2, while Table 3 lists those structures which have been determined at low resolution.

Low-angle *X*-ray scattering can give information about the shape and overall dimensions of more complex structures. Some of these results are summarized in Table 4.

2 Methods and Techniques

The success of *X*-ray analysis is dependent upon the growth of suitable crystals and this can still be an intractable problem. McPherson has examined 22 proteins for their ability to crystallize from poly(ethylene glycol).[1] Use of four different concentrations of five different molecular weight sizes of poly(ethylene glycol) led to the crystallization of 13 of these proteins. Thus poly(ethylene glycol) may be a useful trial reagent for crystallization. Speed and accuracy are

[1] A. McPherson, *J. Biol. Chem.*, 1976, **251**, 6300.

Table 1 *Structure determinations of peptides and derivatives of amino-acids*

Compound	*Space group*	*a*/Å	*b*/Å	*c*/Å	α	β	γ	*Z*	*R*	*Ref.*
α-Glycine [a]	$P2_1/n$	5.1020	11.9709	5.4575		111.705		4	0.03	*b*
t-Butyloxycarbonylsarcosylglycine benzyl ester	$P2_1/c$	12.503	6.312	24.380		102.55		4	0.05	*c*
N-Carboxy-L-alanine anhydride	$P2_12_12_1$	7.749	10.699	6.063				4	0.047	*d*
L-Leucine	$P2_1$	9.61	5.31	14.72		86.2		4	0.13	*e*
D-Alloisoleucine,HCl,H_2O	$P2_12_12_1$	6.13	24.03	6.80				4	0.075	*f*
Allo-4-hydroxy-L-proline,$2H_2O$	$P2_12_12_1$	7.08	22.13	5.20				4	0.054	*g*
N-Acetyl-L-tyrosine-*p*-nitroanilide [h]	$P2_12_12_1$	15.621	19.941	5.253				4	0.058	*i*
Tosyl-L-arginine methyl ester chloride [j]	$P2_12_12_1$	8.084	23.579	9.812				4	0.05	*k*
S-Carboxymethyl-L-cysteine sulphone	$P2_12_12_1$	9.205	5.223	15.891				4	0.045	*l*
S-Carboxymethyl-L-cysteine sulphoxide	$P2_12_12_1$	4.786	8.312	18.914				4	0 035	*m*
NN'-Diglycyl-L-cystine dihydrate	*A*2	12.26	4.84	17.17		124.4		2	0.107	*n*
L-Lysine L-aspartate [o]	$P2_1$	5.539	7.848	15.324		99.1		2	0.069	*p*
N-Pivalyl-L-seryl-methylamide	*A*2	9.66	6.59	17.72		94.03		4	0.044	*q*
N-Phenacetyl-glycyl-DL-phenylalanine	$P2_1/c$	17.915	10.772	9.037		97.52		4	0.045	*r*
Glycyl-DL-phenylalanine [s]	*Pbca*	9.241	28.41	8.602				8	0.041	*t*
N-Acetyl-L-prolinamide	$P2_12_12_1$	6.64	7.67	15.64				4	0.049	*u*
N-Acetyl-(*S*)-thiazolidine-4-carboxamide	$P2_12_12_1$	6.665	7.699	15.451				4	0.059	*u*
N-Acetylactinobolin [v]	$P2_12_12_1$	8.708	8.851	21.718				4	0.064	*w*
Bis-(L-histidinato)cadmium dihydrate [a]	$P4_32_12$	7.397	7.397	30.53				4	0.069	*x*
(L-Valine-L-tyrosine)copper(II),$4H_2O$	$P2_12_12_1$	8.33	14.28	16.23				4	0.098	*y*
(Glycylglycinato)(aquo)(9-methyladenine) [z] copper(II)	$P\bar{1}$	10.419	14.146	6.844	96.87	108.50	68.91	2	0.086	*aa*
α-*S*-Cysteinylthymine [bb]	$P2_12_12_1$	9.499	24.072	5.012				4	0.043	*cc*
Cyclo-L-prolyl-D-phenylalanyl	$P2_1$	8.107	6.638	12.083		96.43		2	0.030	*dd*
Cyclic tetrapeptide dihydrochlamydocin [s]	$P2_1$	12.616	12.355	9.442		99.5		2	0.056	*ee*

Cyclo-(Gly-Tyr-Gly)$_2$ [ff]	$P1$	6.270	8.808	13.350	104.18	97.19	98.46	1	0.051	*gg*
Glycyl-L-methioninatocopper(II)	$P2_12_12_1$	10.354	11.471	8.872				4	0.036	*hh*
Benzyloxycarbonyl-Gly-L-Pro-L-Leu	$P2_1/a$	22.126	9.615	10.185		96.23		4	0.059	*ii*
Enniatin B (depsipeptide ionophore)	$P1$	9.83	9.69	9.83	92.42	99.45	92.45	1	0.12	*jj*
Cyclo-*N*-methyl-L-alanyl-L-alanyl	$P2_12_12_1$	5.978	9.537	14.698				4	0.037	*kk*
Beauvericin (depsipeptide)	$P2_1$	15.936	17.165	8.337		92.14		2	0.096	*ll*
Enterochelin	$P\bar{4}$	27.593	27.593	12.234				8	0.092	*mm*
	$C2/c$	22.887	11.747	23.999		118.19		8	0.054	*mm*
(Phe^4, Val^6)antamanide	$P2_12_12$	14.853	21.773	12.061				2	0.15	*nn*

(*a*) Neutron diffraction study. (*b*) L. F. Power, K. E. Turner, and F. H. Moore, *Acta Cryst.*, 1976, **B32**, 11. (*c*) H. Itoh, T. Yamane, T. Ashida, T. Sugihara, Y. Imanishi, and T. Higashimura, *Acta Cryst.*, 1976, **B32**, 3355. (*d*) H. Kanazawa, Y. Matsuura, N. Tanaka, M. Kakudo, T. Komoto, and T. Kawai, *Acta Cryst.*, 1976, **B32**, 3314. (*e*) M. M. Harding and R. M. Howieson, *Acta Cryst.*, 1976, **B32**, 633. (*f*) K. I. Varughese and R. Srinivasan, *Acta Cryst.*, 1976, **B32**, 994. (*g*) N. Shamala, T. N. Guru Row, and K. Venkatesan, *Acta Cryst.*, 1976, **B32**, 3267. (*h*) Chymotrypsin substrate. (*i*) A. G. Michel and F. Durant, *Acta Cryst.*, 1976, **B32**, 1574. (*j*) Trypsin substrate. (*k*) Y. Barrans and M. Cotrait, *Acta Cryst.*, 1976, **B32**, 2346. (*l*) C. R. Hubbard, A. D. Mighell, J. A. Staffa, C. Zervos, and J. P. Konopelski, *Acta Cryst.*, 1976, **B32**, 2723. (*m*) J. A. Staffa, C. Zervos, A. D. Mighell, and C. R. Hubbard, *Acta Cryst.*, 1976, **B32**, 3132. (*n*) W. C. Stallings and J. Donohue, *Acta Cryst.*, 1976, **B32**, 1916. (*o*) Crystalline complex. (*p*) T. N. Bhat and M. Vijayan, *Acta Cryst.*, 1976, **B32**, 891. (*q*) A. Aubry, J. Protas, M Marraud, and J. Neel, *Acta Cryst.*, 1976, **B32**, 2749. (*r*) P. Blanpain, F. Durant, and G. Evrard, *Acta Cryst.*, 1976, **B32**, 629. (*s*) Non-planar peptide group. (*t*) R. E. Marsh, S. Ramakumar, and K. Venkatesan, *Acta Cryst.*, 1976, **B32**, 66. (*u*) E. Benedetti, A. Christensen, C. Gilon, W. Fuller, and M. Goodman, *Biopolymers*, 1976, **15**, 2523. (*v*) The α-helix conformation in a small peptide. (*w*) R. B. Von Dreele, *Acta Cryst.*, 1976, **B32**, 2852. (*x*) H. Fuess and H. Bartunik, *Acta Cryst.*, 1976, **B32**, 2803. (*y*) V. Amirthalingam and K. V. Muralidharan, *Acta Cryst.*, 1976, **B32**, 3153. (*z*) Model for enzyme–metal–nucleic acid ternary species. (*aa*) T. J. Kistenmacher, L. G. Marzilli, and D. J. Szalda, *Acta Cryst.*, 1976, **B32**, 186. (*bb*) Model for radiation-induced interaction of thymine and cysteine residues. (*cc*) H. M. Berman, D. E. Zacharias, H. L. Carrell, and A. J. Varghese, *Biochemistry*, 1976, **15**, 463. (*dd*) R. Ramani, K. Venkatesan, R. E. Marsh, and W.-J. Hu Kung, *Acta Cryst.*, 1976, **B32**, 1051. (*ee*) J. L. Flippen and I. L. Karle, *Biopolymers*, 1976, **15**, 1081. (*ff*) Crystal structure from packing studies. (*gg*) G. N. Ramachandran, *Acta Cryst.*, 1976, **A32**, 1008. (*hh*) C. A. Bear and H. C. Freeman, *Acta Cryst.*, 1976, **B32**, 2534. (*ii*) T. Yamane, T. Ashida, K. Shimonishi, M. Kakudo, and Y. Sasada, *Acta Cryst.*, 1976, **B32**, 2071. (*jj*) G. N. Tishchenko, Z. Karimov, B. K. Vainshtein, A. V. Evstratov, V. I. Ivanov, and Y. A. Ovchinnikov, *F.E.B.S. Letters*, 1976, **65**, 315. (*kk*) A. Filhol and P. A. Timmins, *Acta Cryst.*, 1976, **B32**, 3116. (*ll*) A. J. Geddes and D. Akrigg, *Acta Cryst.*, 1976, **B32**, 3164. (*mm*) B. F. Anderson, D. A. Buckingham, G. B. Robertson, J. Webb, K. S. Murray, and P. E. Clark, *Nature*, 1976, **262**, 722. (*nn*) I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, and B. Witkop, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1782.

Table 2 *Preliminary X-ray data on proteins*

Protein	*Space group*	*a*/Å	*b*/Å	*c*/Å	*β*	*Mol. wt. asym. unit*	*Ref.*
Anaerobic bacterial rubredoxins							
Desulphovibrio vulgaris	$P2_1$	20.00	41.50	24.41	107.6	6000 (1 molecule)	*a*
Desulphovibrio gigas	$P2_1$	19.75	41.70	24.38	108.1	6000 (1 molecule)	*a*
Thioredoxin induced by bacteriophage T4	$P2_1$	54.1	45.9	40.8	99.4	20 160 (2 molecules)	*b*
Cytochrome c_3, *Desulphovibrio desulphuricans*	$P3_1$ or $P3_2$	44.2	44.2	64.7		13 000 (1 molecule)	*c*
Cytochrome b_2, baker's yeast	$P3_121$	165.51		113.7		230 000 (tetramer)	*d*
Manganese superoxide dismutases							
E. coli	$P222$ or $P222_1$	47	103	47.5		19 500 ($\frac{1}{2}$ molecule)	*e*
	$C222_1$	101	108	180		79 000 (2 dimers)	*e*
	$P2_12_12$	47	51	188		39 500 (1 dimer)	*e*
Yeast mitochondria	$P2_12_12_1$	63	115	125		97 000 (1 tetramer)	*e*
Bacillus stearothermophilus	$P2_1$	50	70	69	111.17	40 000 (1 dimer)	*f*
Iron superoxide dismutase							
Pseudomonas ovalis	$P2$	81.9	49.0	61.9	106	39 500 (1 dimer)	*g*
Horseradish peroxidase	Tetragonal	96.8	96.8	371		32 molecules/unit cell (mol. wt. 40 000)	*h*
Selenoenzyme glutathione peroxidase							
Bovine erythrocytes	$C2$	90.4	109.5	58.6	99	84 000 (tetramer)	*i*
Human plasma albumin	$P2_12_12$	133.3	274.8	58.02		198 921 (3 molecules)	*j*
Rabbit plasma transferrin	$P4_12_12$ or $P4_32_12$	126.9	126.9	145.2		77 000 (1 molecule)	*k*
Subtilopeptidase A	$P2_12_12_1$	76.73	55.35	53.00		27 300 (1 molecule)	*l*
Ribonuclease A (pH9)	$P3_121$	64.34		65.00			*m*
Ribonuclease S (pH9)	$P3_121$	65.01		64.60			*m*
Beta-lytic protease-myxobacter 495	$P2_12_12_1$	54.1	99.6	53.9		38 400 (2 molecules)	*n*
L-Asparaginase, *E. coli*	$P2_122_1$	116.7	62.9	86.6		67 500 ($\frac{1}{2}$ tetramer)	*o*
Pig heart citrate synthase	$P4_12_12$ or $P4_32_12$	77.1		196.8		50 000 ($\frac{1}{2}$ molecule)	*p*

Δ^5-3-Keto-steroid isomerase	$P6_322$	65.38		504		53 600 (4 subunits)	*q*
Pseudomonas testosteroni	$P2_1$	140.4	85.0	94.5	130.1	160 800 (12 subunits)	*r*
Mitogenic lectin, pea seeds	$P2_12_12_1$	51.0	61.7	137.6		49 000 (α_2 β_2 tetramer)	*s*
Anti-tumour protein neocarzinostatin	$P2_12_12_1$	27.4	33.9	102.0		10 700 (1 molecule)	*t*
Non-muscle actin from calf spleen	$P2_12_12_1$	187.4	72.33	38.19		58 000 (1 molecule)	*u*
Elongation factor Tu.GDP from *E. coli*	$P3_1$ or $P3_2$	80	80	327		352 000 (8 molecules)	*v*
	$P3_121$ or $P3_221$	80	80	161		88 000 (2 molecules)	*v*
	$P6_222$ or $P6_422$	80	80	161		44 000 (1 molecule)	*v*
Partially digested elongation factor Tu							*w*
DNA unwinding protein, fd bacteriophage gene 5 product	$C2$	75.8	28.0	42.5	103.2	9800 (1 molecule)	*x*
Ribosomal protein L7/L12 from *E. coli*	$P6_122$ or $P6_533$	71	71	138		24 400 (1 Dimer)	*y*
Bovine liver catalase	$P3_121$ or $P3_221$	142.3		104.0		125 000 ($\frac{1}{2}$ tetramer)	*z*
Glutamine synthetase from *E. coli*	$C2$	235	134	200	103	(1 molecule)	*aa*

(*a*) M. Pierrot, R. Haser, M. Frey, M. Bruschi, J. Le Gall, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, 1976, **107**, 179. (*b*) B.-M. Sjöberg and B.-O. Söderberg, *J. Mol. Biol.*, 1976, **100**, 415. (*c*) M. Frey, R. Haser, M. Pierrot, M. Bruschi, and J. Le Gall, *J. Mol. Biol.*, 1976, **104**, 741. (*d*) F. S. Mathews and F. Lederer, *J. Mol. Biol.*, 1976, **102**, 853. (*e*) K. M. Beem, J. S. Richardson, and D. C. Richardson, *J. Mol. Biol.*, 1976, **105**, 327. (*f*) J. Bridgen, J. I. Harris, and E. Kolb, *J. Mol. Biol.*, 1976, **105**, 333. (*g*) F. Yamakura, K. Suzuki, and Y. Mitsui, *J. Biol. Chem.*, 1976, **251**, 4792. (*h*) A. Braithwaite, *J. Mol. Biol.*, 1976, **106**, 229. (*i*) R. Ladenstein and A. Wendel, *J. Mol. Biol.*, 1976, **104**, 877. (*j*) S. N. Rao, S. P. Basu, C. G. Sanny, R. V. Manley, and J. A. Hartsuck, *J. Biol. Chem.*, 1976, **251**, 3191. (*k*) D. Al-Hilal, E. Baker, C. H. Carlisle, B. Gorinsky, R. C. Horsbrugh, P. F. Lindley, D. S. Moss, H. Schneider, and R. Stimpson, *J. Mol. Biol.*, 1976, **108**, 255. (*l*) G. A. Petsko and D. Tsernoglou, *J. Mol. Biol.*, 1976, **106**, 453. (*m*) P. D. Martin, G. A. Petsko, and D. Tsernoglou, *J. Mol. Biol.*, 1976, **108**, 265. (*n*) W. B. T. Cruse and D. R. Whitaker, *J. Mol. Biol.*, 1976, **102**, 173. (*o*) A. Itai, M. Yonei, Y. Mitsui, and Y. Iitaka, *J. Mol. Biol.*, 1976, **105**, 321. (*p*) G. Wiegand, *F.E.B.S. Letters*, 1976, **62**, 281. (*q*) E. M. Westbrook, *J. Mol. Biol.*, 1976, **103**, 659. (*r*) E. M. Westbrook, P. B. Sigler, H. Berman, J. P. Glusker, G. Bonick, A. Benson, and P. Talalay, *J. Mol. Biol.*, 1976, **103**, 665. (*s*) V. A. Brysgunov, M. D. Lutsik, V. R. Melick-Adamyan, and M. A. Mokulskii, *J. Mol. Biol.*, 1976, **101**, 435. (*t*) L. C. Sieker, L. H. Jensen, and T. S. Anantha Samy, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 358. (*u*) L. Carlsson, L.-E. Nyström, U. Lindberg, K. K. Kannan, H. Cid-Dresdner, S. Lövgren, and H. Jörnvall, *J. Mol. Biol.*, 1976, **105**, 353. (*v*) R. Leberman, A. Wittinghofer, and G. E. Schulz, *J. Mol. Biol.*, 1976, **106**, 951. (*w*) W. H. Gast, R. Leberman, G. E. Schulz, and A. Wittinghofer, *J. Mol. Biol.*, 1976, **106**, 943. (*x*) A. McPherson, I. Molineux, and A. Rich, *J. Mol. Biol.*, 1976, **106**, 1077. (*y*) A. Liljas and C. G. Kurland, *F.E.B.S. Letters*, 1976, **71**, 130. (*z*) W. Eventoff, N. Tanaka, and M. G. Rossmann, *J. Mol. Biol.*, 1976, **103**, 799. (*aa*) W. Kabsch, H. Kabsch, and D. Eisenberg, *J. Mol. Biol.*, 1976, **100**, 283.

Table 3 *Low-resolution structures*

Protein	*Resolution*/Å	*Comments*	*Ref.*
Acid protease from *Rhizopus chinensis*	5.5	Pepsin-like enzyme. Bilobal with large cleft in which inhibitors bind	*a*
Actinidin from Chinese gooseberry	5.5	Sulphydryl protease. Structure similar to that of papain	*b*
Penicillopepsin	6	Acid protease	*c*
Penicillin β-lactamase R-TEM *E. coli*	5.5	No evidence for substrate binding site	*d*
Mouse testicular lactate dehydrogenase	7.5	C_4 Homotetramer. Molecular replacement technique	*e*
Turkey egg-white lysozyme	5	Molecular replacement	*f*
E. coli L-arabinose-binding protein	5	Essential component in the active transport of L-arabinose	*g*
Methemerythrin, sipunculid marine worm	5	Octameric molecule with two layers of four subunits	*h*

(*a*) E. Subramanian, I. D. A. Swan, and D. R. Davies, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 875. (*b*) E. N. Baker, *J. Mol. Biol.*, 1976, **101**, 185. (*c*) I.-Nan Hsu, T. Hofmann, S. C. Nyburg, and M. N. G. James, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 363. (*d*) J. R. Knox, J. A. Kelly, P. C. Moews, and N. S. Murthy, *J. Mol. Biol.*, 1976, **104**, 865. (*e*) W. D. L. Musick, A. D. Adams, M. G. Rossmann, T. E. Wheat, and E. Goldberg, *J. Mol. Biol.*, 1976, **104**, 659. (*f*) R. Bott and R. Sarma, *J. Mol. Biol.*, 1976, **106**, 1037. (*g*) G. N. Phillips, V. K. Mahajan, A. K. Q. Siu, and F. A. Quiocho, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2186. (*h*) R. E. Stenkamp, L. C. Sieker, L. H. Jensen, and J. S. Loehr, *J. Mol. Biol.*, 1976, **100**, 23.

Table 4 *Low-angle X-ray scattering*

Structure	*Mol. wt.*	R_G/Å	V/Å^3	*Comments*	*Ref.*
5S RNA binding proteins from *E. coli* L18		26		Prolate ellipsoid axial ratio 6 : 1	*a*
5S RNA binding proteins from *E. coli* L25		24		Prolate ellipsoid axial ratio 5.7 : 1	*a*
Hen egg-white lysozyme		13.8		Good agreement with crystal structure	*b*
Methionyl-tRNA synthetase from *E. coli*					
Native		43	244 000	Suggests two protomers linked by interactions	*c*
Trypsin modified		24.8	90 000	involving a limited region of the structure	*c*
Rabbit muscle phosphofructokinase dimer	160 000	32.5		Tetramer is biologically active	*d*
tetramer	320 000	51.5			
Protein S4 from *E. coli* ribosomes	24 000	33.6		Rod-like structure with length 140 Å, diameter 10 Å	*e*
L-Asparaginase from *E. coli* monomer	33 000	16.0		Prolate ellipsoid, axial ratio 1.4	*f*
tetramer	132 000	34.0		Distance between subunit centres 43 Å	
Bovine rhodopsin + 156 detergent molecules	156 × 229 + 39 000	29.5	74 600	Elongated molecule which spans flat detergent micelle	*g*
Human immunoglobulin molecule Kol					
Fab		31.5	75 000	Similar shape in crystal and solution but more extended in solution	*k*
Fc		31.5	91 000	More extended in solution and somewhat different in shape	*k*
Apoprotein from porcine high-density Lipoprotein with dimyristoyl lecithin		49		Lipid hydrocarbon chains organized in apolar core with lipid polar head groups and protein in outer shell	*i*
Human serum lipoproteins					
HD3				Maximum diameter 130 Å, hydrocarbon core of 80 Å diameter	*j*, *k*
LDL				Maximum diameter 280 Å, hydrocarbon core of 230 Å diameter	*j*, *k*
Squid retina				Information on photoreceptor structures	

(*a*) R. Österberg, B. Sjöberg, and R. A. Garrett, *F.E.B.S. Letters*, 1976, **65**, 73. (*b*) H. B. Stuhrmann and H. Fuess, *Acta Cryst.*, 1976, **A32**, 67. (*c*) A. Gulik, C. Monteilhet, P. Dessen, and G. Fayat, *European J. Biochem.*, 1976, **64**, 295. (*d*) H. H. Paradies and W. Vettermann, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 520. (*e*) H. H. Paradies and A. Franz, *European J. Biochem.*, 1976, **67**, 23. (*f*) N. S. Murthy and J. R. Knox, *J. Mol. Biol.*, 1976, **105**, 567. (*g*) C. Sardet, A. Tardieu, and V. Luzzati, *J. Mol. Biol.*, 1976, **105**, 383. (*h*) I. Pilz, E. Schwartz, and W. Palm, *European J. Biochem.*, 1976, **71**, 239. (*i*) D. Atkinson, H. M. Smith, J. Dickson, and J. P. Austin, *European J. Biochem.*, 1976, **64**, 541. (*j*) V. Luzzati, A. Tardieu, L. Mateu, and H. B. Stuhrmann, *J. Mol. Biol.*, 1976, **101**, 115. (*k*) A. Tardieu, L. Mateu, C. Sardet, B. Weiss, V. Luzzati, L. Aggerbeck, and A. M. Scanu, *J. Mol. Biol.*, 1976, **101**, 129. (*l*) C. R. Worthington, S. K. Wang, and C. M. Folzer, *Nature*, 1976, **262**, 626.

important when collecting and analysing *X*-ray data from biological molecules, and papers have been published covering various aspects of data collection and data processing. A new simplified method for deriving the equations which relate the angle settings with the rotation around the diffraction vector for four-circle diffractometers has been described.[2] The main sources of background radiation when diffractometers are used to collect data in protein crystallography are scatter from the volume of air irradiated by the direct incident beam and seen by the photon detector, and scatter from the capillary tube in which protein crystals are usually mounted. This background scatter can be reduced by reducing the volume of air which may scatter *X*-rays into the detector and by replacing air in this visible volume by helium contained in a small mylar-walled cylinder around the crystal.[3] These experimental techniques can increase the peak-to-background ratio by a factor of 3—10. Once the data have been collected correction factors for radiation damage can be applied. Hendrickson has derived a general model to describe the rate of radiation decay in protein crystals.[4] The model was tested using data on irradiation effects in myoglobin crystals. It indicates a sequential process of damage and suggests an improved method for correcting diffraction data for the effects of radiation damage. The crystal is assumed to consist of three fractions; A_1 which is essentially undamaged, A_2 which is highly disordered, and A_3 which is effectively amorphous. The ratio of the measured intensity at time t to that at time zero is given by

$$\frac{I(t)}{I(0)} = A_1(t) + A_2(t) \exp(-Ds^2)$$

where $s = \sin^2\theta/\lambda^2$ and $A_1(t) + A_2(t) + A_3(t) = 1$. Rate constants which give the best fit to the experimental data can be found for the conversion of A_1 into A_3, A_1 into A_2, and A_2 into A_3. A sequential model where A_1 was first converted into A_2 (disorder) and then into A_3 (denaturation) gave the best fit to the data and made it possible to make corrections for greater levels of radiation damage than are usually tolerated.

A procedure for the digital processing of fibre diffraction patterns to yield a quasi-continuous map representing a central section through the cylindrically averaged intensity transform of the specimen has been described.[5] No assumptions are made except that the specimen has fibre-type symmetry. The specimen intensity transform can be used to obtain integrated intensities for discrete reflexions from microcrystalline fibres that are potentially as accurate as those obtained from single crystals. Improved procedures for extracting structure amplitudes from specimens with continuous layer lines are also described. This extends the method of Holmes and Barrington Leigh.

Several groups have experimented with synchrotron radiation in order to determine whether this source of *X*-radiation will have advantages over conventional *X*-ray sources for diffraction studies on protein crystals and for small-

[2] B. C. Wang, C. S. Yoo, J. Pletcher, and M. Sax, *Acta Cryst.*, 1976, **A32**, 918.
[3] M. Krieger and R. M. Stroud, *Acta Cryst.*, 1976, **A32**, 653.
[4] W. A. Hendrickson, *J. Mol. Biol.*, 1976, **106**, 889.
[5] R. D. B. Fraser, T. P. Macrae, A. Miller, and R. J. Rowlands, *J. Appl. Cryst.*, 1976, **9**, 81.

angle scattering experiments on biological materials.[6–8] Crystals with unit cell dimensions varying from 33 to 250 Å were used. In general the highly monochromatic beam gave high quality noise-free patterns with an improved signal-to-noise ratio, and crystal decay was considerably less than for Ni-filtered Cu radiation. Another important advantage is the tunable nature of the source which means that the best wavelength for obtaining anomalous scattering data can be selected. Synchrotron radiation will be particularly important for large unit cells where a convergent beam and more powerful sources are advantageous.

Most large molecules are aggregates of smaller molecules or subunits which may be of known structure. Hendrickson and Ward have described a packing function which defines the allowable location of crystallized macromolecules.[9] The principle is that the constituent molecules should not interpenetrate and the method has been applied to the crystal structure of haemerythrin using the molecular shape determined for myohaemerythrin.

Petsko has used a new synthesis for the refinement of heavy-atom parameters.[10] The conventional 'double difference' or 'residual' syntheses used in protein crystallography to detect errors in refined heavy-atom parameters are approximations to the desired vector difference syntheses between the observed and calculated heavy-atom structure factors. By using the measured anomalous differences between Friedel related pairs of reflexions it is possible to calculate the coefficients for the vector difference synthesis. This leads to a better signal-to-noise ratio.

The satellite tobacco necrosis virus (STNV) is one of the smallest viruses known, so small that it lacks some functions necessary for replication and must co-infect with the larger, fully competent tobacco necrosis virus in order to propagate. The virus particle is an aggregate of 60 copies of the single coat-protein molecule, mol. wt. 23 000, arranged on a $T = 1$ icosahedral surface lattice and a single-stranded ribonucleic acid molecule, mol. wt. 400 000, which codes for that coat protein. Because of the relatively small size of the virus, the high order of the crystals, and the unusual occurrence of an entire particle as the asymmetric unit, an effort is being made to determine the high-resolution structure of this intact, infectious virion. The structure can be determined by the conventional multiple isomorphous replacement method using heavy-atom derivatives of the coat protein for phase determination because, although the number of sites is expected to be very large, the set of sites should retain the icosahedral point symmetry of the coat. Argos and Rossmann have shown that once the orientation of the virus has been established, then Patterson self-vectors can be calculated between heavy atoms attached specifically to equivalent points in each subunit. A systematic search of all reasonable positions within the subunit and a comparison with the known difference Patterson map would thus establish the heavy-atom positions relative to the centre of the virus particle.[11] This vector search method has been used to find the heavy-atom

[6] J. C. Phillips, A. Wlodawer, M. M. Yevitz, and K. O. Hodgson, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 128.
[7] A. Harmsen, R. Leberman, and G. E. Schulz, *J. Mol. Biol.*, 1976, **104**, 311.
[8] J. Bordas, I. H. Munro, and A. M. Glazer, *Nature*, 1976, **262**, 541.
[9] W. A. Hendrickson and K. B. Ward, *Acta Cryst.*, 1976, **A32**, 778.
[10] G. A. Petsko, *Acta Cryst.*, 1976, **A32**, 473.
[11] P. Argos and M. G. Rossmann, *Acta Cryst.*, 1976, **B32**, 2975.

binding sites in two derivatives of crystalline STNV.[12] One major site has been found for each derivative in each of the 60 subunits. Once scale factor, occupancy, positional, and thermal parameters were refined single isomorphous replacement phases from either derivative were sufficiently accurate to locate the substitution site in the difference Fourier map of the other derivative. These phases are consistent with those given by the molecular replacement method. Rossmann has derived the necessary algebra for the least-squares refinement of heavy-atom parameters in such a series of heavy-atom derivatives where the heavy-atom derivatives are related by non-crystallographic symmetry.[13] In addition, the simultaneous refinement of the particle (or molecular) orientation and position is considered permitting the precise adjustment of the system of non-crystallographic rotation axes. This method has been applied to pig H_4 lactate dehydrogenase, STNV, and southern bean mosaic virus.

Bricogne has continued to develop his programs for the direct-space exploitation of non-crystallographic symmetry to improve the quality of the electron-density map.[14] The procedure is to compute an electron-density map using the currently available phases, average the electron density of all crystallographically independent molecules, rebuild the crystal from this averaged subunit setting the density outside the molecular boundaries to its average value, obtain phase information from the resultant structure and combine it with isomorphous replacement information to produce phases for the next iteration. This procedure converges very quickly and includes a rapid, general method for real-space averaging. It has been applied to glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* and to the disc of TMV coat protein. Fletterick and Steitz have improved the quality of an electron-density map of yeast hexokinase by combining independent phase information obtained from separate structure determinations.[15] They present a procedure for combining in reciprocal space the information contained in protein structures which have been independently determined using the multiple isomorphous replacement technique. Its application to two crystal forms of yeast hexokinase B, which had been solved at 3.5 Å resolution, resulted in an electron density map which was a striking improvement over either isomorphous replacement map or an averaged map. The background was lower, the apparent resolution was better and side-chains became visible. The method consists of determining a linear transformation matrix and vector relating the subunits of the two crystals, orienting the electron density of one crystal form in the unit cell of the other and transforming this map to give a set of calculated F's and phases. These calculated phases from the first crystal were combined with those of the second to produce a hybrid phase set.

Several authors have described methods for refining structures in direct or reciprocal space and for phase extension.[16–20] Two papers describe a method of

[12] P. J. Lentz, B. Strandberg, T. Unge, I. Vaara, A. Borell, K. Fridborg, and G. Petef, *Acta Cryst.*, 1976, **B32**, 2979.
[13] M. G. Rossmann, *Acta Cryst.*, 1976, **A32**, 774.
[14] G. Bricogne, *Acta Cryst.*, 1976, **A32**, 832.
[15] R. J. Fletterick and T. A. Steitz, *Acta Cryst.*, 1976, **A32**, 125.
[16] A. R. Davies and J. S. Rollett, *Acta Cryst.*, 1976, **A32**, 17.
[17] J. Gassmann, *Acta Cryst.*, 1976, **A32**, 274.
[18] A. D. Podjarny, A. Yonath, and W. Traub, *Acta Cryst.*, 1976, **A32**, 281.
[19] J. H. Konnert, *Acta Cryst.*, 1976, **A32**, 614.
[20] R. E. Stenkamp and L. H. Jensen, *Acta Cryst.*, 1976, **A32**, 255.

obtaining a stereochemically acceptable protein model which fits a set of atomic co-ordinates.[21, 22] The method consists of expressing all constraints in terms of distances between pairs of atoms and it is possible to enforce any desired stereochemistry in a physically realistic manner, and at the same time substantially reduce computing time. The equations have many unknowns but few coefficients and an effective iterative method can be used. The method has been used to refine the structures of insulin and thermolysin. Diamond has described a technique whereby co-ordinate sets for related molecules may be compared in such a way as to express rigorously the relationship between them in terms of position, orientation, homogeneous strain, and curvature.[23] It is thought that such curvatures may be useful in characterizing hinge regions in allosteric enzymes. Previous methods have used linear transformations which cannot describe a region in which bending or twisting occurs.

The problem of finding an initial starting model for such refinements without having to build a physical model has been tackled by Greer.[24] He has expanded his earlier work on the automated interpretation of protein electron-density maps to produce atomic co-ordinates for the main chain of the molecule. He has designed procedures to select the main-chain segments, determine the directionality of the chain, and fit α-carbon and β-carbon co-ordinates at the appropriate positions. The orientation of the peptide plane is derived for some residues by pre-building secondary structure regions. The method was developed using the 2.0 Å map of ribonuclease S and has been applied to a newly calculated 3 Å resolution electron-density map of Bence-Jones protein Rhe.[25] The automated system produced a single polypeptide chain which accounts for the whole molecule. Much of the secondary structure was detected and provisional main-chain co-ordinates obtained.

Major problems in the study of enzyme mechanisms are the rapidity of the reaction and the low concentration of intermediates present in steady-state conditions. *X*-Ray crystallography is one of the few techniques that can yield structural data about such intermediates at the necessary resolution. However, *X*-ray diffraction is a static procedure and requires stable, crystalline compounds. Sub-zero temperatures can be used to trap intermediates in enzyme-catalysed reactions using suitable cryosolvents, and the feasibility of obtaining such intermediates in the crystalline state has been demonstrated with several proteases, using specific substrates and optimal pH.[26] The structure of a specific acyl-enzyme intermediate in the elastase-catalysed hydrolysis of *N*-benzoxycarbonyl-L-alanyl-*p*-nitrophenol ester has been determined at 3.5 Å resolution.[27] The acyl-enzyme was stabilized by cooling the crystal to -55 °C during substrate addition and data collection. It is not possible at this resolution to use these results to answer detailed questions about the mechanism of serine protease catalysis or the nature of the catalytic power of enzymes. Some insight into these

[21] E. J. Dodson, N. W. Isaacs, and J. S. Rollett, *Acta Cryst.*, 1976, **A32**, 311.
[22] L. F. Ten Eyck, L. H. Weaver, and B. W. Matthews, *Acta Cryst.*, 1976, **A32**, 349.
[23] R. Diamond, *Acta Cryst.*, 1976, **A32**, 1.
[24] J. Greer, *J. Mol. Biol.*, 1976, **100**, 427.
[25] J. Greer, *J. Mol. Biol.*, 1976, **104**, 371.
[26] A. L. Fink and A. I. Ahmed, *Nature*, 1976, **263**, 294.
[27] T. Alber, G. A. Petsko, and D. Tsernoglou, *Nature*, 1976, **263**, 297.

questions should come from the extension of this work to much higher resolution and several different substrates. For example, since the rate-limiting step for the hydrolysis of *p*-nitroanilide substrates is the formation of the acyl-enzyme rather than its breakdown, binding a substrate of this type to the crystalline enzyme at low temperatures may yield a pre-acyl-enzyme or the Michaelis complex. This would give a second static picture of a different point in the overall reaction pathway. This work demonstrates that it is feasible to use low-temperature protein crystallography to stabilize and study actual enzyme–substrate complexes.

3 Insulin

The structure of insulin in tetrazinc insulin has been determined at a resolution of 2.8 Å.[28] There are four zinc atoms per insulin hexamer rather than two. The hexamer has a crystallographic three-fold axis of symmetry so that the asymmetric unit is the dimer. One zinc atom lies on the three-fold axis and the remainder occupy general sites surrounding the three-fold axis. The overall structure is very similar to that of dizinc insulin but this basic similarity conceals surprisingly large changes in the conformation of one only of the two insulin molecules in the asymmetric unit. These changed molecules interact with the zinc atoms in general positions (one per dimer). The change in structure is effected by a major rearrangement of the first eight residues of the B chain. These, originally extended, now follow a generally helical course and the zinc atom is between two histidine residues. This means that the local two-fold axis relating the two molecules in the dimer is completely violated by the arrangement of these first eight B-chain residues. The two A chains are similar in conformation but not in position relative to the B chains. It is possible that the flexible character of this region may be an important factor in generating a biological response. However, another paper on insulin suggests that other areas of the molecule could be more important than residues B1—B8.[29] It is hoped that once the nature of the receptor-binding region is understood it will be possible to design synthetic insulin analogues for the treatment of diabetes. Studies of the biological activities and sequences of insulins from different animals suggested that a largely invariant region on the surface of the insulin monomer is a good candidate for the receptor-binding region. Studies of high-resolution *X*-ray diffraction, circular dichroism, receptor binding, and biological potency in fat cells of native insulin and various chemically modified insulins have clarified the factors leading to decreased potency and have helped to define the nature of the receptor-binding region. The results of these experiments suggest that the conformation of the insulin molecule is critical to the formation of both the zinc insulin hexamer and the insulin–receptor complex and that the receptor-binding region includes many of the hydrophobic residues important to dimerization as well as more polar surface residues. There is a possibility of the formation of an antiparallel sheet structure between the insulin and receptor molecules in the complex similar to that between monomers in the insulin dimer. This suggested mode for insulin–receptor binding implies that a change in the three-dimensional structure would

[28] G. Bentley, E. Dodson, G. Dodson, D. Hodgkin, and D. Mercola, *Nature*, 1976, **261**, 166.
[29] R. A. Pullen, D. G. Lindsay, S. P. Wood, I. J. Tickle, T. L. Blundell, A. Wollmer, G. Krail, D. Brandenburg, H. Zahn, J. Gliemann, and S. Gammeltoft, *Nature*, 1976, **259**, 369.

decrease receptor binding, that full receptor binding is attained by interaction of several insulin residues with the receptor so that the loss of any one residue would decrease rather than abolish receptor binding, and that receptor binding is additive through hydrophobic interactions and formation of specific hydrogen bonds. The importance of hydrophobic interactions may be a general feature in hormone receptor binding. It is likely that some residues of the hormone which interact with the receptor are important for the biological response rather than the stability of the insulin–receptor complex.

4 Electron Transport and Oxygen Carriers

Ferredoxin.—The structure of *Peptococcus aerogenes* ferredoxin has been refined using native data to a resolution of 2 Å and a starting model obtained from the 2.8 Å electron-density map.[30] The *R*-factor refined to 0.188 for an unconstrained model and 0.206 for a constrained model. Standard deviations for the positions of the Fe and S atoms varied between 0.04 and 0.05 Å and those for O, N, and C atoms between 0.14 and 0.26 Å; 146 water oxygens were included in the refinement.

Cytochromes.—Timkovich and Dickerson have published the structure of *Paracoccus denitrificans* cytochrome c_{550} at 2.45 Å resolution.[31] This cytochrome is evolutionarily homologous with photosynthetic cytochrome c_2 from purple non-sulphur bacteria and mitochondrial cytochrome *c* from eukaryotes. All these cytochromes have the same basic folding pattern with surface insertions of extra amino-acids in c_{550}. Radical structural changes at all regions on the molecular surface except the haem crevice argue for the importance of the crevice and the exposed edge of the haem in the transfer of electrons to and from the cytochrome molecule. At one time electron transfer from one aromatic ring to a neighbour was proposed as a mechanism for reduction of the haem iron, with tyrosine 74 as the outermost ring in this π-electron 'channel'. Its replacement by valine (in c_2) and leucine (in c_{550}) in molecules of similar redox properties effectively eliminates this mechanism. The sequence of cytochrome c_{550} was determined by ordering the peptides from the trypsin digest by means of the electron-density map.[32] From a comparison of known primary sequences and tertiary structures the authors suggest that the respiratory electron-transport chain in prokaryotes and eukaryotes is a relatively late evolutionary offshoot of the photosynthetic electron-transport chain in purple non-sulphur bacteria. Dickerson *et al.* have expanded this theme in a paper where they propose that bacterial and eukaryotic oxygen respiration arose from the dual-function photosynthetic and respiratory electron chain in purple non-sulphur bacteria by the loss of photosynthetic capabilities.[33] They present a detailed outline which involves first a symbiotic sulphur cycle, then development of the Krebs cycle machinery and finally the evolution of the present-day symbiotic oxygen cycle between plants and animals.

Salemme has described a hypothetical structural complex of cytochromes *c* and b_5.[34] This structure, which optimizes intermolecular complementary charge

[30] E. T. Adman, L. C. Sieker, and L. H. Jensen, *J. Biol. Chem.*, 1976, **251**, 3801.
[31] R. Timkovich and R. E. Dickerson, *J. Biol. Chem.*, 1976, **251**, 4033.
[32] R. Timkovich, R. E. Dickerson, and E. Margoliash, *J. Biol. Chem.*, 1976, **251**, 2197.
[33] R. E. Dickerson, R. Timkovich, and R. J. Almassy, *J. Mol. Biol.*, 1976, **100**, 473.
[34] F. R. Salemme, *J. Mol. Biol.*, 1976, **102**, 563.

and steric interactions, was generated by a least-squares fitting process using the known structures of the individual cytochromes. The interactions formed in this complex constrain the two haem prosthetic groups to be nearly coplanar at a closest approach distance for resonant heterocyclic porphyrin ring atoms of 8.4 Å.

Haemoglobin.—Perutz's stereochemical mechanism for the co-operative effects in haemoglobin was based on comparisons between the structures of deoxy and met, rather than deoxy and oxyhaemoglobin. The structure of oxyhaemoglobin is not yet accessible to *X*-ray analysis, because autoxidation occurs faster than *X*-ray data can be collected. However, carbonmonoxyhaemoglobin is stable, and is also isomorphous with oxy- and met-haemoglobin. Oxygen and carbon monoxide have different affinities for, and different reaction kinetics with, haemoglobin, but their co-operative, Bohr, and 2,3-diphosphoglycerate effects are alike; oxy- and carbonmonoxy-haemoglobin are both low-spin complexes of ferrous iron and have closely similar properties. Thus carbonmonoxyhaemoglobin may be regarded as a close relative of oxyhaemoglobin. The difference electron-density map at a resolution of 2.8 Å between horse carbonmonoxyglobin and horse methaemoglobin shows that carbonmonoxyhaemoglobin has the same quaternary structure as methaemoglobin but differs from it by slight changes in tertiary structure in the immediate vicinity of the haems.[35] The distal histidines are slightly further away from the haem ligands and closer to the molecular surface in carbonmonoxyhaemoglobin and there are differences in the position of the thiol group of cysteine F9(93)β and the position of tyrosine HC2(145)β. These differences indicate that, in the quaternary oxy-structure, transition to higher spin and/or loss of ligand increases the occupancy of the pockets between helices F and H by the tyrosine side-chains in preparation for making the *C*-terminal salt bridges in the quaternary deoxystructure. The carbon monoxide oxygen lies off the haem axis in both subunits and it seems possible that the carbon may be displaced from the haem axis in the same direction as the oxygen. The haem pocket is so constructed that it fits an oxygen molecule in the bent conformation, but not a carbon monoxide molecule which has its axis normal to the haem plane, because of steric hindrance. A bent oxygen molecule would fit into the pocket as readily as the haem-linked water molecule, except that histidine E7 would move away from its position in methaemoglobin, where it is hydrogen-bonded to the water molecule, to van der Waals' distance from the oxygen molecule. The difference map indicates that in methaemoglobin the α-haem is ruffled and that on transition from met- to carbonymonoxy-haemoglobin it becomes flattened; in the β-haem the iron appears to move towards the porphyrin plane. The resolution is not sufficient to determine the exact position of the iron atoms and the proximal histidines relative to the porphyrins. All these changes are consistent with the proposed mechanism. Extensive refinement of the model of horse aquomethaemoglobin against the 2.0 Å *X*-ray data suggested that an alanine and a glycine residue in the published sequence were interchanged.[36] This was confirmed by chemical

[35] E. J. Heidner, R. C. Ladner, and M. F. Perutz, *J. Mol. Biol.*, 1976, **104**, 707.
[36] R. C. Ladner, G. M. Air, and J. H. Fogg, *J. Mol. Biol.*, 1976, **103**, 675.

analysis so that E12(63)α should be glycine and E14(65)α should be alanine. In calculating the Fourier synthesis of human deoxyhaemoglobin at 2.5 Å resolution a new procedure was used for weighting the calculation of phases in the isomorphous replacement method.[37] This method separates the effects of statistical errors from those of systematic errors and improves the contribution of anomalous dispersion data to the phase calculation. By studying the diffuse *X*-ray scattering curve of adult human oxyhaemoglobin in an aqueous solution, Damaschun *et al.* have obtained evidence for long-range order in globular proteins with a quaternary structure.[38]

Moffat and co-workers have investigated the structural effects produced by altering the nature of the haem group or of the ligand.[39–42] Metal substitution offers a direct means of studying the function of the haem iron in haemoglobin, for the effect of this replacement on both functional and structural properties can be measured. Haemoglobin has been reconstituted with haem groups containing manganese. The resultant manganoglobin binds ligands reversibly, undergoes a change in quaternary structure, and possesses the normal range of co-operative effects. However, the detailed stereochemistry of Mn-porphyrin differs from that of Fe-porphyrin due to differences in spin state, ionic radius, and co-ordination preferences. Study of manganoglobin should help to determine the effect of such factors as metal spin state, the location of the metal with respect to the mean plane of the porphyrin, the extent of non-planarity of the porphyrin, and the ligand bulk and binding geometry on haemoglobin function. Using difference techniques the structure of $Mn^{III}Hb$ has been compared with that of $Fe^{III}Hb$.[39] The quaternary structures are identical and the tertiary structures are similar. However, there is extensive perturbation of the β-haem contrasted with almost complete lack of perturbation of the α-haem. Either in $Mn^{III}Hb$ the β-haems are six-co-ordinated but undergo a very marked ruffling or the β-haems are only five-co-ordinated in crystals of $Mn^{III}Hb$, but the α-haems remain six-co-ordinated. Numerous small perturbations in the β-globin are transmitted across the $\alpha_1\beta_1$ interface to the α-globin while the $\alpha_1\beta_2$ interface is only slightly perturbed. Metmanganoglobin thus displays some, but not all, of the structural features to be expected in a partially liganded haemoglobin: those arising directly from ligand loss from the β-haems, and contraction of the ligand pocket in the β-chain, but not others such as those which accompany a marked alteration in the distance of the proximal histidine from the mean plane of the porphyrin. The structure of horse methaemoglobin reconstituted with deuteriohaem, in which the haem vinyl substituents are replaced by hydrogens, has also been compared with that of native horse methaemoglobin.[40] The only observed structural changes are small perturbations in the globin which occur in direct response to the missing vinyl groups. These perturbations are highly localized and do not propagate beyond the immediate vicinity of the haem groups. It is possible that these perturbations are of sufficient magnitude to produce the

[37] L. F. Ten Eyck and A. Arnone, *J. Mol. Biol.*, 1976, **100**, 3.
[38] G. Damaschun, J. J. Müller, Ch. Gedicke, and B. A. Federov, *J. Mol. Biol.*, 1976, **104**, 735.
[39] K. Moffat, R. S. Loe, and B. M. Hoffman, *J. Mol. Biol.*, 1976, **104**, 669.
[40] D. W. Seybert and K. Moffat, *J. Mol. Biol.*, 1976, **106**, 895.
[41] J. F. Deatherage, R. S. Loe, C. M. Anderson, and K. Moffat, *J. Mol. Biol.*, 1976, **104**, 687.
[42] J. F. Deatherage, R. S. Loe, and K. Moffat, *J. Mol. Biol.*, 1976, **104**, 723.

slightly altered functional behaviour of deuterioHb. The tertiary structures of horse cyanide methaemoglobin and methaemoglobin differ significantly.[41] The conformations of the haem groups and their interactions with the globin are altered. Any movement of the iron atoms is not directly apparent although there is evidence that some changes in tertiary structure are induced by the shortening of the iron–pyrrol nitrogen bond lengths. These results suggest that the structural changes responsible for co-operativity in haemoglobin may be induced by changes in the non-covalent interactions of the globin with the ligand or porphyrin as well as by an alteration in the covalent porphyrin–proximal histidine linkage. The structure of fluoride methaemoglobin, where the iron is essentially high spin as in methaemoglobin, also showed that the globin structure is extremely sensitive to small changes in the nature of the ligand and demonstrated again that not all liganded haemoglobins are identical in tertiary structure.[42] However, the changes are small.

Abnormal human haemoglobins Nancy β145(HC2) Tyr → Asp and Cochin-Port-Royal β146(HC3) His → Arg have mutations in residues thought to be essential for the full expression of allosteric action in haemoglobin. The structure of deoxyhaemoglobin Nancy shows severe disordering of the β-chain carboxy-terminal tetrapeptide and a possible movement of the β haem iron atom towards the plane of the porphyrin ring.[43] These structural perturbations lead to a high oxygen affinity, reduced Bohr effect, and lack of co-operativity. Inositol hexaphosphate does not influence the $R \rightleftharpoons T$ equilibrium as much as in HbA. The structure of deoxyhaemoglobin Cochin-Port-Royal differs from that of deoxyhaemoglobin A only in the position of the side-chain of residue 146β.[43] The intrasubunit salt bridge between His-146β and Asp-94β is lost. This results in a reduced Bohr effect but no change in Hill coefficient, response to 2,3-diphosphoglycerate, or oxygen affinity. The intersubunit linkage between Lys-40α and the α-carboxy-group is still present. Thus the intrasubunit salt bridge is not required for full co-operativity.

Haemerythrin.—The co-ordination of ligands about the iron atoms in methaemerythrin from *Themiste dyscritum* has been deduced from the 2.8 Å electron-density map.[44] This molecule is an octamer which transports oxygen; each subunit contains two iron atoms and is capable of binding one molecule of oxygen. The co-ordination of the iron atoms is quite different from any of the proposed models. The co-ordination about each iron atom is octahedral and the complex can be described in terms of two trigonal antiprisms about the pair of iron atoms in each subunit; the antiprisms have one face in common. Thus each iron has six ligands, three of which are shared by the other iron atom. Ligands at eight of the nine co-ordination positions are protein side-chains, the ninth is presumably water. The sequence information and the interpretation of the electron-density map suggest six aromatic side-chain ligands and two non-aromatic side-chain ligands. The latter provide atoms at two of the three vertices of the face shared by the two antiprisms and these, along with the presumed water at the third vertex, form bridges between the iron atoms of each pair. The

[43] A. Arnone, G. Gacon, and H. Wajcman, *J. Biol. Chem.*, 1976, **251**, 5875.
[44] R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 349.

low-resolution structure of myohaemerythrin from sipunculan worm erythrocytes has also been determined.[45]

5 Immunoglobulins

Antibodies are capable both of recognizing foreign cells and macromolecules, and of triggering the events which lead to their elimination. Specific recognition requires surface structures complementary to the antigen, which is the part of the foreign body which is recognized and is usually a small area on the surface of a large protein or an associated carbohydrate molecule. Such recognition implies a great variety of antibody molecules while the biological response would be produced most efficiently if all antibody molecules were identical. An IgG antibody molecule consists of two identical light (L) chains and two identical heavy (H) chains. Each chain is subdivided into constant regions and variable regions where sequences vary between antibodies specific for different antigens. The recognition function lies in the Fab fragment, which consists of a light chain and half a heavy chain, and the effector function resides in the Fc fragment or the *C*-terminal halves of the heavy chains. There is interaction between the two parts of the molecule so that effector functions leading to the biological response are triggered by antigen binding. The crystal structures of an intact human IgG antibody molecule Kol and of a human Fc fragment have been determined at 4 and 3.5 Å resolution, respectively.[46, 47] The electron-density maps were interpreted in terms of immunoglobulin domains of known structure. The Fab parts of Kol have a different quaternary structure from that observed in isolated crystalline Fab, and the Fc part is disordered. It is suggested that the Kol molecule is flexible in solution, whereas fragments are rigid. In the Fc fragment the carbohydrate moiety is bound at a fixed position. A hypothetical liganded antibody molecule was constructed by combining the fragment molecules. This resulted in a rigid structure and the authors suggest that antigen binding results in a stiffening of the flexible antibody molecule by means of the formation of inter-domain contacts.[47] The liganded molecule has a T-shaped structure rather than the Y-shaped structure of the non-liganded Kol molecule. Thus there is a ligand-induced conformational change which could trigger the biological response.

There are striking similarities between the structure of the immunoglobin domain and that of the subunit in Cu,Zn superoxide dismutase.[48] These functionally unrelated, sequentially non-homologous proteins contain structurally similar domains composed of anti-parallel β-strands forming a bilayer structure or flattened cylinder. In superoxide dismutase external loops occur in places equivalent to hypervariable region loops in the immunoglobulins.

6 Concanavalin A

The biological activities of the mitogenic lectin concanavalin A depend on the binding of the protein to receptors on the cell surface, and this binding can be

[45] I. M. Klotz, G. L. Klippenstein, and W. A. Hendrickson, *Science*, 1976, **192**, 335–344.
[46] P. M. Colman, J. Deisenhofer, R. Huber, and W. Palm, *J. Mol. Biol.*, 1976, **100**, 257.
[47] R. Huber, J. Deisenhofer, P. M. Colman, M. Matsushima, and W. Palm, *Nature*, 1976, **264**, 415.
[48] J. S. Richardson, D. C. Richardson, K. A. Thomas, E. W. Silverton, and D. R. Davies, *J. Mol. Biol.*, 1976, **102**, 221.

inhibited by specific saccharides. Two papers describe the location of this carbohydrate binding site which is about 13 Å away from the Mn^{2+} binding site.[49, 50]

7 Neurotoxins

The post-synaptic neurotoxins are among the most lethal proteins in snake venom. They act by blocking the nicotinic acetylcholine receptor of the muscle

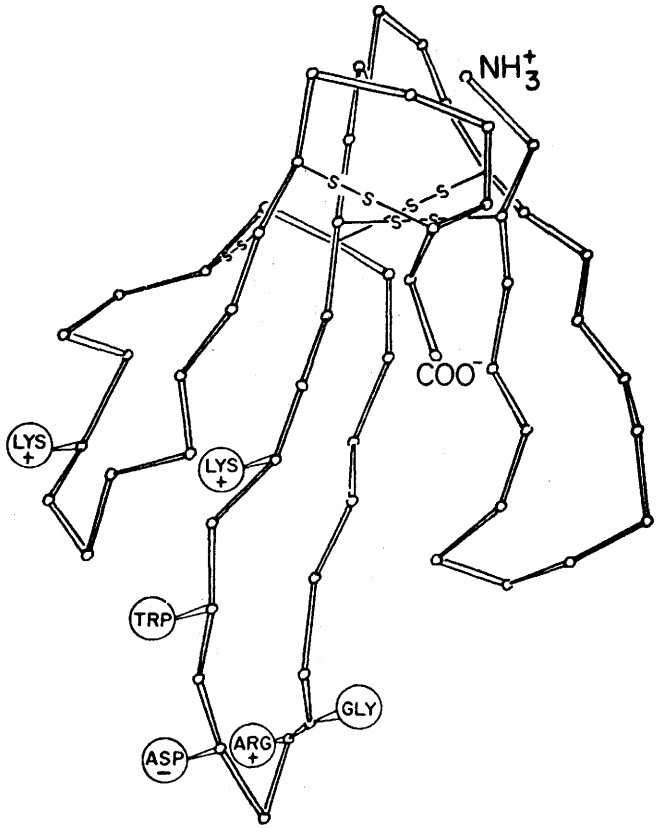


Figure 1 *Perspective drawing of the course of the polypeptide chain of the sea snake neurotoxin. The circles indicate approximate α-carbon positions. The six residues believed essential for toxicity are indicated*
(Reproduced by permission from *F.E.B.S. Letters*, 1976, **68**, 1)

motor end plate, thus preventing transmission across the cholinergic synapse. Their binding to the receptor is extremely specific and tight and they have been used to isolate the receptor proteins. The structures of the closely related

[49] K. D. Hardman and C. F. Ainsworth, *Biochemistry*, 1976, **15**, 1120.
[50] J. W. Becker, G. N. Reeke, B. A. Cunningham, and G. M. Edelman, *Nature*, 1976, **259**, 407.

neurotoxin *b* and erabutoxin *b* from sea snake venom have been determined at high resolution.[51, 52] The molecule, which contains 62 residues and four disulphide bridges, is extremely flat and extended in one direction. The central core of the molecule is the four sulphide bridges and three long loops of chain emerge from this central core (Figure 1). About 40% of the molecule forms a twisted antiparallel β-sheet. Comparison of amino-acid sequences has led to the identification of 12 invariant residues as essential for maintaining tertiary structure. At least six other residues are thought to be involved in neurotoxic activity. All of these observations are explained by the structure. Of the 'toxic' residues five are contained in the extended loop of antiparallel pleated sheet pointing straight down in Figure 1. Five of them are also pointing in the same direction forming an 'active surface'. If it is assumed that this active surface is the principal site of interaction with the acetylcholine receptor a number of inferences can be drawn about the nature of this interaction. These are that the binding takes place through a combination of ionic and hydrophobic interactions, the principal driving forces being the binding of the arginine residue (33) at the tip of the loop to the acetylcholine cation binding site, and the burying of the exposed hydrophobic side-chain of the nearby tryptophan (29). The remarkable shape of the toxin structure and the concentration of key side-chains on one extended loop suggest that the toxin binds by inserting the loop into a cleft or channel in the membrane-bound receptor. This channel must be at least 19 Å deep and 7 Å wide.

8 Transfer-RNA Synthetases

tRNA synthetases provide the key for the translation of the genetic code and play a central role in the assembly of amino-acids into peptide chains. They are capable of binding amino-acid, tRNA, and ATP. The structure of tyrosyl-tRNA synthetase from *Bacillus stearothermophilis* has been determined at 2.7 Å resolution.[53] The molecule is an elongated dimer of mol. wt. 90 000. The monomer has dimensions 60 × 60 × 40 Å while the maximum dimension of the dimer is *ca.* 130 Å. The monomer contains a central core of six-stranded pleated sheet together with five α-helices more than 12 Å in length, as well as several shorter helices. There is a narrow waist *ca.* 35 Å in diameter at the centre of the dimer and the catalytic site is about 18 Å from the two-fold axis at the centre of the dimer. Methionyl tRNA synthetase from *E. coli* is a dimer of mol. wt. 172 000. Controlled proteolysis by trypsin gives a well-defined single-chain fragment of mol. wt. 64 000. This fragment retains full specificity towards Met and $tRNA^{Met}$ and has unimpaired activity. The structure at 4 Å resolution shows that the fragment is an elongated ellipsoid with overall dimensions 90 × 43 × 43 Å. It has two domains, the volume of one domain being twice that of the other, and the two domains are separated by a large cleft.[54]

[51] D. Tsernoglou and G. A. Petsko, *F.E.B.S. Letters*, 1976, **68**, 1.
[52] B. W. Low, H. S. Preston, A. Sato, L. S. Rosen, J. E. Searl, A. D. Rudko, and J. S. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2991.
[53] M. J. Irwin, J. Nyborg, B. R. Reid, and D. M. Blow, *J. Mol. Biol.*, 1976, **105**, 577.
[54] C. Zelwer, J. L. Risler, and C. Monteilhet, *J. Mol. Biol.*, 1976, **102**, 93.

9 Proteolytic Enzymes

Trypsin.—The high-resolution structure of bovine trypsinogen has been determined by Patterson search techniques, using the refined model of bovine trypsin, and by subsequent refinement.[55] The overall chain folding is very similar to that of trypsin. The chain segments which form the specificity pocket in trypsin are flexible in trypsinogen and the autolysis loop is partially mobile between residues 142 and 152. The *N*-terminus may be only weakly fixed to the rest of the molecule or may even float freely in solution. The general structural concept of zymogen activation which emerges is that of a fixation of flexible regions to form the specificity pocket. Bode and Huber have shown that this fixation or trypsinogen–trypsin transition can be induced by peptides sequentially similar to the *N*-terminus of trypsin.[56] Normally bovine trypsinogen is activated by the enzymatic removal of its *N*-terminal hexapeptide. The newly formed amino-terminal group forms an internal salt bridge which is necessary for the transition to the active enzyme conformation. This conversion of trypsinogen to the ordered trypsin-like conformation can be triggered by an Ile-Val dipeptide which is capable of binding and forming the internal salt bridge.

Chymotrypsin.—An improved refined model of chymotrypsinogen shows that the catalytic Ser-195 side-chain is in the acyl orientation and its Oγ atom is in position to form a normal hydrogen bond with Nε2 of His-57.[57] In contrast, the corresponding hydrogen bond in α-chymotrypsin is severely distorted as a consequence of a 1.5 Å shift in the relative positions of the two cylindrical domains composing most of the molecule. There is evidence that the hydrogen bond between the histidine and serine side-chains of the charge-relay system is distorted in all serine proteases and this could facilitate the transfer of a proton from the catalytic serine side-chain to the leaving group of the substrate.

Subtilisin.—The structure of the complex formed when subtilisin BPN′ is inactivated with the covalent inhibitor L-phenylalanyl-L-alanyl-L-lysine chloromethyl ketone shows that the polypeptide chain of the lysine inhibitor forms an antiparallel β-pair network of hydrogen bonds with an enzyme backbone segment.[58] A novel structural feature is that the aliphatic portion of the inhibitor's P_1 lysine side-chain fits into the entrance of the S_1 specificity cavity with its side-chain extending out to interact with the side-chain carboxylate group of Glu-156. The inhibitor forms a stabilized hemiketal with the catalytic serine side-chain, and it is proposed that this structure represents the productive binding geometry for substrates with lysine or arginine at P_1 and that the first step of the mechanism for halogenomethyl ketone inactivation of serine proteases is the utilization of the enzyme's catalytic apparatus to form a hemiketal. The second and rate-limiting step is alkylation of the catalytic histidine (Figure 2).

Papain.—The binding of chloromethyl ketone substrate analogues to papain has also been analysed.[59] The active site of papain, which is a cysteine protease,

[55] W. Bode, H Fehlhammer, and R. Huber, *J. Mol. Biol.*, 1976, **106**, 325.
[56] W. Bode and R. Huber, *F.E.B.S. Letters*, 1976, **68**, 231.
[57] J. J. Birktoft, J. Kraut, and S. T. Freer, *Biochemistry*, 1976, **15**, 4481.
[58] T. L. Poulos, R. A. Alden, S. T. Freer, J. J. Birktoft, and J. Kraut, *J. Biol. Chem.*, 1976, **251**, 1097.
[59] J. Drenth, K. H. Kalk, and H. M. Swen, *Biochemistry*, 1976, **15**, 3731.

is a groove on the molecular surface in which the essential thiol group of Cys-25 is situated next to the imidazole ring of His-159. The interpretation of the difference maps gave models for the acyl-enzyme structure and for the tetrahedral intermediate. The binding of the carbonyl oxygen of the P_1 residue is very

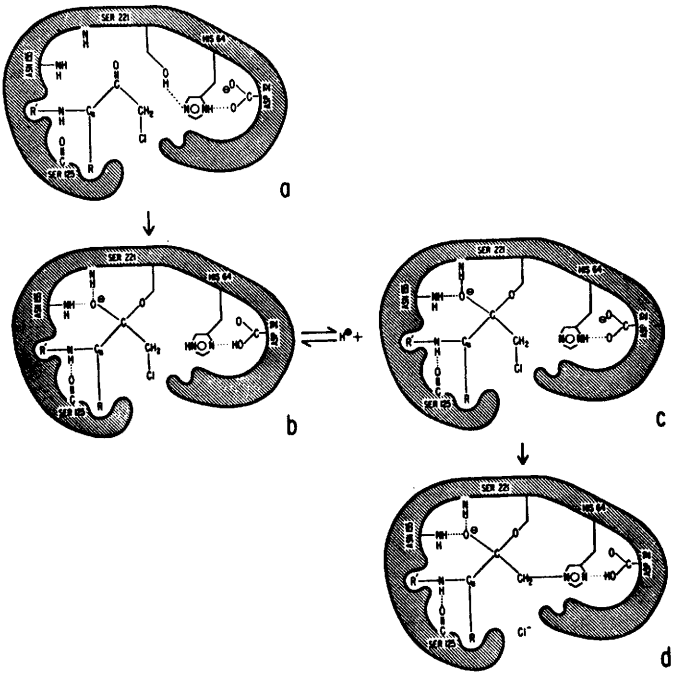


Figure 2 *Schematic representation of a proposed mechanism of inactivation of subtilisin by polypeptide chloromethyl ketones.* (a) *Michaelis complex*; (b) *tetrahedral hemiketal with protonated charge-relay systems*; (c) *tetrahedral hemiketal with deprotonated charge-relay system*; (d) *final alkylated product with chloride ion diffusing away*
(Reproduced by permission from *J. Biol. Chem.*, 1976, **251**, 1097)

similar to that found for the proteolytic serine enzymes. The nitrogen atom of the scissile peptide bond is close to the imidazole ring of His-159, suggesting a role for this ring in protonating the N atom of the leaving group.

Phospholipase A_2.—This enzyme catalyses the hydrolysis of the fatty acid ester bond at the 2-position of 1,2-diacyl *sn*-phosphoglycerides. The precursor, which contains 132 residues of known sequence and seven disulphide bridges, is converted into active phospholipase A_2 by fission of the peptide bond between Arg-7 and Ala-8 and removal of the *N*-terminal heptapeptide from the molecule. The 3 Å resolution structure of porcine pancreatic prephospholipase A_2 shows that the molecule contains neither regular helices nor β-pleated sheets.[60] Assuming that the phosphate moiety of the substrate is bound to Arg-108 and a Ca ion in a manner similar to that observed in staphylococcal nuclease the hydrophobic

[60] J. Drenth, C. M. Enzing, K. H. Kalk, and J. C. A. Vessies, *Nature*, 1976, **264**, 373.

part of the lecithin molecule could fold along a highly hydrophobic surface of the enzyme. The geometry of the active site is very similar to that found in other proteolytic enzymes; the residues Asp-56, His-55, and Tyr-35 are highly conserved and could act as the nucleophile, the stabilizer of the tetrahedral intermediate, and the proton donor, respectively.

Lysozyme.—The structures of the triclinic and tetragonal crystal forms of hen egg-white lysozyme have been compared.[61] There are appreciable conformational differences in the main chain as well as side-chain conformations but these all occur in surface regions involved in intermolecular contacts. Both mono- and di-*N*-acetylglucosamine have been bound to triclinic lysozyme.[62] As in tetragonal lysozyme the side-chain of Trp-62 moves towards the bound inhibitor and the carbonyl oxygen atom of Ala-107 shifts slightly. There is no suggestion of a movement in the lobes of the molecule as was evident in the tetragonal crystals. The molecular replacement method has been used to calculate the structure of human lysozyme.[63]

Ribonuclease S.—Crystals of solid-phase-derived semisynthetic ribonuclease-S′ have been compared with those of the native enzyme.[64] The structure of the non-covalent complex of synthetic fragment 1—20 and native 21—124 is identical with that of native ribonuclease-S′.

Carboxypeptidase B.—An improved, refined structure of carboxypeptidase B was obtained by using the known structure of carboxypeptidase A as a starting model.[65] The resulting differences correlate well with chemical and physical differences between the two molecules observed by other techniques. Any differences in the folding of the main chain are confined to the chain termini and external loops. The active sites are alike with the Zn atom and its ligands, Arg-145 (substrate carboxy-group binding) and Glu-270 (peptide bond cleavage) all in nearly identical positions. Asp-255 in the centre of the binding pocket explains the difference in substrate specificities. Tyr-247, the proposed proton donor, was found to be disordered.

10 Glycolytic Enzymes, Dehydrogenases, and Kinases

Now that the *X*-ray technique is fairly routine more ambitious projects are being undertaken. One of these is a concerted effort by several laboratories to determine the structures of all the enzymes involved in glycolysis in the hope that some patterns in structural and functional properties might emerge. Detailed structural information is now available for many of these enzymes.

Phosphorylase.—Glycogen phosphorylase from muscle catalyses the first step in the breakdown of glycogen producing α-D-glucose-1-phosphate from glycogen and P_i. Skeletal muscle contains two forms of the enzyme which are interconvertible. Phosphorylase b, found in resting muscle, requires AMP for activity

[61] J. Moult, A. Yonath, W. Traub, A. Smilansky, A. Podjarny, D. Rabinovich, and A. Saya, *J. Mol. Biol.*, 1976, **100**, 179.
[62] K. Kurachi, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, 1976, **101**, 11.
[63] P. E. Dixon and A. C. T. North, *Acta Cryst.*, 1976, **A32**, 320, 325.
[64] M. Pandin, E. A. Padlan, C. DiBello, and I. M. Chaiken, *Proc. Acad. Nat. Sci. U.S.A.*, 1976, **73**, 1844.
[65] M. F. Schmid and J. R. Herriott, *J. Mol. Biol.*, 1976, **103**, 175.

and is inhibited by ATP or glucose-6-phosphate, so that it is normally inactive. Phosphorylase a is formed during muscle contraction, or hormonal stimulation, by the phosphorylation of Ser-14 of each monomer of the phosphorylase b dimer. Phosphorylase a no longer requires AMP for activity nor is it inhibited by ATP or glucose-6-phosphate, although it is inhibited by glucose. The a form is predominantly tetrameric *in vitro*, although its catalytically active form, when bound to glycogen, is dimeric. The b enzyme crystallizes as a dimer with one subunit of molecular weight 100 000 in the asymmetric unit. In the presence of glucose to cause dimer formation phosphorylase a will crystallize in the same space group. Low-resolution electron-density maps for both forms have been calculated.[66] The monomer–monomer interface region within the dimer shows many close contacts and interpenetration of the polypeptide chain. Extensive structural differences between the two forms are evident, although these differences are mainly confined to two regions. One of these regions is the interface, where an apparent shift of a 30 Å length of main-chain is visible. Fletterick *et al.* have published the 3 Å resolution structure of the a form.[67] The active site, of which there are two per dimer, is shared between the two subunits at their interface. Within the active site region are the binding sites for the substrates and the allosteric effector, AMP. The site of metabolic control, Ser-14 phosphate, is 15 Å from the AMP binding site. A second region of the molecule contains a polysaccharide binding site which may represent a storage site where by phosphorylase is bound to glycogen in the muscle cell. The polypeptide chain in another region has the same topological structure as the nucleotide binding domains in the dehydrogenases. This domain is partially buried inside the molecule. Glutaraldehyde cross-linked crystals have been shown to be catalytically active.[68]

Hexokinase.—The structure determination of yeast hexokinase has been extended to 3.5 Å resolution for the dimer and to 2.7 Å resolution for the monomer.[69] In the dimer the interaction of the subunits is heterologous or non-symmetric and this is associated with small differences in structure, particularly at the subunit interface. This accounts for some of the enzyme's non-symmetric interactions with substrates and activators.

Glucose Phosphate Isomerase.—The structure of glucose-6-phosphate isomerase has been determined at 3.5 Å resolution and the conformation of the active site described.[70] The enzyme is a dimer with a subunit mol. wt. *ca.* 60 000. The structure of each subunit consists of two domains, each with a central core of β-sheet surrounded by helices. As in phosphorylase the active site is formed by parts of both subunits. The binding site for the powerful competitive inhibitor, arabinose-5-phosphate, is formed partly in the slight cleft between the two β-sheet domains of one subunit, and partly by portions of chain from the second subunit. These portions of chain from the second subunit move closer to the

[66] R. J. Fletterick, J. Sygusch, N. Murray, N. B. Madsen, and L. N. Lohnson, *J. Mol. Biol.*, 1976, **103**, 1.
[67] R. J. Fletterick, J. Sygusch, H. Semple, and N. B. Madsen, *J. Biol. Chem.*, 1976, **251**, 6142.
[68] P. J. Kasvinsky and N. B. Madsen, *J. Biol. Chem.*, 1976, **251**, 6852.
[69] T. A. Steitz, R. J. Fletterick, W. F. Anderson, and C. M. Anderson, *J. Mol. Biol.*, 1976, **104**, 197.
[70] P. J. Shaw and H. Muirhead, *F.E.B.S. Letters*, 1976, **65**, 50.

inhibitor as it binds (Figure 3). Based on the structure a mechanism is proposed which is consistent with the formation of a *cis*-ene-diol intermediate.

Aldolase.—The folding and quaternary structure of trimeric 2-keto-3-deoxy-6-phosphogluconic aldolase from *Pseudomonas putida* has been described.[71] The

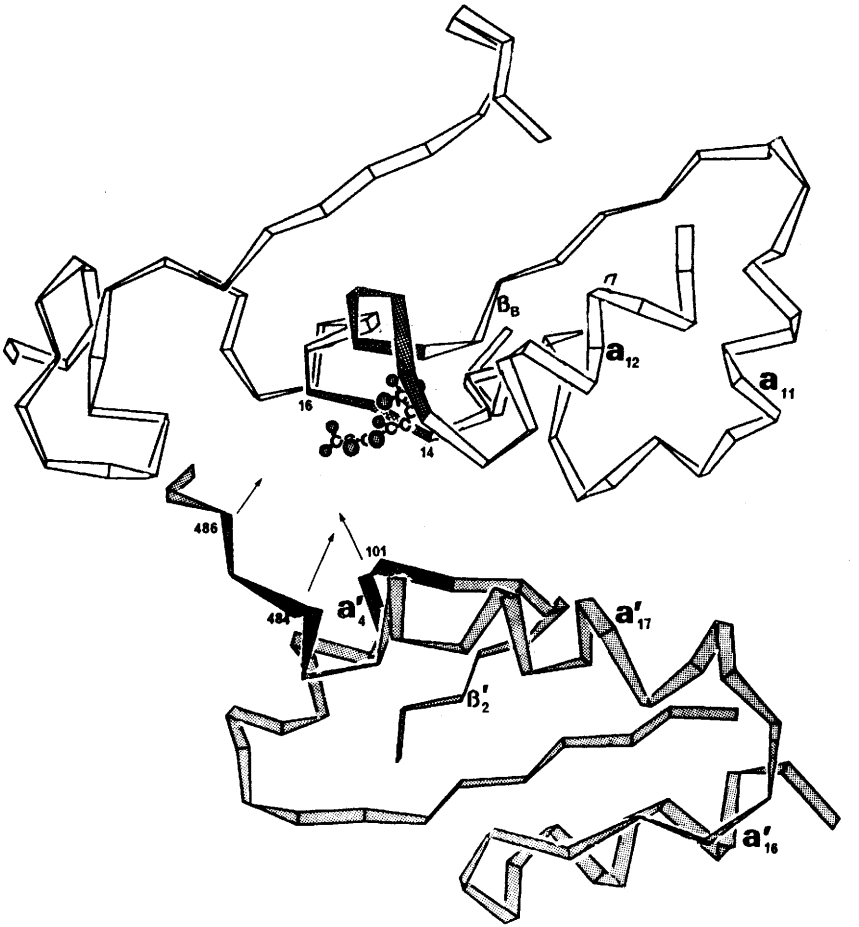


Figure 3 *The folding of the polypeptide chain around the active site of glucose phosphate isomerase. The inhibitor, arabinose-5-phosphate, is shown in the site. The chain is shown in its conformation in the native enzyme, the movements of protein that accompany the binding of the inhibitor are indicated by arrows. The amino-acid side-chains implicated in binding and catalysis are numbered, and the portions of the chain forming the active site pocket are indicated by dark shading. The chain in one subunit has been lightly shaded, that in the other left blank. Secondary structural elements have been labelled* a (α-*helices*) *and* β (*parallel* β-*sheet*). *The labelling scheme adopted for the subunit was* β_1—β_6 *for the* β-*sheet in one domain and* β_A—β_D *for the other*
(Reproduced by permission form *F.E.B.S. Letters*, 1976, **65**, 50)

[71] I. M. Mavridis and A. Tulinsky, *Biochemistry*, 1976, **15**, 4410.

reaction catalysed by this aldolase is similar or identical with that effected by fructose-1,6-diphosphate aldolase found in higher animals. Each subunit contains nine helical regions, two parallel chains, and a long empty channel.

Triose Phosphate Isomerase.—The atomic co-ordinates for the chicken muscle enzyme have been published.[72] Histidine residues in the enzyme have been studied by proton magnetic resonance spectroscopy and crystallography.[73] The results suggest that a combination of such techniques will enable conformational changes to be defined very precisely.

D-Glyceraldehyde-3-Phosphate Dehydrogenase.—Two anion-binding sites have been located in the active centres of each of the four subunits of D-glyceraldehyde-3-phosphate dehydrogenase.[74] The positions of the two sites can be associated with the binding sites of the terminal phosphate group of the substrate and the participatory organic phosphate. Cofactor-depleted crystals at pH 5.0 contained only one NAD^+ per tetramer and were isomorphous with those of the holoenzyme.[75] There were no significant conformational changes. Study of crystals of the apo-enzyme plus brominated coenzyme showed that the coenzyme was in the anti conformation in all four subunits.[75] These experiments did not verify the presence of possible molecular asymmetry. Twinning in crystals of the holoenzyme from human skeletal muscle has been discussed.[76]

Lactate Dehydrogenase.—The structure of apo dogfish M_4 LDH has been compared with those of its ternary complexes.[77] The differences in structure all occur in regions of the protein around the active site, particularly in the flexible loop covering the active centre pocket and the *C*-terminal helix. The changes suggest that the closing of the loop on binding coenzyme and substrate triggers the movement of the critical reactive residues into their catalytically active positions.

Liver Alcohol Dehydrogenase.—The crystal structure analysis of horse liver alcohol dehydrogenase has been extended to 2.4 Å resolution.[78] The coenzyme binding domain consists of a central six-stranded pleated-sheet structure plus helices. The coenzyme analogue ADP-ribose binds to this domain in a position and orientation very similar to coenzyme binding to lactate dehydrogenase. The catalytic domain is mainly built up from three distinct antiparallel pleated-sheet regions (Figure 4) and provides ligands for the catalytic zinc, and 19 residues form a lobe that binds the second zinc atom of the subunit. The lobe forms one side of a significant cleft in the enzyme surface, suggesting that this region might

[72] D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, and I. A. Wilson, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 146.
[73] C. A. Browne, I. D. Campbell, P. A. Kiener, D. C. Phillips, S. G. Waley, and I. A. Wilson, *J. Mol. Biol.*, 1976, **100**, 319.
[74] K. W. Olsen, R. M. Garavito, M. N. Sabesan, and M. G. Rossmann, *J. Mol. Biol.*, 1976, **107**, 571.
[75] K. W. Olsen, R. M. Garavito, M. N. Sabesan, and M. G. Rossmann, *J. Mol. Biol.*, 1976, **107**, 577.
[76] W. D. Mercer, S. I. Winn, and H. C. Watson, *J. Mol. Biol.*, 1976, **104**, 277.
[77] J. L. White, M. L. Hackert, M. Buehner, M. J. Adams, G. C. Ford, P. J. Lentz, I. E. Smiley, S. J. Steindel, and M. G. Rossmann, *J. Mol. Biol.*, 1976, **102**, 759.
[78] H. Eklund, B. Nordström, E. Zeppezauer, G. Söderlund, I. Ohlsson, T. Boiwe, B.-O. Söderberg, O. Tapia, C.-I. Brändén, and A. Akeson, *J. Mol. Biol.*, 1976, **102**, 27.

form a second catalytic centre of unknown function. Both subunits contribute residues to each of the two substrate binding pockets of the molecule. Comparisons of primary structures of mammalian, yeast, and bacillar alcohol dehydrogenase show that residues involved in subunit interactions, ADP-ribose binding, and the possible enzymatic mechanism are conserved as well as all zinc ligands.[79]

Adenylate Kinase.—The structure of the principal human adenylate kinase is the same as that of porcine adenylate kinase and incorporates the nucleotide binding fold.[80]

11 Protein Conformation

Structural resemblances between proteins have led to the suggestion that proteins with a similar chain-fold may have evolved from a common gene, although there are no longer similarities between their amino-acid sequences. Rossmann and Argos have developed a technique for exploring these structural homologies between proteins.[81] A search function represents the number of sequentially equivalenced amino-acids for each orientation of one protein structure with respect to another. The structurally equivalent residues with the highest probabilities are then selected for the best common topology. The method has been used to predict the similarity of the NAD binding domains of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, the haem binding fold of cytochrome b_5 and the globins, and the substrate binding sites of hen egg-white lysozyme and phage lysozyme. However, such similar structures could arise either by divergent evolution from a common gene or by convergent evolution of stable associations of secondary structural elements. Several authors have considered the arrangements of α-helices and β-sheets in globular proteins and have shown that structural similarity may be an expression of a structural rule.[82–84] For example, consider the β-α-β unit which consists of two parallel but not necessarily adjacent β-strands which lie in a β-sheet and are connected by one or more α-helices. This unit could adopt either a right-handed or a left-handed conformation. About 98% of such units found in proteins of known structure are right-handed. This can be explained in terms of the twist of the β-sheet which favours a right-handed unit to avoid steric hindrance in the loop regions. This handedness is an important element in understanding the folding of proteins. Levitt and Chothia have defined four clearly separate classes to describe such arrangements of α-helices and β-sheets. They are (i) all-α proteins that have only α-helix secondary structure, (ii) all-β proteins that have mainly β-sheet secondary structure, (iii) $\alpha + \beta$ proteins that have α-helix and β-strand secondary structure segments that do not mix but tend to segregate along the polypeptide chain, and (iv) α/β proteins that have mixed or approximately

[79] H. Eklund, C.-I. Brändén, and H. Jörnvall, *J. Mol. Biol.*, 1976, **102**, 61.
[80] I. von Zabern, B. Wittmann-Liebold, R. Untucht-Grau, R. H. Schirmer, and E. F. Pai, *European J. Biochim.*, 1976, **68**, 281.
[81] M. G. Rossmann and P. Argos, *J. Mol. Biol.*, 1976, **105**, 75.
[82] J. S. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2619.
[83] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1976, **105**, 367.
[84] M. Levitt and C. Chothia, *Nature*, 1976, **261**, 552.

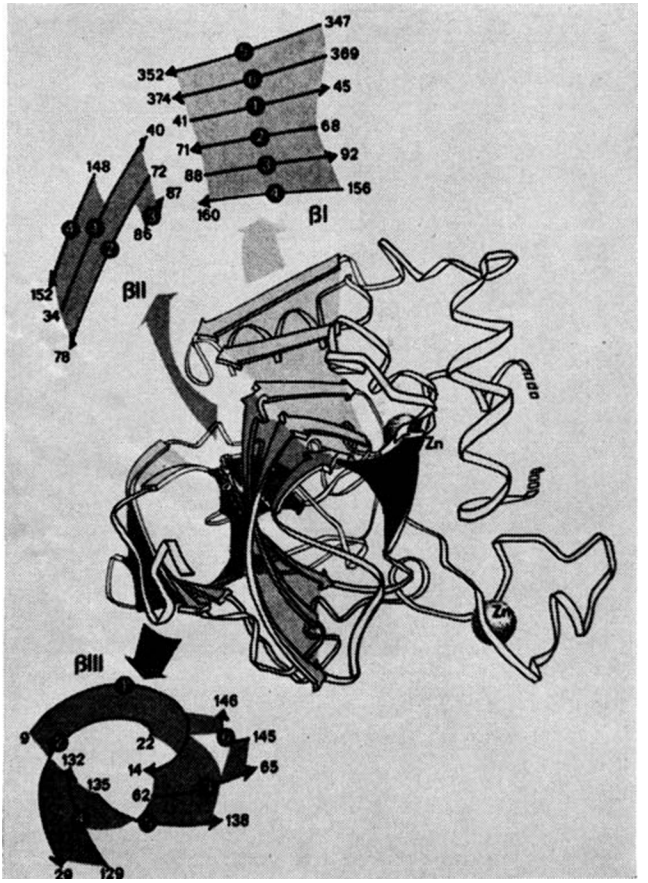


Figure 4 *Schematic diagram of the three different pleated-sheet structures in the catalytic domain of liver alcohol dehydrogenase. The strands of region* βII, *in common with some of the strands of* βI, *enclose an approximate cylinder with a tightly packed hydrophobic core. Sheet* βIII *forms a somewhat irregular closed cylinder enclosing a second core of hydrophobic residues*

(Reproduced by permission from *J. Mol. Biol.*, 1976, **102**, 27)

alternating segments of α-helical and β-strand secondary structure. The folding in some α/β proteins is shown in Figure 5. Many of these proteins have similar or closely related structures. The degree of significance of structural homologies can only be established when the structural rules leading to these folding patterns are fully understood.

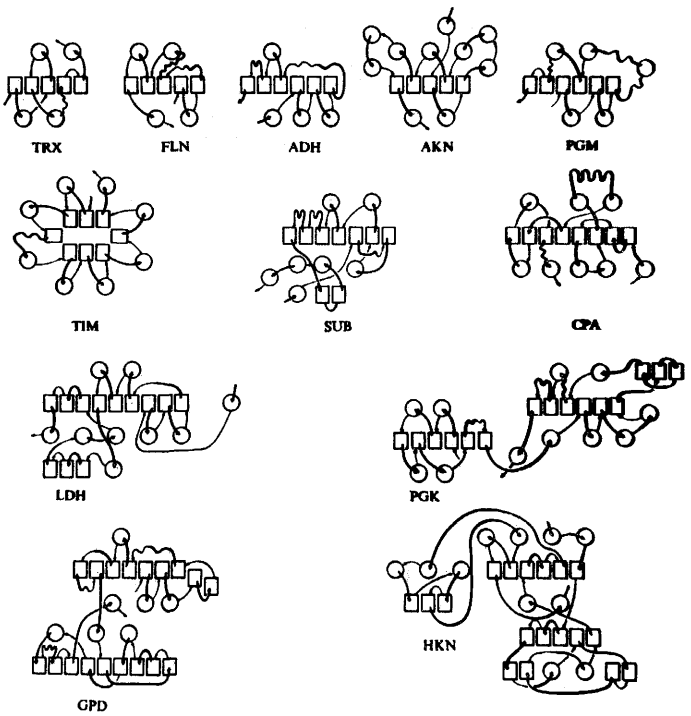


Figure 5 *Topology/packing diagrams of* 12 α/β *proteins in order of increasing size*: *thioredoxin* (TRX); *flavodoxin* (FLN); *alcohol dehydrogenase coenzyme domain* (ADH); *adenyl kinase* (AKN); *phosphoglycerate mutase* (PGM); *triose phosphate isomerase* (TIM); *subtilisin* (SUB); *carboxypeptidase* (CPA); *lactate dehydrogenase* (LDH); *phosphoglycerate kinase* (PGK); D-*glyceraldehyde-3-phosphate dehydrogenase* (GPD); *and hexokinase* (HKN). *The rectangles represent* β-*strands and the circles* α-*helices*

(Reproduced by permission from *Nature*, 1976, **261**, 552)

12 Muscle

The tropomyosin molecule consists of two α-helices, which form a coiled-coil a little over 400 Å long. The molecular packing in magnesium paracrystals of α-tropomyosin has been examined by electron microscopy.[85] The paracrystals are composed of antiparallel arrays of molecules. The overlap between the *C*-terminal of the molecules in the unit cell is *ca.* 175 residues and the overlap of the *N*-termini is between 107 and 122 residues. It is proposed that the Mg^{2+}

[85] M. Stewart and A. D. McLachlan, *J. Mol. Biol.*, 1976, **103**, 251.

ions form bridges between opposing negative charges and make an important contribution to the stability of the structure, that the troponin binding site is *ca.* 130 Å from the *C*-terminus, and that Cys-190 is within 10 to 15 Å of the dyad relating two *C*-termini. This troponin binding site is probably near residues 197 to 217.[86] A Fourier analysis of the different types of amino-acid in the sequence of tropomyosin showed a strong 14-fold periodicity.[87] Anomalies in charge distribution found near to the molecular ends and close to Cys-190 were attributed to end-to-end overlap sites and the troponin binding site. The periodicity was explained by proposing alternative sets of sites which bind equivalently to complementary groups of sites in seven actins in the 'relaxed' and 'inactive' states of muscle, respectively. At each site a negative zone probably attaches to actin by magnesium bridges and a hydrophobic zone by direct interaction. A quarter rotation of the whole tropomyosin molecule would detach one set of seven sites and attach the other set, allowing a highly co-operative mechanism.

The cross-striated adductor muscle of the scallop is similar in many respects to other striated muscles.[88] The major difference is in the structure of the thick filaments and particularly in the arrangement of the projections. It is proposed that the thick filaments, which contain myosin and paramyosin, have a core of paramyosin around which the myosin molecules pack. The data are consistent with a six-stranded helix with projections along the strands corresponding to the heads of one or two myosin molecules. In rigor muscle these projections move away from the backbone and attach to the thin filaments. *X*-Ray diffraction studies indicate that the sarcomeric unit cell of the thick filament lattice of crayfish muscle fibres stripped of the sarcolemma does not shorten isovolumetrically.[89] This is explained in terms of the variation of negative charge within the A-band with the interdigitation of the thin filaments.

β,γ-Imido-ATP is an ATP analogue which will bind to glycerol-extracted insect flight and rabbit back muscle fibres.[90] It binds tightly to the enzymatic site of myosin, but is not hydrolysed there. The binding causes some change in the myosin conformation, as if the head was rotated relative to the actin, but it is not detached from the actin. This complex could be an analogue of the hypothetical tension-bearing attached state of active muscle. An attempt was made to observe *X*-ray diffraction changes within a short time after the nucleotide was added at different nucleotide concentrations by using synchrotron radiation.[91] The results showed that within a short time of the binding of imido-ATP to myosin a structural change occurs in the muscle of magnitude directly proportional to the amount of nucleotide bound. The effect is both rapid and specific and may well be relevant to the state of the myosin during its contractile cycle. Two papers describe attempts to analyse the low-angle *X*-ray diffraction pattern from resting and activated muscle in terms of the effect on the cross

[86] A. D. McLachlan and M. Stewart, *J. Mol. Biol.*, 1976, **106**, 1017.
[87] A. D. McLachlan and M. Stewart, *J. Mol. Biol.*, 1976, **103**, 271.
[88] B. M. Millman and P. M. Bennett, *J. Mol. Biol.*, 1976, **103**, 439.
[89] E. W. April and D. Wong, *J. Mol. Biol.*, 1976, **101**, 107.
[90] S. B. Marston, C. D. Rodger, and R. T. Tregear, *J. Mol. Biol.*, 1976, **104**, 263.
[91] R. S. Goody, J. B. Leigh, H. G. Mannherz, R. T. Tregear, and G. Rosenbaum, *Nature*, 1976, **262**, 613.

bridges.[92, 93] The movement of the cross bridges is thought to involve both radial and azimuthal components. However, it seems to be impossible to reach any detailed conclusions.

13 Viruses

Tobacco Mosaic Virus—The hand of the helical array of subunits in the stacked-disk form of TMV protein has been determined from electron microscopy.[94] The near-axial intersubunit contacts between disks are similar to those within a single disk but quite different from those in the virus. TMV coat protein has been repolymerized in the absence of RNA to give a new helical aggregate.[95] Two different helical surface lattices are obtained, with only small differences in polypeptide chain folding. The structure of the protein rod in one lattice is almost identical with that in intact TMV, while in the absence of RNA the lattice with an extra subunit per turn is predominant. These structures could be important in the self-assembly of TMV.

Bacteriophage.—The structures of the contractile tail sheaths of T4 and Mu have been studied using the technique of three-dimensional image reconstruction.[96, 97] The structures are similar. The assembly of the head of bacteriophage P22 has been examined using electron microscopy and small-angle *X*-ray diffraction to study the principal structures found in the head assembly pathway.[98] The proheads consist of a shell of coat protein of radius 256 Å containing an internal scaffold which is a thick shell or solid ball of outer radius 215 Å. The DNA appears to interact with coat protein subunits in a specific manner, and the prohead to head transformation involves, in addition to loss of the internal scaffold and its relacement by DNA, a structural transition in the outer shell. The clustering of the coat protein does not change radically during this transition or expansion.

Filamentous Bacterial Virus.—The filamentous bacterial virus Pfl exhibits two distinct fibre diffraction patterns. The transition between these patterns can be explained by a fractional change in the number of units per turn in the coat protein helix.[99] The transition is energetically feasible.

14 Fibrous Proteins

The structure of a polypeptide with a collagen-like sequence has been investigated[100] and various models for the structure of collagen have been described.[101–103] The fine detail to a resolution of 4 Å of the α-keratin microfibril

[92] R. J. Poddsky, R. St. Onge, L. Yu, and R. W. Lymn, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 813.
[93] J. C. Haselgrove, M. Stewart, and H. E. Huxley, *Nature*, 1976, **261**, 606.
[94] A. C. Bloomer, J. N. Champness, and P. N. T. Unwin, *J. Mol. Biol.*, 1976, **105**, 453.
[95] E. Mandelkow, K. C. Holmes, and U. Gallwitz, *J. Mol. Biol.*, 1976, **102**, 265.
[96] P. R. Smith, U. Aebi, R. Josephs, and M. Kessel, *J. Mol. Biol.*, 1976, **106**, 243.
[97] G. Admiraal and J. E. Mellema, *J. Ultrastructure Res.*, 1976, **56**, 48.
[98] W. Earnshaw, S. Casjens, and S. C. Harrison, *J. Mol. Biol.*, 1976, **104**, 387.
[99] E. J. Wachtel, F. J. Marvin, and D. A. Marvin, *J. Mol. Biol.*, 1976, **107**, 379.
[100] A. Del Pra, M. Palumbo, and M. Goodman, *Biopolymers*, 1976, **15**, 503.
[101] D. W. L. Hukins, J. Woodhead-Galloway, and D. P. Knight, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 1049.
[102] J. Woodhead-Galloway and P. A. Machin, *Acta Cryst.*, 1976, **A32**, 368.
[103] J. Woodhead-Galloway, *Acta Cryst.*, 1976, **B32**, 1880.

has been obtained by direct mapping of the cylindrically averaged intensity transform.[5, 104] There are two possible models involving either two-strand or three-strand rope segments. A family of double helices of alternating poly-(γ-benzyl-DL-glutamate) has been studied as a stereochemical model for the antibiotic gramicidin A which is a linear pentadecapeptide.[105] The models are based on double helices made of antiparallel strands.

15 Other Biological Structures

Electron-density profiles obtained for hydrated egg lecithin and cholesterol bilayers have been analysed in terms of the molecular configurations of the component lipids.[106] The cholesterol molecule is largely immersed in the hydrocarbon core of the bilayer with its hydroxy-group near the water interface. The phosphorylcholine headgroup of the lecithin lies approximately in the bilayer plane.

The *X*-ray diffraction pattern has been obtained for both intact and collapsed halobacterium halobium gas vesicles.[107] The wall is remarkably thin, its average thickness being *ca.* 20 Å. A large part of the protein in the wall consists of β-sheet. This β-sheet is in two layers, one on top of the other. The two layers together can stiffen the wall and hence strengthen the vesicle against collapse. The structure of the gas vesicle wall from *Anabaena flos-aquae* has a similar structure.[108]

X-Ray diffraction patterns from nerve myelin show broadening of the Bragg reflexions and diffuse intensity between them.[109] These observations indicate disordered stacking of the myelin membrane and the model indicates that both the cytoplasmic and extracellular spaces are somewhat variable.

PART III: Conformation and Interaction of Peptides and Proteins in Solution

edited by R. H. Pain, with contributions by T. Brittain, E. A. Carrey, D. P. E. Dickson, J. G. Hoggett, C. Mitchinson, D. J. Osguthorpe, H. W. E. Rattle, B. Robson, R. M. Stephens, P. J. Sealey, and E. J. Wood

1 Theoretical Aspects of Protein Conformation

Contributed by B. Robson and D. J. Osguthorpe

In a recent review Schulz [1] has noted that some 40 proteins have been structurally analysed by *X*-ray crystallography to atomic resolution, and that the general course of the backbone is known in about 10 more cases. He concludes that 'the pioneering era has undoubtedly come to an end' and that this 'challenges us to understand the architecture of the observed structures and thus to trace biology back to its physical roots on a much more fundamental scale than was

[104] R. D. B. Fraser, T. P. Macrae, and E. Suzuki, *J. Mol. Biol.*, 1976, **108**, 435.
[105] B. Lotz, F. Colonna-Cesari, F. Heitz, and G. Spach, *J. Mol. Biol.*, 1976, **106**, 915.
[106] N. P. Franks, *J. Mol. Biol.*, 1976, **100**, 345.
[107] A. E. Blaurock and W. Wober, *J. Mol. Biol.*, 1976, **106**, 871.
[108] A. E. Blaurock and A. E. Walsby, *J. Mol. Biol.*, 1976, **105**, 183.
[109] A. E. Blaurock and J. C. Nelander, *J. Mol. Biol.*, 1976, **103**, 421.
[1] G. E. Schulz, *Angew. Chem. Internat. Edn.*, 1977, **16**, 24.

previously possible'. Inasmuch as previous reviews in this series [2–6] have also been in this spirit, we have decided to review recent work in the context of his appraisal. We shall confine attention principally to work not considered by Schulz, or published subsequent to his review.

Theoretical Implications of Experimental Protein Folding.—The implications of the experimental observations on protein folding have been discussed by several authors.[7–11] The unfolding of a globular protein involves breaking all the non-covalent bonds between groups in the protein, and refolding involves reforming all those non-covalent bonds. These bonds may be reformed correctly, leading to a recovery of the native, biologically active structure, in the absence of ribosomes or any other biological structures, and this requires only conditions of temperature, pressure, pH, and ionic strength typically found on the surface of the Earth. Sometimes covalent disulphide bonds may be broken by reduction on unfolding, and reformed by oxidation on refolding. This may also result in a correctly refolded protein.

On the basis of such experiments Schulz conforms to current dogma that all the information for the tertiary structure of a globular protein is contained in the amino-acid sequence. This is important because it opens the possibility of computing the tertiary structure from the amino-acid sequence alone, using a quantitative knowledge of non-covalent forces. However, this is only a valid interpretation if all residual tertiary structure has been removed prior to experimental refolding, else the protein 'cheats',[12] as exemplified by recent kinetic studies.[13]

The Concept of Native Structure.—Schulz also conforms with the increasingly popular notion that, since the protein simply does not have enough time to try every possible conformation and so discover the most thermodynamically favourable one, the native structure must be kinetically determined. The native structure is obviously, however, conserved by steep energy barriers, else one would see proteins losing their biological activity by further conformational changes in their normal lifetime. The stability of the native structure is expressed by Schulz through equation (1), where ΔH is the enthalpy arising from

$$\Delta G = \Delta H_{\text{bond}} - T\Delta S_{\text{water}} - T\Delta S_{\text{chain}} \quad (1)$$

intramolecular non-covalent interactions, ΔS_{water} is the solvent entropy determined by the degree of exposure of water-ordering non-polar side-chain groups, and ΔS_{chain} is the conformational entropy of the protein. By emphasizing the role of the two entropy terms, Schulz supports the 'oil droplet' model of a protein

[2] B. Robson, in this series, 1972, Vol. 4, p. 224.
[3] B. Robson, in this series, 1973, Vol. 5, p. 180.
[4] A. T. Hagler and B. Robson, in this series, 1974, Vol. 6, p. 206.
[5] B. Robson and D. Osguthorpe, in this series, 1975, Vol. 7, p. 176.
[6] M. J. Asher, D. Osguthorpe, and B. Robson, in this series, 1976, Vol. 8, p. 181.
[7] C. B. Anfinsen, *Brookhaven Symp. Biol.*, 1962, **15**, 184.
[8] C. B. Anfinsen, *Science*, 1973, **181**, 223.
[9] D. B. Wetlaufer and S. Ristow, *Ann. Rev. Biochem.*, 1973, **42**, 135.
[10] C. B. Anfinsen and H. A. Scheraga, *Adv. Protein Chem.*, 1975, **29**, 205.
[11] B. Robson, *Trends in Biochem. Sci.*, 1976, **1**, No. 3, 49.
[12] B. Robson, *Nature*, 1976, **262**, 447.
[13] P. J. Hagerman and R. L. Baldwin, *Biochemistry*, 1976, **15**, 1462.

as a fine balance between protein order and solvent order, the protein order winning by a narrow margin of *ca.* −10 kcal. It is highly probable that any low-energy protein structure, whether kinetically accessible in reasonable time or not, conforms to this general 'oil droplet' picture, so that the observed native structure is one of several possible stable 'oil droplet' arrangements. However, Schulz also notes that the native structure is characterized by very efficient packing, for which an organic crystal rather than an oil droplet would be a good analogue. Hence, the native structure should be envisaged as one of several possible efficiently packed 'oil droplets'.

However, too simple-minded a viewpoint of native structure could spring from neglect of the true dynamic situation. Cooper [14] has emphasized that, in the statistical mechanical distribution of protein conformations, the *mode* is not necessarily equivalent to the *mean.* In other words, while the most probable state of a protein molecule may be an efficiently packed globule, experimental properties measured in solution may reflect the large number of possibilities for different, relatively open structures, which individually have low probabilities but which collectively add up to a high probability. The structure determined by *X*-ray crystallography is 'frozen' by the constraint of crystal periodicity, and thus provides a picture of the most probable kinetically accessible structure, rather than of the time-averaged kinetically accessible structures. For example, grouping all the conformational states of a penicillinase [15] into three (native, open with helical regions, and random) fits experimental data [15, 16] and shows that some 5—10% of the population of penicillinase molecules in aqueous solution exist in the open form with helical regions.

Solvent Structure.—Equation (1) shows that water structure is an important factor, probably the most important factor, favouring the compact 'oil droplet' structure of a protein. Since water structure implies a consideration of the interactions between water molecules, theoretical as well as experimental studies of water structure continue to be of fundamental interest to a consideration of protein conformation, stability, and folding.

Considering pure water, the relatively exact *ab initio* calculation has been carried out for the conformations of single water molecules [17] and the interactions between members of a pair.[18] The latter pair interaction has been used to develop empirical potential functions for water, from which bulk water properties can be calculated.[19, 20] The latter calculations employed the Monte Carlo method, which, like the related molecular dynamic approach, could be extended to study the interactions between protein groups and water in detail. Recently water surface properties have also been examined by such techniques [21, 22] and may be relevant to the hydrophobic drive principally responsible for 'oil droplet'

[14] A. Cooper, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2740.
[15] B. Robson and R. H. Pain, *Biochem. J.*, 1976, **155**, 125.
[16] B. Robson and R. H. Pain, *Biochem. J.*, 1976, **155**, 331.
[17] B. J. Rosenberg, W. C. Ermler, and I. Shavitt, *J. Chem. Phys.*, 1976, **65**, 4072.
[18] O. Matsuoka, E. Clementi, and M. Yoshimine, *J. Chem. Phys.*, 1976, **64**, 1351.
[19] G. C. Lie, E. Clementi, and M. Yoshimine, *J. Chem. Phys.*, 1976, **64**, 2314.
[20] G. C. Lie and E. Clementi, *J. Chem. Phys.*, 1976, **64**, 5308.
[21] J. Miyazaki, J. A. Barker, and G. M. Pound, *J. Chem. Phys.*, 1976, **64**, 3364.
[22] M. Rao and D. Levesque, *J. Chem. Phys.*, 1976, **65**, 3233.

formation. They have also been applied to anisotropic [23] and polar fluids [24] in general.

While *ab initio* studies lead to an understanding of solvent forces, and Monte Carlo studies lead to an understanding of solvent properties in terms of those forces, both these steps involve a great deal of computing time. General models of water structure therefore continue to be proposed [25–27] in the hope that useful rules of thumb will emerge.

Recently, interest has been shown [28–31] in theoretical studies on the effect of ions on water structure. This is of interest not only because protein solvents are usually salt solutions (including those used in preparing protein crystals for *X*-ray analysis), but because of the relevance to ordering of water around charged protein groups.

Despite the enormous potential of the above theoretical studies, at present experimental thermodynamic parameters relating to hydrophobic bonding are likely to be of most direct interest to protein theoreticians. The situation up to 1976 has been reviewed.[32] Transfer experiments, in which the solubilities of small molecular weight analogues of protein groups are compared in water and non-polar solvent, are probably the most relevant of all. The recent studies of Nandi,[33] employing *N*-acetylaminoacyl esters, should be of particular value.

The gap between the more fundamental water structure studies and the experimental studies on hydrophobicity must still be bridged, however, by experiments which enhance theoretical understanding [34–39] and by theoretical models of hydrophobicity.[40]

Secondary Structure.—In his review Schulz lays great emphasis on the hierarchical organization of protein structure, and on secondary structure in particular as the most fundamental architectural component in this hierarchy. Schulz describes secondary structure as regular chain arrangements permitting optimal hydrogen bonding (α-helix, β-pleated sheet, and reverse turn), typically involving 75% of the protein backbone. In fact, there is some difficulty in nomenclature here as not all chain features contemporarily referred to as 'secondary structure' are regular; in particular, some reverse turns. Further, the IUPAC–IUB Commission on Biochemical Nomenclature currently defines

[23] J. Kushick and B. J. Berne, *J. Chem. Phys.*, 1976, **64**, 1362.
[24] G. N. Patey and J. P. Valleau, *J. Chem. Phys.*, 1976, **64**, 170.
[25] D. R. Herrick and F. H. Stillinger, *J. Chem. Phys.*, 1976, **65**, 1345.
[26] A. H. Lemberg and F. H. Stillinger, *Mol. Phys.*, 1976, **32**, 353.
[27] R. A. Pierotti, *Chem. Rev.*, 1976, **76**, 717.
[28] M. R. Mruzik, F. F. Abraham, D. E. Schreiber, and G. M. Pound, *J. Chem. Phys.*, 1976, **64**, 481.
[29] G. L. Briant and J. J. Burton, *J. Chem. Phys.*, 1976, **64**, 2888.
[30] R. O. Watts, *Mol. Phys.*, 1976, **32**, 659.
[31] E. Clementi, R. Barsotti, J. Fromm, and R. O. Watts, *Theor. Chim. Acta*, 1976, **43**, 101.
[32] H. Edelhoch and J. C. Osborne, *Adv. Protein Chem.*, 1976, **30**, 183.
[33] P. K. Nandi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 253.
[34] M. Lucas, *J. Phys. Chem.*, 1976, **80**, 359.
[35] M. Lucas and R. Bury, *J. Phys. Chem.*, 1976, **80**, 999.
[36] R. Tenne and A. Ben-Naim, *J. Phys. Chem.*, 1976, **80**, 1120.
[37] D. W. James, R. F. Armishaw, and R. L. Frost, *J. Phys. Chem.*, 1976, **80**, 1346.
[38] C. N. R. Rao, S. Singh, and V. P. Senthilnathan, *Chem. Soc. Rev.*, 1976, **5**, 297.
[39] S. C. Valvani, S. H. Yalkowsky, and G. L. Amidon, *J. Phys. Chem.*, 1976, **80**, 829.
[40] H. A. Sallouta and G. M. Bell, *Mol. Phys.*, 1976, **32**, 931.

secondary structure as a description of the progress of the backbone through space in terms of bond rotation angles. This encompasses all chain conformations, *i.e.* 100% of any polypeptide chain must involve secondary structure. Nevertheless, the implication is still the same: the local arrangement of chain described in secondary structural terms is likely to be at least partly determined by local interactions between atoms and groups fairly close together in the covalent representation of the molecule. In other words, the stability of, for example, α-helix extending from residue i to residue $i + m$ will probably be influenced by interactions within and between residues $i - 5$ through to $i + m + 5$. Schulz presumably feels that local chain conformations, which he does not call secondary structure, have a much less important role in determining their own conformation.

Schulz notes that local determination of secondary structure has two important implications: (i) some secondary structure features are likely to appear early during folding, and (ii) such secondary structure features are likely to be readily predictable from amino-acid sequence alone. He classifies predictions of secondary structure into two types: those which depend on energy calculations or experimental data from simple polypeptide systems, and those which depend on statistical analysis of proteins of known sequence and conformation.

Energy Calculations. Quantum mechanical methods are perhaps the most obvious procedures for carrying out energy calculations, though being very complex and time-consuming their application is largely restricted to small biological molecules.[41] However, if low molecular weight molecules such as amino-acid derivatives are used as analogues of protein backbone fragments, they are ideally suited for analysis of some of the factors involved in secondary structure formation. When larger fragments of backbone, or the whole protein, must be considered, semi-empirical methods which assume that interactions can be represented as summations of the energies resulting from independent van der Waals repulsive forces, London dispersion forces, electrostatic forces, and hydrogen-bonding contributions, are usually the only practical alternative. Nevertheless, quantum mechanics may be used indirectly even here, in order to choose semi-empirical function forms or to assign parameters.[42]

For interactions between atoms close together in the covalent structure, such as those separated by three covalent bonds as A and D in A–B–C–D, quantum mechanics has considerable advantages over a semi-empirical approach. Such interactions are generally poorly accounted for by van der Waals contributions and so on, and if a semi-empirical calculation is employed it is frequently necessary to introduce an intrinsic rotational potential around bond B–C to bridge the discrepancy between the semi-empirical and quantum mechanical approaches. This discrepancy has been quantified by the application of the relatively exact quantum mechanical *ab initio* calculation in the case of the backbone $N'—C^{\alpha}$ and $C^{\alpha}—C'$ bonds.[43] Interesting observations relevant to the preferred rotations of other bonds found in small molecular weight analogues of the protein backbone have also been made by Hehre *et al.*[44] Agreement with

[41] A. Pullman and H. Berthod, *Chem. Phys. Letters*, 1976, **41**, 205.
[42] A. T. Hagler and A. Lapiccirella, *Biopolymers*, 1976, **15**, 1167.
[43] A. T. Hagler, L. Leiserowitz, and M. Tuval, *J. Amer. Chem. Soc.*, 1976, **98**, 4600.
[44] W. J. Hehre, J. A. Pople, and A. J. P. Devaquet, *J. Amer. Chem. Soc.*, 1976, **98**, 664.

experiment is of course still important, despite the confidence placed in the best of the quantum mechanical techniques,[46] and the validity of torsional potentials derived for a number of molecules by Lombardi *et al.*[45] may be judged in the light of experiments like those of Hoogasian *et al.*,[46] who employed neutron magnetic resonance spectroscopy. In fact, coupling constants obtained by this kind of spectroscopy are likely to continue to play a fundamental role in assessing theoretical calculations,[47, 48] although other kinds of experimental data, such as those based on electrical properties,[49, 50] can and should be taken into account.

Despite the fundamental superiority of quantum mechanical calculations of the interactions involving atoms close together in the covalent structure, there are one or two advantages in the semi-empirical approach even here. For example, the semi-empirical approach is more readily adjusted to bring about agreement with experiment. Rather than being a point of criticism, this is a basic *raison d'être* for the semi-empirical functions. Further, there are some interactions which are difficult and time-consuming for quantum mechanical approaches to analyse, and for this reason the otherwise very exact *ab initio* approach usually neglects the dipole-induced dipole interactions inherent in the London dispersion forces.[51] Whatever the claims for the accuracy of a calculation which authors may make, it is therefore important to note whether or not such interactions have been taken into account.

In considering rotational potentials, studies on the peptide N′—C′ bond continue to occupy a special place. This is because in theoretical calculations of all kinds, this bond is normally assumed to be fixed, planar, and *trans*. Scott *et al.*[52] have discussed the relative stability of *cis*, *trans*, and non-planar forms. Certainly the traditional assumption of the *trans* form is justified by its enormous predominance in the 40 or so proteins whose structures have been experimentally determined in detail. Those rare occasions in which the *cis* form is observed have been discussed by Mitra *et al.*[53] Probably the possibility of the *cis* form is a cause for major concern only in synthetic *N*-methylated polypeptides: recently, the significance of the *cis* form in poly(*N*-methyl glycine) has been investigated using polymer Monte Carlo techniques.[54]

Experimental Data from Simple Polypeptides. The amount of α-helix or β-pleated sheet in a protein or simple polypeptide can be determined by circular dichroism,[55, 56] optical rotary dispersion, or by laser Raman spectroscopy.[57] Many homopolypeptides undergo a helix–coil transition in response to changes in temperature, pH, or solvent composition. In some circumstances, a coil to pleated-sheet transition can also be induced.

[45] E. Lombardi, G. Tarantini, L. Pirola, and P. Torsellini, *J. Chem. Phys.*, 1976, **64**, 5229.
[46] S. Hoogasian, C. H. Bushweller, W. G. Anderson, and G. Kingsley, *J. Phys. Chem.*, 1976, **80**, 643.
[47] W. G. Espersen and R. B. Martin, *J. Phys. Chem.*, 1976, **80**, 741.
[48] M. Barfield, V. J. Hruby, and J. P. Meraldi, *J. Amer. Chem. Soc.*, 1976, **98**, 1308.
[49] J. Kirchenerova, P. G. Farrell, and J. T. Edward, *J. Phys. Chem.*, 1976, **80**, 1974.
[50] J. A. Walder, *J. Phys. Chem.*, 1976, **80**, 2777.
[51] A. R. Gregory and M. N. Paddon-Row, *J. Amer. Chem. Soc.*, 1976, **98**, 7521.
[52] S. Scott Zimmerman and H. A. Scheraga, *Macromolecules*, 1976, **9**, 408.
[53] A. K. Mitra and G. N. Ramachandran, *J. Mol. Biol.*, 1976, **107**, 85.
[54] A. R. Srinivason and P. K. Ponnuswamy, *Polymer*, 1977, **18**, 107.
[55] M. Goodman, R. T. Ingwall, and S. St. Pierre, *Macromolecules*, 1976, **9**, 1.
[56] M. Rinaudo and A. Domard, *J. Amer. Chem. Soc.*, 1976, **98**, 6360.
[57] J. L. Lippert, D. Tyminski, and P. J. Desmeules, *J. Amer. Chem. Soc.*, 1976, **98**, 7075.

The simplest interpretation of a helix–coil transitions in homopolypeptides is that the tendency to form a helix is not strongly determined by interactions between side-chains, but only by interactions between the side-chain of a residue and its own backbone and between the backbones of different residues. In this way, parameters obtained conceptually relate to amino-acid type but not to the amino-acid sequence, and hence data from homopolypeptides are assumed to be transferable in the first approximation from homopolypeptides to proteins. Roughly half of the 20 amino-acids found in proteins have thus been assigned helix–coil transition parameters which are of value in predicting the helical regions of globular proteins. Recently parameters have been obtained for the tendency of tyrosine [58] and lysine [59] to form α-helices in water, and in case ethanol is a better analogue of the environment of a residue in a folding protein, comparative studies in this solvent [60] are also of considerable interest. Thermodynamic [61] and kinetic [62] parameters have also been measured in detail for helix–coil transitions. The kinetic studies [62] are of considerable interest in considering the rate of helix growth which Schulz, following others, considers and early stage of protein folding.

The helix–coil transition has also been studied [63] by computer simulation using the polymer Monte Carlo technique which can take into account interactions between residues which are not near neighbours in the amino-acid sequence. However, models which are refinements of the more traditional analytical approach continue to be proposed.[64]

Most of the experimental work has concerned the helix–coil transition rather than the pleated sheet–coil transition, but recently this transition has been studied, and parameters obtained, for polytyrosine.[65] A similar transition has also been observed for a lysine–isoleucine copolymer.[66]

Statistical Analysis of Proteins of Known Sequence and Conformation. The time-consuming nature of both energy calculations and, in particular, the experimental studies on simple polypeptides, as well as the practical difficulties in extending them to complex heteropolypeptides including proteins, means that obtaining parameters by statistical analysis of proteins of known sequence and conformation is still a very attractive alternative.

The potential in the amino-acid sequence for coding conformation is implied by studies of sequence homology [67] and non-randomness.[68] This potential is exploited in a series of detailed studies by Tanaka and Scheraga [69–72] with the

[58] R. K. Scheule, F. Cardineaux, G. T. Taylor, and H. A. Scheraga, *Macromolecules*, 1976, **9**, 23.
[59] M. K. Dygert, G. T. Taylor, F. Cordineaux, and H. A. Scheraga, *Macromolecules*, 1976, **9**, 794.
[60] E. Patton and H. E. Auer, *Macromolecules*, 1976, **9**, 529.
[61] Y. C. Fu, H. E. Van Wart, and H. A. Scheraga, *Biopolymers*, 1976, **15**, 1795.
[62] Y. Tsuji, T. Yasunaga, T. Sano, and H. Ushio, *J. Amer. Chem. Soc.*, 1976, **98**, 813.
[63] D. A. Neves and R. A. Scott, *Macromolecules*, 1976, **9**, 554.
[64] S. Caboni, A. Paci, and V. Rizzo, *Biopolymers*, 1976, **15**, 113.
[65] T. P. McKnight and H. E. Aver, *Macromolecules*, 1976, **9**, 939.
[66] B. Walter and G. D. Fasman, *Biopolymers*, 1977, **16**, 17.
[67] M. G. Rossmann and P. Argos, *J. Mol. Biol.*, 1976, **105**, 75.
[68] J. A. Black, R. N. Harkins, and P. Stenzel, *Internat. J. Peptide Protein Res.*, 1976, **8**, 125.
[69] S. Tanaka and H. A. Scheraga, *Macromolecules*, 1976, **9**, 142.
[70] S. Tanaka and H. A. Scheraga, *Macromolecules*, 1976, **9**, 159.
[71] S. Tanaka and H. A. Scheraga, *Macromolecules*, 1976, **9**, 168.
[72] S. Tanaka and H. A. Scheraga, *Macromolecules*, 1976, **9**, 812.

aim of developing a further predictive method. Robson and Suzuki[73] have analysed 25 proteins to obtain parameters for α-helix, pleated sheet and extended chain not in pleated sheet, reverse turns, and loops in the protein backbone. The convergence of these parameters with sample size was demonstrated, and the effect of different residues not only on their own conformations but the conformations of sequence neighbours at different distances along the chain was considered. These parameters can also be used to make predictions,[73] and a model has been proposed by which helix–coil transition parameters can be deduced from them in the absence of experimental data.[74] A fairly clear picture of the origin of statistical correlations between sequence and conformation is beginning to emerge from these and related studies.[75–77]

Tertiary Structure.—*Calculation of the Structure of Small Polypeptide Hormones and Related Molecules.* Small polypeptide hormones are not only of intrinsic pharmacological interest, but represent mini-proteins and hence a testing ground for theoretical approaches. Conformational calculations have been carried out for small peptides containing 2—4 residues.[78–81] However, most of the polypeptide hormones studied recently have been 4—10 residues long, and so present considerable problems for a detailed conformational analysis. Usually it is not practical to sample accurately the whole conformational energy surface, but a minimization technique can be applied in the hope of locating the conformation of lowest energy. Momany *et al.*[82, 83] have studied luteinizing hormone-releasing factor by employing not only minimization but other simplifying assumptions implying independent behaviour between certain regions of the chain. If the energy surface as a whole is sampled, as by Yamada *et al.*[84] for gastrin, then this is done only very coarsely (followed by a more refined scan when low-energy regions are located). An alternative approach, which constitutes a random sampling of the conformational energy surface, is to use a Monte Carlo procedure. This is a promising technique which is recently exemplified by calculastion on chain reversal regions of chymotrypsin.[85]

Tertiary Structure of Proteins. In his hierarchical description of protein conformation, Schulz separates tertiary structure into three levels of organization: (i) secondary structure aggregates, (ii) domains, and (iii) the packing of domains to make up the globular structure of the protein. The first two levels are probably less readily distinguishable in equivalent terms, although the first is generally studied as the mutual orientation and linkage of secondary structure features,

[73] B. Robson and E. Suzuki, *J. Mol. Biol.*, 1976, **107**, 327.
[74] E. Suzuki and B. Robson, *J. Mol. Biol.*, 1976, **107**, 387.
[75] V. S. Ananthanarayanan and J. Bandeker, *Internat. J. Peptide Protein Res.*, 1976, **8**, 615.
[76] T. M. Birshtien, A. M. Skvortsov, and V. I. Alexanyan, *Biopolymers*, 1976, **15**, 1061.
[77] A. V. Finkelstein, O. B. Ptitsyn, and S. A. Kozitsyn, *Biopolymers*, 1977, **16**, 497.
[78] V. Madison, P. E. Young, and E. R. Blout, *J. Amer. Chem. Soc.*, 1976, **98**, 5358.
[79] V. Madison, P. E. Young, and E. R. Blout, *J. Amer. Chem. Soc.*, 1976, **98**, 5365.
[80] S. Kong and R. Walter, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1203.
[81] D. L. Miles, D. W. Miles, P. Redington, and H. Eying, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4257.
[82] F. A. Momany, *J. Amer. Chem. Soc.*, 1977, **98**, 2990.
[83] F. A. Momany, *J. Amer. Chem. Soc.*, 1977, **98**, 2996.
[84] T. Yamada, H. Wako, N. Saito, Y. Isogai, and H. Watari, *Internat. J. Peptide Protein Res.*, 1976, **8**, 607.
[85] F. I. Hurwitz and A. J. Hopfinger, *Internat. J. Peptide Protein Res.*, 1976, **8**, 543.

while the second is generally studied in terms of the density of packing of atoms and groups into large clusters. When helix and pleated sheets lie in proximity, the connections between the chain making up the helix and the strands of the sheet show a preferential behaviour which can be described in terms of the 'handedness' of the aggregate.[86] Preferences are also shown in the mutual orientations and connections of the chain making up pleated sheets alone.[87, 88] Parameters obtained from analysis of the packing of globular proteins are probably directly relevant to the formation of domains.[89] Finally, the packing of domains to make up the overall globular structure probably has rules entirely analogous to those for the packing between globular proteins to form quaternary complexes, which are discussed below.

The hierarchical arrangement probably reflects the folding process, secondary structure forming first, then aggregating, then tightening into domains, and finally the domains binding together to form the overall structure.

Adonts *et al.*[90] have described this in terms of a transition between fully denatured dynamic random coil, helical regions, and tertiary structure. This increasingly popular viewpoint receives considerable experimental support from the thermodynamic and kinetic studies on a penicillinase by Robson and Pain.[16] Further, these investigators propose that the absence of data of a similar kind for other proteins does not exclude for those proteins either the initial formation of helical regions or the considerable stability of an intermediate helix–aggregate complex, both of which may be masked if the overall globular structure is stable by -10 kcal or more with respect to the unfolded protein. Finkelstein *et al.*[91] have recently begun a series of studies based on a similar viewpoint to that of Adonts *et al.*[90]

It is of interest to note that many of the philosophies and calculations involved in studies of the above kinds are also applicable to supercoiling and chain interactions in fibrous proteins.[92]

Prediction of Tertiary Structure. If the complexity of the conformational energy surface of small polypeptide hormones poses problems, then the enormous complexity of the conformational energy surface of a protein would seem to pose insurmountable problems. Certainly the distinction between the prediction of small polypeptide hormone structure and protein structure is that the latter is necessarily a science of simplification, both in the sense of simplifying the problem by using external information to apply biasing or constraints to the search of the energy surface and in the sense of using considerably simplified representations of molecular structure.

The most obvious kind of external information is the initial prediction of secondary structure by means of energy calculation, application of experimentally determined parameters from simple polypeptides, or statistical techniques as

[86] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1976, **105**, 367.
[87] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1976, **110**, 269.
[88] J. S. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2619.
[89] S. Tanaka and H. A. Scheraga, *Macromolecules*, 1976, **9**, 945.
[90] V. G. Adonts, T. M. Birshtein, A. M. Elyashevich, and A. M. Skvortsov, *Biopolymers*, 1976, **15**, 1037.
[91] A. V. Finkelstein and O. B. Ptitsyn, *Biopolymers*, 1977, **16**, 469.
[92] K. Nishikava and H. A. Scheraga, *Macromolecules*, 1976, **9**, 395.

described above. Thus, Stelluragen [93] has attempted a prediction of the tertiary structure of aldolase by first carrying out a statistical prediction of secondary structure, and Johnson and Wu [94] have used a comparable approach for alamethicin. Further, Gabel *et al.*[95] have searched for low-energy conformations of neurotoxic protein by means of secondary structure predictive rules, tests for atomic clashes, and finally an energy minimization. On the other hand, the use of simplified representations of molecular structure is exemplified by the recent work of Kuntz *et al.*[96] Levitt [97] has considered in detail the various possibilities by which protein molecules can be represented as simple structures suitable for simulating folding, and continues to apply them.[98] The practical implications of not grossly simplifying the structural representation are illustrated by the work of Honig *et al.*,[99] who minimized the known tertiary structure of subtilisin only after locking extensive regions of the protein in the observed conformation and allowing no part to move more than 6 Å away from its observed position.

Quaternary Structure.—If quaternary structure, describing the interaction and interrelation of globular proteins in higher structures, is indeed comparable with the interactions of domains to form a single globular protein, then this area of study is likely to remain popular with workers interested in protein folding. Chothia has used surface area calculations to estimate the binding between haemoglobin subunits and has indicated how these are relevant to an understanding of the allosteric effect.[100] He has also investigated the protein–protein interaction between trypsin and trypsin inhibitor.[101] Damaschun [102] had discussed the long-range order in proteins which have quaternary structure.

Enzyme Function.—Predictions of protein conformation are even more useful if the resulting conformation can be related to biological function. Quantum mechanical calculations of enzyme–substrate interactions have been carried out for papain,[103] chymotrypsin,[104] and the serine proteases.[105] In these calculations, most of the protein is neglected and only the known active site structure considered.

The binding of substrates to lysozyme has been studied by Pincus *et al.*[106, 107] using semi-empirical calculations; in this case the whole lysozyme structure was considered. Various substrates and inhibitors were placed in the active site and the binding energy minimized.

[93] E. Stelluragen, *J. Mol. Biol.*, 1976, **106**, 903.
[94] M. Johnson and T. T. Wu, *J. Theor. Biol.*, 1976, **60**, 183.
[95] D. Gabel, D. Rasse, and H. A. Scheraga, *Internat. J. Peptide Protein Res.*, 1976, **8**, 237.
[96] I. D. Kuntz, G. M. Crippen, P. A. Kollman, and D. Kimelman, *J. Mol. Biol.*, 1976, **106**, 983.
[97] M. Levitt, *J. Mol. Biol.*, 1976, **104**, 59.
[98] A. Warshel and M. Levitt, *J. Theor. Biol.*, 1976, **106**, 421.
[99] B. Honig, A. Ray, and C. Levinthal, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1974.
[100] C. Chothia, S. Wodak, and J. Janin, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3793.
[101] J. Janin and C. Chothia, *J. Mol. Biol.*, 1976, **100**, 197.
[102] G. Damaschun, J. J. Miller, C. H. Gedicke, and B. A. Fedorov, *J. Mol. Biol.*, 1976, **104**, 735.
[103] R. Broer, P. Th. Von Duijnen, and N. C. Nieuwpoort, *Chem. Phys. Letters*, 1976, **42**, 525.
[104] H. P. Kitayama and H. Fukutome, *J. Theor. Biol.*, 1976, **60**, 1.
[105] S. Scheiner and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 432.
[106] M. R. Pincus, A. W. Burgess, and H. A. Scheraga, *Biopolymers*, 1976, **15**, 2485.
[107] M. R. Pincus, S. Zimmerman, and H. A. Scheraga, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4261.

Relation to Drug Design.—Studies of the type carried out by Pincus *et al.*[106–107] are of obvious application to drug design providing the three-dimensional structure of the receptor protein is known. Certainly conformational energy calculations are useful for the analysis of drug conformation even when the receptor structure is not known, but this is a large field which would be out of place in a review of this kind except in instances when the drug itself is a polypeptide. Conformational studies on polypeptide hormones already mentioned above fall into this class. Recent conformational energy calculations on the structure of aspartyl phenylalanyl methyl ester [108] and the implications for interaction with the sweet taste bud receptor further illustrates the relevance of this area of study to the food and drug industry.

Conclusions: Towards a Dynamic Viewpoint.—None of the investigations which have been reported subsequent to the review of Schulz contest his general conclusions. Perhaps the only problem concerns his overall impression to the reader that most of the important questions about protein structure can ultimately be disposed of without introducing dynamics, except in connection with folding. While this may well be true in general, the difficulty is that the considerations of investigators like Cooper,[14] taken to the limit, could demand a broader approach to native structure in certain situations.

Techniques for providing time-averaged descriptions of conformation are already being developed, particularly in connection with Monte Carlo analyses of polymer behaviour.[109–112] The dynamics of secondary [113, 114] and tertiary [115] structures are being considered, and vibrational freedom of polypeptides continues to be a fairly popular area of inquiry.[116, 117] The most sophisticated study to date, however, is undoubtedly the molecular dynamics simulation of the protein pancreatic trypsin inhibitor by Karplus and colleagues.[117a] It is therefore very reasonable to suppose that by the time the assumption of relative rigidity is found to hinder, rather than help, further development, the groundwork for a more dynamic viewpoint will be firmly established.

2 Folding of Globular Proteins

Contributed by C. Mitchinson

Some of the current theories about protein folding have been briefly reviewed by Robson.[118]

Native State.—*Multiple Conformations.* Despite the normal assumption of a unique native conformation for a given primary sequence, there seems some

[108] F. Lelj, T. Tancredi, P. A. Temussi, and C. Toniolo, *J. Amer. Chem. Soc.*, 1976, **98**, 6669.
[109] J. G. Curro, *J. Chem. Phys.*, 1976, **64**, 2496.
[110] R. A. White, *J. Chem. Phys.*, 1976, **64**, 3510.
[111] J. C. Conrad and P. J. Flory, *Macromolecules*, 1976, **9**, 41.
[112] A. Baram and W. M. Gelbart, *J. Chem. Phys.*, 1977, **66**, 617.
[113] M. Gō and N. Gō, *Biopolymers*, 1976, **15**, 1119.
[114] Y. Suezaki and N. Gō, *Biopolymers*, 1976, **15**, 2132.
[115] Y. Ueda and N. Gō, *Internat. J. Peptide Protein Res.*, 1976, **8**, 551.
[116] W. H. Moore and S. Krimm, *Biopolymers*, 1976, **15**, 2439.
[117] W. H. Moore and S. Krimm, *Biopolymers*, 1976, **15**, 2465.
[117a] J. A. McCammon, B. R. Gelin, and M. Karplus, *Nature*, 1977, **267**, 585.
[118] B. Robson, *Trends in Biochem. Sci.*, 1976, **1**, 49.

evidence for conformational isomers within the native state; haptoglobulins, although shown to be homogeneous by many physical methods, exhibit heterogeneity in their binding affinity for haemoglobin;[119] thermal and solvent perturbation studies suggest two conformational states of the native molecule (Hp* and Hp_x) with no detectable difference in their amino-acid composition (not excluding the possibility of a point mutation at one or two residues). These apparent conformational isomers[120] with different free energies are not in equilibrium at room temperature suggesting a possible high potential barrier between them. It is suggested that the tryptophan fluorescence of human lysozyme and α-lactalbumin indicates multiple conformations in solution.[121]

Crystal State. In the past there have been very few detailed tests of the effect of crystalline environment on protein conformation. The differences between free and crystallized carboxypeptidase A suggest[122] the existence of multiple, readily interconvertible conformations in solution, the rearrangements between these possibly being reduced in crystals. The stability of lysozyme to the combined effects of temperature and denaturants in the dissolved and crystalline state[123] shows differences in the action of urea and polyhydroxylic alcohols which can be explained by decreased accessibility of polar groups in the crystal state. Comparison of tetragonal and triclinic crystal forms of egg-white lysozyme shows that, whereas there is no appreciable difference in the internal portions of the protein molecule, there are considerable conformational variations, involving regions of the main chain as well as side-chains, on the surface.[124] These apparently arise from the different modes of crystal packing, and such intermolecular interactions are thought to be responsible for the local conformational differences between the crystal and solution forms of basic pancreatic trypsin inhibitor.[125] It is suggested that the conformational probe used here, nuclear magnetic resonance of ε-CH_2 protons of lysine, may have wider applications. The thermal stability of pepsin in the crystalline state[126] and comparison with its denaturation in solution[127] shows that the crystal lattice increases the transition co-operativity.

Choice of Solvent. Some proteins have been shown to acquire native structure only under certain conditions; gonadotropin-releasing hormone has a random coil state in aqueous solution but forms a β-structure in trifluoroethanol.[128]

I.r. spectroscopy on histones H1, H2A, and H4 in solution in ^2H_2O shows that anti-parallel pleated-sheet structure is only formed under conditions favouring aggregation,[129] although only H2A and H4 show such structure under physiological conditions. In all the four major sub-fractions of calf-thymus histone

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H1 [130] addition of salt to aqueous solutions at neutral pH causes a highly co-operative folding with the formation of α-helix and β-structure, a similar transition taking place in the absence of salt in raising the pH to about pH 9. Spectroscopic studies on the very lysine-rich histone Φ1 from marine invertebrates [131] show the attainment of a specifically folded structure, with 25—30% α-helix, on salt addition in two cases. Non-histone proteins of the high-mobility groups HMG1 and HMG2 form 40—50% α-helix [132] on salt addition, which also stabilizes against acid denaturation. The care needed in defining a structure as 'native' is shown by the fact that many of the treatments applied in isolating histone fractions are seen to cause their irreversible denaturation.[133]

Ordered, Non-native States.—In certain solvents proteins can be rearranged into non-native but regularly ordered conformations, the best known example of this being complexes with sodium dodecyl sulphate (SDS). The circular dichroism of 14 proteins in the presence of SDS and dithiothreitol has been shown to be consistent with a mixture of random coil and α-helix,[134] the differences in spectral intensities being quantitatively explained by an enhanced probability of Arg, His, and Lys residues to propagate helical segments in the presence of the detergent. A model of the protein–SDS complexes as being flexible, constituted of random coil and α-helix, with clusters of SDS along the chain in a non-draining complex is discussed. The electro-optical properties of reduced protein–SDS complexes [135] rule out a number of models but offer no firm conclusions with respect to the free draining model. A two-stage mechanism of SDS action [136] involving unfolding of the globular protein and then refolding into a non-native conformation is proposed. In all cases non-helical proteins form moderately helical conformations and interchain disulphides restrict such rearrangements, the structure of highly helical proteins being little altered. The action of SDS in promoting β-structure in lysine oligomers [137] and β-structure in poly-(L-Lys) and α-helix in poly-(L-Orn) [138] has been investigated. Whilst certain reports [134, 137] stress the importance of the detergent head-group, others place emphasis on the hydrophobic tail.[136]

Similar investigations with poly-amino-acids have explored the structure-promoting effects of tetra-alkylammonium chloride [139] and sodium trichloroacetate,[140] whereas the α-helix-inducing effect of 2-chloroethanol–water mixed solvent on bovine serum albumin [141] and the formation of more ordered structures

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[141] E. Maes, *Biopolymers*, 1976, **15**, 293.

in lysozyme and chymotrypsinogen in high concentrations of ethylene glycol [142] have also been reported.

The importance of hydrophobic effects on the action of urea and alkyl-ureas in chymotrypsinogen [143] and the combined effect of urea and alcohols on the heat-induced reversible denaturation of ribonuclease [144] is also discussed.

Denaturation.—*General.* A review has been published [145] of the thermodynamic basis of the stability of proteins and nucleic acids, placing special emphasis on the calorimetry of proteins, non-polar model compounds and denaturation, and a similar review by C. N. Pace [146] also discusses the denatured state, mechanism of denaturation and, especially, methods of estimating $\Delta G_D^{H_2O}$, the free energy change on denaturation in absence of denaturant. A theory linking the Hofmeister series of ions to their effects on the empty volume of solvents is proposed [147] and applied to salt effects on the T_m of ribonuclease. Microcalorimetric studies of the enthalpies of mixing of ribonuclease, trypsin, β-lactoglobulin, ovalbumin, and bovine serum albumin (BSA) have given enthalpies of interaction in the range -45 to -75 J g^{-1} protein, corresponding to an average binding enthalpy of -23 kJ mol^{-1} bound urea.[148]

Reversibility. It is now generally held that all proteins can be reversibly unfolded to their native state, though this may be complicated, *in vitro*, by such problems as aggregation and interdomain interactions (see ref. 208) which may not occur in the sequentially produced polypeptide chain *in vivo*. There has been a kinetic study [149] of the subunit dissociation, unfolding, and reassembly of rabbit muscle phosphofructokinase but, on the other hand, the randomized structure obtained in 8M-urea for an α-D-galactopyranosyl-binding lectin does not renature to its native form on dialysing away the denaturant.[150]

Experimental Studies. The thermal denaturation of a bacteriorhodopsin,[151] BSA,[152] ovalbumin,[153] human serum albumin,[154] and bovine eye-lens leucine aminopeptidase [155] has been studied. In the guanidinium hydrochloride (GuCl) denaturation of the holo- and apo-enzymes of pig-heart lipoamide dehydrogenase [156] the more sensitive α-helical structure in the apoenzyme is stabilized by FAD and two hydrophobic probes. The acid denaturation of human transcortin [157] and the stages of dissociation and denaturation of sesame globulin

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in SDS [158] have been reported, as have the effects of urea, pH, ammonium sulphate, chloroform, and temperature on the conformation of a post-synaptic acetylcholinesterase.[159]

That denaturation experiments can be affected by conditions is well illustrated by the fact that the previously reported thermal destabilization of *E. coli* nucleoprotein, in the absence of DNA, derives from incomplete removal of edta.[160]

Conformational Probes. There have been many reports both of adaptations of existing methods of following protein conformational changes and of newly developed methods.

Seagle and Cowgill [161] have described a method of nitrating peptides and proteins with tetranitromethane which they used for the specific formation of 3-aminotyrosine residues in ribonuclease A: the fluorescence is easily distinguishable from that of unmodified tyrosines and can be used as a probe of conformational change. The formation of protein–polynucleotide scattering centres is used as a structure probe in the thermal denaturation of ribonuclease and chicken lysozyme,[162] the denatured enzymes showing enhanced interaction with nucleic acids. Fluorescence-detected magnetic circular dichroism [163] can be applied even to non-fluorescent molecules by using adsorbed fluorophores. The conformational changes of serum albumin in the predenaturation temperature range have been followed by means of a spin radical adsorbed to the protein.[164]

N-Acetylbenzotriazole is a useful protein reagent [165] reacting with side-chain amino and phenolic groups. Dansyl fluoride apparently exhibits greatly increased selectivity over dansyl chloride [166] in a number of proteins. A method has been developed for the large-scale economic preparation of selectively deuteriated amino-acids suitable for incorporation into bacterial proteins for n.m.r. studies.[167] The solvent absorption effects in circular dichroism have been countered using a 0.01 nm quartz cell and a computerized time-averaging system.[168]

In the light of improved instrumentation and protein purifications now available the circular dichroism and optical rotation of muscle proteins [169] have been re-examined and estimates of their α and β content re-evaluated. The use of Freund's adjuvant in preparing antibodies as structural probes is supported by the report [170] that its enhancement of the immunogenicity of emulsified haemoglobin does not involve a major conformational effect.

Immobilized Enzymes. There have been many recent studies on the stability of enzymes attached to insoluble supports, including those on the thermal stability

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of immobilized chymotrypsin[171] and trypsin[172] as well as the characterization of immobilized baker's yeast glucose-6-phosphate dehydrogenase[173] and cytochrome *c*.[174]

Denatured State. That proteins may have more than one denatured state has been suggested by experiments on human luteinizing hormone[175] (with different conformations in 0.1N-HCl and 4M-GuCl) and on rhodanese[176] in which the states produced by GuCl and by urea and SDS differ in their number of free thiols. It has often been supposed that, for some proteins, the thermally denatured form includes regions of ordered residual structure which can be destroyed by GuCl. This is opposed by Pfeil and Privalov in a series of papers[177] in which direct methods of obtaining thermodynamic standard functions for native and denatured proteins, from the experimental data of scanning and isothermal calorimetry and potentiometric titrations, are applied to lysozyme. These functions, in the ranges 0—100 °C, pH 1.5—7.0, are represented in three-dimensional form. It is shown[177] that if the preferential binding of GuCl is considered then the heat and GuCl-denatured states of lysozyme are thermodynamically indistinguishable.

The fluorescence decay of tryptophan residues in 17 proteins of vastly different primary structures[178] suggests that in the denatured states, in 6M-GuCl, all tryptophan residues have similar types of environment tending to argue against the existence of specifically folded residual structures. Despite the fact that cyanogen bromide fragment F-CB3 from fibrinogen α chain retains considerable serological activity after reduction and alkylation of its single disulphide bridge, it is probable[179] that the antigenic determinants concerned are dependent on sequence and not on a residual conformation.

There does, however, seem to be some evidence for residual structure in certain denatured proteins; reduced sarcoplasmic reticular Ca^{II} ATPase and its two tryptic fragments show retention of folded regions in GuCl.[180] It is suggested that whilst this is rare amongst soluble proteins it may be characteristic of most intrinsic membrane proteins. The controlled sequence of deamination observed when ribonuclease A is exposed to a highly acid (pH <1) environment for long periods[181] is suggested to depend upon residual ordered structure maintained by disulphide bonds, and pepsin seems to have such structure even in 7.6M-urea.[182]

[171] L. Bessmertnaya, L. V. Kozlov, and V. K. Antonov, *European J. Biochem.*, 1976, **63**, 591; A. F. Chernysheve, K. Marinek, A. M. Klibanov, A. T. Meukh, and I. V. Berezin, *Bull. Acad. Sci. U.S.S.R. Chem. Sci.*, 1975, **24**, 1648; K. Martinek, V. S. Goldmakher, A. M. Klibanov, V. P. Torchilin, V. N. Smirnov, E. I. Chazov, and I. V. Berezin, *Doklady Akad. Nauk S.S.S.R.*, 1976, **228**, 1468.

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[182] H. Aoshima, A. Naito, and H. Hatano, *Internat. J. Peptide Protein Res.*, 1976, **8**, 131.

The fact that corticotropin exists in solution as a structureless polypeptide chain and may be a suitable model for aperiodic regions of globular proteins [183] may be of use in the characterization of the denatured state. Calculations of the free energy of transfer of ribonuclease in urea and GuCl from isopiestic vapour pressure measurements [184] suggests that the denatured state (in 6M-GuCl or 8M-urea) has 36% accessible surface area, compared with 11% in the native state (in 1M-GuCl or 2M-urea). Regions of protein sequence maintaining residual structure are often proposed as nucleating regions for protein folding.

Folding.—*Nucleation.* The possibility of nucleation in protein folding has been discussed by Robson,[185] and Karplus and Weaver [186] discussed the random-search nucleation and chain propagation and the diffusion–collision models of protein folding.

The chain folding of ferricytochrome *c* [187] followed by stopped-flow fluorescence and temperature jump in urea and GuCl shows four relaxation times, suggesting at least five species in an extended form of a nucleation-dependent simple sequential model of refolding. The biphasic kinetics of ribonuclease A refolding from GuCl or urea are explained by Garel, Nall, and Baldwin [188] by the existence of fast- and slow-refolding species (U_2 and U_1, respectively) in the GuCl-unfolded state. The slow ($U_2 \rightleftharpoons U_1$) phase is proposed as a proline isomerization, and in a later paper by Hagerman and Baldwin [189] a further intermediate (I) is observable only at higher temperatures, suggesting a folding pathway

$$U_1 \rightleftharpoons U_2 \rightleftharpoons I \rightleftharpoons N$$

in which the $U_2 \rightleftharpoons I$ transition is much too fast for a proline isomerization. It is suggested that the first two transitions could involve a topologically difficult change in overall configuration, perhaps involving the disulphide-bonded loop.

The existence of minor, *cis*-proline isomers, has been demonstrated in n.m.r. studies of Pro^3,Pro^5-angiotensin II [190] and in an angiotensin hexapeptide.[191] A 'minimal isomerizamodel' of protein folding,[192]

$$N \rightleftharpoons D \rightleftharpoons D'$$

in which D and D′ are unfolded states (D having all peptide bonds in the native isomeric conformation and D′ having undergone isomerization at one or more bonds) is a very simplified model based on that of Brandts [193] and uses only one state for D′. It is shown to give a satisfactory description of the equilibrium and kinetic data on reversible unfolding of globular proteins. The prediction of two unfolded species with similar lifetimes is possibly confirmed in the case of

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chymotrypsinogen A,[194] where rapid quenching of protein solutions from the thermal unfolding transition to 0 °C allows the electrophoretic separation of three species N, X, and D. Species X constitutes 5—20% of the protein (dependent on temperature) and could either be a transition region intermediate or a stable equilibrium species in the denatured base-line region.

Multi-state Transitions. Although it has been shown [195] that many proteins have a true two-state transition, there are cases where this is not so and it has been noted [196] that even in proteins where the transition can be thermodynamically described as two-state this does not necessarily preclude the existence of intermediates.

Because of its insolubility at low denaturant concentration it has not been possible reversibly to denature ovalbumin, but the unfolding of its major fraction, A_1, by GuCl has been shown to be fully reversible over the ranges 1—7M-GuCl, 10—60 °C and pH 1—7.[197] The native state is only marginally more stable than the unfolded even under native conditions and the transition is deemed to be a two-state one, although undetectable intermediates are not precluded. Similarly, in the fluorescence study of the reversible pressure denaturation of riboflavin-binding protein and its FMN complex,[198] although described in terms of a two-state ($N \rightleftharpoons D$) transition, it is conceded that the chances of observing intermediate states are 'not good'. In Apo A1 from human high-density lipoproteins, as in ovalbumin A_1, the two-state reversible denaturation of a relatively unstable protein is described,[199] but in the case of muscle parvalbumins [200] over the range 5—80 °C there are no conformational transitions, only a gradual alteration of the whole molecule, possibly by expansion.

The circular dichroism monitored GuCl transition in α-lactalbumin [201] indicates the presence of at least one intermediate which can be identified with the acid-expanded, quasi-native species A.[202] The lower stability of α-lactalbumin as compared to egg-white lysozyme is not thought to be in conflict with their postulated similarity in structure, and the increased chemical reactivity of lactalbumin groups can be explained by a dynamic equilibrium with a rapid transition between N and the more exposed A state.[201]

A fluorescence study of the GuCl unfolding of some apo-myoglobins [203] assumed to have the same basic structure shows that a two-state process is followed by the buffalo and bovine proteins whereas in tuna and sperm-whale apo-myoglobins there is a loosening of folding preceding tryptophan exposure, suggesting an appreciable concentration of intermediate(s) in their unfolding equilibrium mixtures. In the GuCl denaturation of high-density lipoproteins AI and AII [204] the unfolding of AI is clearly not two-state, though that of AII

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[204] J. A. Reynolds, *J. Biol. Chem.*, 1976, **251**, 6013.

could be. The sensitivity of these two proteins to 6M-GuCl is compared with the high thermodynamic stability of the ligand-free, water-soluble forms of intrinsic membrane proteins.

Pathways – Experimental. The validity of a kinetic analysis of the renaturation of rabbit muscle aldolase after acid dissociation has been demonstrated by showing that the reassociation product is indistinguishable from the native state.[205] Such an analysis shows that denaturation leads to an equilibrium state differing from a random coil, that the rates of denaturation and renaturation differ by several orders of magnitude, and that the rates of renaturation and reassociation depend upon enzyme concentration.[205] Fluorescence studies reveal a multi-step renaturation involving a rapid initial regain of most of the native monomeric secondary and tertiary structure followed by a slow 'reshuffling' showing that the final recovery of full enzymatic activity and native fluorescence is dependent upon association. Similarly, the renaturation of two formyltetrahydrofolate synthetases from urea and GuCl shows rapid refolding of the chains followed by a much slower reassociation of the refolded subunits to form the native tetramer.[206]

Laser raman spectroscopy of the reversible thermal denaturation of ribonuclease A [207] at pH 5.0 is compatible with the six overlapping stages of unfolding suggested by Burgess and Scheraga.

Disulphide-containing Proteins. The need for correct disulphide bond formation can add further complications to the folding of proteins. An extreme example is BSA where random disulphide formation amongst the 35 half-cystines could give 2.2×10^{20} possible conformers: obviously there must be some restriction on disulphide formation. Using antibodies as a structural probe to follow the refolding of reduced BSA [208] the rates of glutathione or rat-liver disulphide-exchange enzyme-catalysed refolding, whilst much faster than simple air oxidation, still cannot approach the *in vivo* rate of BSA synthesis.

In the regeneration of reduced egg-white lysozyme (which has only four disulphides) by complete air oxidation at pH 8.0, 37 °C,[209] active and inactive forms are produced. The latter have enlarged molecular domains, disordered conformations, at least 24% contain incorrect disulphides and all can be converted into the native form by thiol–disulphide exchange. The active forms consist of completely renatured lysozyme and three other major species, each one apparently having one of the native disulphide bonds open whilst still maintaining the compact, native tertiary structure. It is here suggested that regeneration of the native form does not follow an obligatory pathway, as in pancreatic trypsin inhibitor, but involves oxidative pairing of cysteines followed by thiol-catalysed rearrangement of incorrect disulphides. A more restricted pathway of glutathione regeneration of reduced lysozyme is proposed [210] in

[205] M. Engelhard, R. Rudolph, and R. Jaenicke, *J. Biochem.*, 1976, **67**, 447; R. Rudolph, M. Engelhard, and R. Jaenicke, *ibid.*, p. 455.
[206] C. K. Garrison, J. A. K. Harmony, and R. H. Himes, *Biochim. Biophys. Acta*, 1976, **446**, 301.
[207] M. C. Chen and R. C. Lord, *Biochemistry*, 1976, **15**, 1889.
[208] J. M. Teale and D. C. Benjamin, *J. Biol. Chem.*, 1976, **251**, 4603.
[209] A. S. Acharya and H. Taniuchi, *J. Biol. Chem.*, 1976, **251**, 6934.
[210] W. L. Anderson and D. B. Wetlaufer, *J. Biol. Chem.*, 1976, **251**, 3147.

which the disulphide bond formation is directed by a nucleating structure, and it has been shown that such a β-structure [211] may direct an initial disulphide reshuffling favouring the initial formation of two specific disulphides, the others forming more slowly and the native state being formed with a concomitant loss of β- and gain in α-structure.

Disulphide bonds can be very important in stabilizing native structures as in chymotrypsin and trypsin inhibitor and the trypsin inhibitor of black-eyed peas,[212] barley trypsin inhibitor,[213] and snake venom toxins.[214]

Pathways – Theoretical. Theoretical studies of protein conformation are dealt with elsewhere within this volume but some will be mentioned here. Teller [215] has reassessed Chothia's data on the accessible surface area of 12 monomeric proteins to give expressions relating the total accessible areas of polypeptide chains, in their fully folded or extended states, to their molecular weights. The treatment suggests that no surface is buried in the folding of a chain of less than 450 mol. wt. It is suggested [216] that α helices are important in the initial stages of folding, β bends only appearing when local interactions facilitate chain bending, with structural 'hair-pins' and sheets being formed at a later stage. The suggestion [216] that all native helices pre-exist in the unfolded chain as fluctuating embryos, some of which do not appear in the native state, resembles Lim's [217] proposed pathway of folding *via* a highly coiled globular intermediate which contains all native helices plus excess coiling which unfolds in the formation of the native state.

Sequence–Conformation Relationship.—*Thermostable Proteins.* In many cases there seems to be very little difference between thermophilic proteins and the homologous mesophilic counterparts to explain their stability to heat and, in some cases, denaturants.[218] No significant structural differences are found between the α-amylase from the thermophilic bacterium V2 and the mesophilic enzyme [219] but its stability against heat and denaturant treatment probably derives, by a remarkable decrease in activation entropy, from its one intrinsic calcium atom per molecule.[220] A similar explanation has been suggested for thermolysin [221] to explain its stability in contrast with the homologous [222] neutral protease A (*B. subtilis*). By comparison [221] the stability of thermomycolase relative to other serine proteases is largely due to an intrinsic contribution of the

[211] F. H. White, jun., *Biochemistry*, 1976, **15**, 2906.
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protein chain, and another report [223] suggests that the stabilizing effect of Ca^{2+} is the same in neutral protease and thermolysin, the stability of the latter being assigned to intrinsic factors.

Individual Amino-acids. An assessment of secondary structure prediction methods based on primary sequence has been made,[224] and the influence of specific amino-acids is being investigated in poly-amino-acid systems. The helix–coil transition in poly-(L-Orn) [225] and poly-(α-L-Glu) [226] as well as the disordered chain to β transition in poly-(L-Tyr) [227] have been investigated. The existence of local ordered structure in poly-lysine, often used as a model for disordered regions of proteins,[228] is noted.

Structural importance has been assigned to specific residues Glu-9 and/or Asp-15 of secretin in folding and helix formation by salt bridge formation,[229] and the observed differences in stability and chemical reactivity of groups between rat and liver ornithine-oxo-acid aminotransferases [230] may be due to the detected differences in amino-acid content in these proteins, previously thought to be identical. In contrast the *in vivo* methylation of baker's yeast iso-1 ferricytochrome *c* has no apparent stabilizing effect on native structure towards acid, GuCl or thermal denaturation.[231]

Primary Sequence Alterations. Substitutions. There is a general review [232] of the technique used by Miller and Lu to give systematic amino-acid substitutions in bacterial and phage proteins. A study [233] of the effect of substituting any of five amino-acids in position 8 of ribonuclease S-peptide suggests that residue conformational parameters play an important, but not exclusive, role in α helix stability since side-chain hydrophobicity has a strong influence. The capabilities of the S-peptide analogues to bind S-protein (forming ribonuclease S′) show that conformational parameters play no part, the hydrophobic character and side-chain interactions being the determining binding factors. The limited use of conformational parameters in ribonuclease may be exceptional since the normal Phe-8 side-chain interacts with 10 residues distributed along three-fifths of the protein. Thus, the conformational information for correct folding residing in the S-peptide derives from long-range interactions which are also thought to be important in haemoglobin [234] and carp myogen [235] folding.

Partial sequences. N.m.r. titration [236] shows a pH-dependent equilibrium between folded and unfolded ribonuclease S-protein and a close similarity between folded S-protein and ribonuclease, despite the absence of 20 *N*-terminal residues in the

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[230] C. C. Kalita, J. D. Kerman, and H. J. Strecker, *Biochim. Biophys. Acta*, 1976, **429**, 780.
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[235] A. Warshel and M. Levitt, *J. Mol. Biol.*, 1976, **106**, 421.
[236] H. Shindo and J. S. Cohen, *J. Biol. Chem.*, 1976, **251**, 2648.

former, showing that the complete sequence is not required for folding and arguing against folding from the *N*-terminus. Several steps were shown in the acid denaturation of the S-protein. The very similar rates and yields of the glutathione regeneration of reduced lysozyme and reduced lysozyme (1—127) demonstrate that the *C*-terminal Arg-Leu is not essential for folding.[237] In glucagon peptides [238] folding takes place only in Trp-25 containing peptides over a critical length. The C-peptides of ox, pig, and duck proinsulins are all unstructured in aqueous solution [239] as are those of corticotropin (1—32),[240] where only the *N*-terminal peptides show any α helix and then only in trifluoroethanol (the holopeptide shows some helical organization even in water).

Cleaved proteins. The importance of polypeptide chain continuity in maintaining native conformation has been investigated: the subtilisin-produced S-peptide of rabbit and sheep liver fructose 1,6-bisphosphatases [241] is non-dissociating under neutral conditions, but the changes in catalytic activity involved are similar to those observed in the native enzyme in 5M-urea, suggesting that cleavage produces a more relaxed conformation. Ribonuclease T_1 (split at the single Arg peptide bond) consists of two chains of 77 and 27 residues, covalently linked by the Cys-6-Cys-103 disulphide and shows no activity, no 3′-GMP binding capacity and an extensively destroyed conformation showing the importance of sequence continuity at the Arg residue.[242] Epidermal growth factor, which is very stable for its size possibly due to its three disulphides, when cyanogen bromide cleaved at residue 21 [243] shows a 70% loss of stability, attributed almost entirely to a change in entropy. In contrast human serum alkaline phosphatase [244] is stabilized against histidine-induced heat inactivation by tryptic digestion. Bovine plasma albumin cleaved within the Cys-390-Cys-434 disulphide loop has an essentially unaltered overall structure despite damage to one domain and a weakening of interdomain interaction.[245] Cleavage of penicillinase into three large peptides produces a compact globular form closely similar to native penicillinase [246] but with a considerably lower conformational free energy. This penicillinase C shows completely reversible unfolding in GuCl.

Amino-acid modification. Modification of some of the residues of bovine liver dihydrafolate reductase in the denatured form [247] partially inhibits the renaturation of the fully active enzyme. The varying importance of cysteine residues can be seen by the fact that modification of two thiol groups in a bacterial His-tRNA synthetase leads to destabilization of the native structure [248]

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but modification of four cysteine residues in asparaginase [249] gives very little change in α-helix, antigenicity, quaternary structure, or reactivity, all of which are greatly affected by modification of four tryptophans. The modification of ovalbumin by succinylation of its lysine residues [250] results in a conformational change proportional to the degree of modification, but with retention of significant residual structure even when 16 lysines are modified.

Domains. General. The existence of compact, folded, covalently interconnected portions of polypeptide chains can give important clues to protein folding mechanisms, especially when, as in BSA, they fold independently, as shown [251] by the renaturation of fragments A and B, on air reoxidation, to give catalytically active products. Limited proteolysis of bovine milk xanthine oxidase [252] shows that each of the two A chains is composed of three interconnected globular domains (C, E, F) identified with the three cofactor sites. Limited trypsinolysis of β-haemocyanin (*Helix pomatia*) [253] shows that each twentieth is made up of nine domains each of *ca.* 50 000 molecular weight, a strikingly similar result being obtained for the α-haemocyanin [254] in which each of the 20 polypeptide chains apparently consists of about seven domains of 50 000—55 000 molecular weight. This conclusion is confirmed by electron microscopy but it is not known whether the domains are chemically or functionally alike or not.

The reversible, independent unfolding of the two domains of phosphoglycerate kinase, a nucleotide-binding protein, has been reported recently.[255]

Nucleotide-binding domains. Domains of this type, with a main motif of a central β-pleated sheet flanked by α-helices, are a common supersecondary structure occurring even in proteins that do not bind nucleotides under physiological conditions. It is suggested, therefore, that there are protein folding constraints favouring these conformations and that they arise by convergent as well as divergent evolution. Such conclusions were reached in studies on yeast hexokinase,[256] bovine liver rhodanese,[257] and liver alcohol dehydrogenase.[258] In the latter case part of the folding pathway has been proposed, assuming that the nucleotide-binding and catalytic domains fold independently and that there are two nucleation sites per co-enzyme binding domain. The occurrence and evolutionary significance of such domains has been discussed [259] and such structures have been predicted in aldolase,[260] ribosomal protein L25,[261] and human adenylate kinase.[262]

[249] T. Saito, M. Obata, K. Todoroko, M. Okubo, M. Minowa, S. Yamazaki, Y. Tamaura, and Y. Inada, *F.E.B.S. Letters*, 1976, **65**, 11.
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Immunoglobulins. These are the best known example of domain-containing proteins. The aromatic chromophores of the C_H2 domains of human IgG are perturbed when the isolated domains are incorporated into the Fc fragment [263] and this arises either from an alteration of domain folding or a change of chromophore environment in the inter-domain contact region. A study of the conformational equilibria and kinetics of the approach to equilibrium, as a function of GuCl, of IgGl myeloma (Wes) fragments [264] is consistent with the idea of a two-region structure for Fab. One region is the V_L and V_H and the other the C_L and C_H domains, the C region being 10^2—10^4 times as stable as the V region, largely due to a stronger and more extensive interaction between its domains.

A fluorescence study of over 75 Bence-Jones protein light chains shows no two to have identical spectra and significant spectral differences were found between proteins with greater than 85% sequence homology.[265] Because of the essential identity of C domain primary structure in these cases the diversity reflects variation in the tryptophan environment in the V domain. The same study shows that selective cleavage of a light chain produces a V domain with luminescence properties similar to the intact chain, the C domain showing distinct difference. The mechanism of assembly of Fab from Fd and L chains has been investigated [266] and an increased conformational mobility in IgGl molecules due to loss of interchain disulphides reported.[267]

Intermolecular Influences in Folding.—The renaturation from 8M-urea of phage Qβ RNA replicase [268] suggested that, when present initially, native EF-Tu or EF-Ts act either as cofactors stabilizing a productive intermediate in refolding or to prevent formation of kinetically trapped intermediates. It was then reported [269] that these elongation factors act as the EF-Tu,EF-Ts complex in the renaturation and that GDP and high concentrations of kirromycin, which inhibit complex formation, also inhibit renaturation. In *E. coli* RNA polymerase [270] the interaction of the subunit with the core enzyme traps a unique conformation of the polymerase.

Similarly, a peptide-stabilizing factor is thought to shift the PFK-L_2 conformer equilibrium of rat-liver phosphofructokinase [271] in favour of a form more resistant to lysosomal and thermal inactivation. The irreversible change seen above pH 4 in potato virus X coat protein subunits is not seen in the intact virus [272] and it is suggested that the coat proteins are 'locked' into a conformation similar to that of the subunits at pH 7. In tobacco mosaic virus protein all modes of aggregation are seemingly determined by intrasubunit interactions

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between aromatic residues,[273] intersubunit interactions competing with a specific intrasubunit Trp-17-Trp-52 interaction and double-disc formation apparently increasing subunit conformational flexibility.

The thermodynamics of self-association of reduced carboxymethylated human serum ApoA-II from high-density lipoproteins,[274] whose monomers resemble random coils, reveal a structural change comparable in magnitude to those observed in thermal transitions of smaller globular proteins. The fluorescence study of thymidylate synthetase [275] GuCl denaturation reveals a swelling or elongation below 2.3M-GuCl, and the absence of a break in the denaturation curve from 2.3 to 5M-GuCl suggests a highly co-operative association of unfolding and dissociation of the two subunits. The role of subunit association in the renaturation of lactate dehydrogenases is discussed [276] (see also refs. 205 and 206).

3 Immunological Probes of Protein Conformation

Contributed by E. A. Carrey

Introduction.—The binding of soluble antibodies (immunoglobulin G) to antigenic sites on the surface of a protein is important to protein chemists interested in conformational probes since it is a specific recognition process occurring with great precision between the two complex molecules.[277] Antigenic sites on the protein immunogen may also occur on a chemically modified protein or a related protein from another organism.[278] Where several antigenic determinants are found on one protein molecule, a mixed population of antibodies, each bivalent, will cross-link between two identical sites on different molecules, forming a three-dimensional lattice or precipitate. Precipitin reactions, including qualitative double diffusion in agar gels, and the more sensitive micro-complement fixation and radioimmunoassay, are used to measure the extent of antibody–antigen binding.

Changes and Similarities between Protein Conformations.—The integrity of antigenic sites in proteins depends very heavily on the correct arrangement of amino-acid side-chains and thus on the backbone conformation in the area where the interaction takes place with the antigen-combining site [277] on the antibody. The individual antigenic sites of lysozyme and myoglobin, both of known three-dimensional structure, have been studied extensively.[278, 279] Antigenic sites are found on the surface of the proteins, commonly at exposed sites (in myoglobin) [280] or in regions depending on interactions from several regions of the polypeptide

[273] D. Vogel and R. Jaenicke, *European J. Biochem.*, 1976, **61**, 423.
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chain,[279, 281] and conformational constraints such as disulphide bridges [282] (in lysozyme) and the presence of prosthetic groups such as the haem group in myoglobin [283, 284] maintain the structure of the determinants.

The small molecular weight effectors and subunit interactions which control the activity of allosteric enzymes act through conformational changes which affect the catalytically active site, and it is reasonable to suppose that there will be a difference in antigenicity between two forms of such an enzyme. For instance, antigenic sites which are present in a subunit of a multimeric enzyme may disappear when the subunits combine, and new determinants be created.[285] The regulatory and catalytic subunits of aspartate transcarbamylase have different antigenic characteristics from the same subunits when found in the intact enzyme,[286] and antisera raised against the subunits or against the enzyme have been used to monitor the effects of ligands on the conformation of the subunit to which they bind and on the subsequent dissociation of regulatory from catalytic subunits.[287] Purified antibodies raised against *E. coli* alkaline phosphatase have been used to study the exposure or burial of antigenic determinants in the monomer–dimer equilibrium and in the interactions with Zn^{2+} and phosphate ligands.[288] The majority of antigenic determinants carried by the aspartokinase region of the bifunctional tetrameric enzyme aspartokinase–homoserine dehydrogenase have been shown to be destroyed on binding the effector molecule L-threonine, while other determinants become available.[289]

The conformational change occurring in arginine kinase on binding the nucleotide substrate Mg-ATP has been shown to prevent 50% of the inactivating effect of antibodies raised against the nucleotide binding site;[290] the binding of arginine at the catalytically active site did not have a conformational effect resulting in inhibition of the binding of antibodies. Antibodies raised against human carbonic anhydrase B have been shown to inhibit the enzyme activity, giving different Lineweaver–Burk plots according to the identity of the substrate,[291] and the inference is that the enzyme is in the native conformation when the substrate is carbon dioxide or *p*-nitrophenyl acetate, but that bicarbonate induces a conformational change.

While a change in antigenicity is a sensitive indication of rearrangements of surface groups, often a result of conformational changes in the protein, it is possible that some small conformational changes may occur which have no

[281] M. Sela, B. Schechter, I. Schechter, and F. Borek, *Cold Spring Harbor Symp. Quant. Biol.*, 1967, **32**, 537.
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[291] E. Shapira and Y. Ben-Yoseph, *J. Biol. Chem.*, 1976, **251**, 4867.

effect on the small part of the surface comprising an antigenic site. Atassi and Zablocki[292] have reported, for instance, that a lysozyme derivative chemically modified at Trp-123 was more accessible to trypsin and to disulphide reagents while the antigenicity and the secondary structure measured by o.r.d. and c.d. are identical with the native protein.

Molecular Evolution.—The classical use of immunological cross-reaction to estimate similarities between related proteins, usually in conjunction with sequence determination, is subject to a similar restriction to that raised in the previous paragraph; namely, that while cross-reaction is a good indication of similar conformations since a change in conformation will usually alter antigenic sites, the surface features of a protein are subject to very much less stringent evolutionary control than the conformation-determining residues, apart from the general polar-for-polar residue substitution rules.[293] This means that proteins with very similar overall conformations may show no cross-reaction at all because of differences in their surface groups;[294] it has been proposed that no cross-reaction can be expected between proteins differing by more than about 30% between their primary sequences.[295] Nevertheless, phylogenetic trees have been drawn up based on the cross-reaction between enzymes having the same function but found in different organisms, such as lysozymes,[295, 296] cytochromes,[297] ribonucleases,[298] and several metal-liganded plant proteins.[299]

The comparison by cross-reaction of enzymes with the same function but occurring in different organs or cell compartments (isoenzymes) can show whether the proteins are related, bearing in mind the limits placed on deductions of conformational similarity. Alkaline phosphatases found in the human liver and bone are immunologically indistinguishable,[300] but do not cross-react with the enzymes from kidney or placenta.[301] Monoamine oxidases from several organs have been shown to be immunologically identical,[302] and the rat and human liver enzymes have both been shown to contain two enzyme activities in the same antigenic molecule.[303] Two functionally distinct haemoglobins found in trout, however, show no cross-reaction.[304] Some differences between glutamate dehydrogenases from the nucleus and mitochondria have been shown by the use

[292] M. Z. Atassi and W. Zablocki, *J. Biol. Chem.*, 1976, **251**, 1653.
[293] N. Arnheim, ref. 285, p. 378; T. T. Wu, W. M. Fitch, and E. Margoliash, *Ann. Rev. Biochem.*, 1974, **43**, 539.
[294] R. Arnon and E. Maron, *J. Mol. Biol.*, 1970, **51**, 703.
[295] E. M. Prager and A. C. Wilson, *J. Biol. Chem.*, 1971, **246**, 5978; *ibid.*, p. 7010.
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[297] E. Margoliash, A. Nisonoff, and M. Reichlin, *J. Biol. Chem.*, 1970, **245**, 931.
[298] G. W. Welling, G. Groen, J. J. Beintema, M. Emmens, and F. P. Schröder, *Immunochemistry*, 1976, **13**, 653.
[299] D. G. Wallace and D. Boulter, *Immunochemistry*, 1976, **13**, 831; J. G. R. Hurrell, N. A. Nicola, W. J. Broughton, M. J. Dilworth, E. Minasian, and S. J. Leach, *European J. Biochem.*, 1976, **66**, 389; E. Tel-Or, R. Cammack, K. K. Rao, L. J. Rogers, W. D. P. Stewart, and D. O. Hall, *Biochim. Biophys. Acta*, 1977, **490**, 120.
[300] L. Korngold, *Internat. Arch. Allergy Appl. Immunol.*, 1970, **37**, 366.
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[302] H. Hidaka, B. Hartman, and S. Udenfriend, *Arch. Biochem. Biophys.*, 1971, **147**, 85.
[303] R. G. Dennick and R. J. Meyer, *Biochem. J.*, 1977, **161**, 167.
[304] A. L. Tan-Wilson, M. Reichlin, M. Brunori, and R. W. Noble, *European J. Biochem.*, 1976, **71**, 125.

of immunoabsorbents;[305] the nuclear enzyme contains specific determinants in addition to those also found on the mitochondrial enzyme. Cross-reactions between the subunits of transcarboxylase[306] suggest that the subunits, which are thought to have evolved from a common ancestor, are structurally similar.

The immunological similarity between enzymes and their proenzymes has been investigated using the same approach;[285] as might be expected, the closest correlations occur when only a small peptide is removed on activation of the proenzyme, and when enzyme and proenzyme are both active and stable at the pH to which the immune system is exposed and at which antibodies are stable.

Conformational Changes induced by the Binding of Antibody.—The 'lock and key' model which was proposed for enzyme–substrate recognition is a useful approximation to a model for antibody–antigen binding but one which is increasingly being treated with caution.[277] It is being shown that specific antibodies binding with high affinity to one form of a protein may 'pull' the conformational equilibrium towards higher concentrations of that form, whether by stabilizing the conformation or by 'induced-fit' of a flexible antigenic determinant.[278]

Specific antibodies may, for example, affect the combination of subunits of enzymes such as alkaline phosphatase[307] and β-galactosidase[308] by preferential binding to the associated and more active form, resulting in an apparent increase in enzyme activity. The complementation of peptide fragments from β-galactosidase is increased by the presence of antibodies to the active form.[309] Other examples include the release of ferrihaem from metmyoglobin on binding antibodies to the haem-free protein,[310] the stabilization of enzymes against denaturation,[311] and the activation of defective mutant enzymes by binding antibodies to the wild-type enzyme.[312] Even the inactivation of enzymes by antibodies, commonly used as a measure of bound antibody,[313] is not necessarily exerted by steric hindrance at the active site,[314] and may be a conformational effect.[315] Celada and Strom[316] have reviewed some of the specific examples referred to above.

Reports of cross-reactions between pairs of partly unfolded molecules such as lysozymes and α-lactalbumin,[317] or creatine kinase and arginine kinase,[318]

[305] G. di Prisco and L. Casola, *Biochemistry*, 1975, **14**, 4679.
[306] M. Berger and H. G. Wood, *J. Biol. Chem.*, 1976, **251**, 7021.
[307] J. M. Pages, S. Varenne, and C. Lazdunski, *European J. Biochem.*, 1976, **67**, 145.
[308] R. A. Roth and B. Rotman, *J. Biol. Chem.*, 1975, **250**, 7759; *Biochem. Biophys. Res. Comm.*, 1975, **67**, 1384.
[309] F. Celada, A. Ullman, and J. Monod, *Biochemistry*, 1974, **13**, 5543.
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[312] M. R. Pollock, J. Fleming, and S. Petrie, in 'Antibodies to Biologically Active Molecules', ed. B. Cinader, Pergamon, Oxford, 1967, p. 139; F. Melchers and W. Messer, *European J. Biochem.*, 1973, **35**, 380.
[313] D. H. Sachs, A. N. Schechter, A. Eastlake, and C. B. Anfinsen, *Biochemistry*, 1972, **11**, 4268.
[314] L. Corman and N. O. Kaplan, *Biochemistry*, 1965, **4**, 2175.
[315] J. M. Conroy and A. A. Marucci, *Immunochemistry*, 1976, **13**, 599.
[316] F. Celada and R. Strom, *Quart. Rev. Biophys.*, 1972, **5**, 395.
[317] R. Arnon and E. Maron, *J. Mol. Biol.*, 1971, **61**, 225; N. Arnheim, J. H. Sobel, and R. E. Canfield, *J. Mol. Biol.*, 1971, **61**, 237.
[318] Y. Robin, Y. Benyamin, and N. V. Thoai, *F.E.B.S. Letters*, 1976, **63**, 174; Y. Benyamin, Y. Guillou, J. P. Desormeau-Bedot, and Y. Robin, *European J. Biochem.*, 1976, **68**, 489.

which do not cross react in the native conformation, probably reflect the release of parts of the sequence which then become free to take up alternative conformations in solution, among them the native conformation or one which can be 'induced' to bind to the antibody.

Conformational Equilibria in Protein Fragments and in Regions of the Intact Molecule.—Protein fragments are usually required in large excess before they significantly inhibit the precipitation of the intact protein with antibodies.[280] The antigenic sites which are most successfully inhibited are those which are not composed of distant regions in the primary sequence brought together by specific folding.[279] The polypeptide in solution has a greater flexibility than the corresponding sequence in the intact protein, and physical chemical methods commonly demonstrate an apparently disordered structure, but some peptides have a small finite probability of local interactions leading to formation of the 'native format'.[319] It is, of course, possible that the antigenic structure of such fragments may arise partly because the antibody induces folding into the antigenic conformation,[278] so that estimates of the proportion of such fragments in the native conformation will be overestimates. A sensitive means of measuring the equilibrium constant for the native–unfolded transition in such immunologically active conformations has been developed by Sachs *et al.*[320] The very low values (less than 0.1%) of native format calculated for several enzymically inactive staphylococcal nuclease fragments indicate that induced folding is not significant in this system. A similarly low level of enzyme activity is found in the nuclease peptide (1—126), and immunological methods were used to demonstrate that this is due to the presence of the native conformation in this peptide.[321]

When applied to native staphylococcal nuclease, the immunological approach estimated that one in 2900 molecules are in the unfolded conformation.[322] The stabilizing effect of ligands and the increase of unfolding at extreme temperatures were also precisely measured. Recently the approach has been applied to three defined antigenic sites in the native myoglobin molecule,[323] and has demonstrated that the region near the haem group is very much more rigid than other parts of the molecule. The equilibrium constant for an isolated portion of the polypeptide chain indicates 1% native conformation in solution.

Larger polypeptide fragments containing disulphide bridges which may retain several 'native' antigenic determinants will show more extensive cross-reaction with antisera raised against the intact protein.[309, 324] Such fragments have been obtained from proteins such as serum albumins [325] which demonstrate the presence of discretely folded domains similar to those found in immunoglobulins.[326] A fragment of bovine serum albumin comprising one-third of the

[319] G. S. Omenn, D. A. Ontjes, and C. B. Anfinsen, *Biochemistry*, 1970, **9**, 313; D. H. Sachs, A. N. Schechter, A. Eastlake, and C. B. Anfinsen, *J. Immunol.*, 1972, **106**, 1300.
[320] D. H. Sachs, A. N. Schechter, A. Eastlake, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 3790.
[321] D. H. Sachs, A. N. Schechter, A. Eastlake, and C. B. Anfinsen, *Nature*, 1974, **251**, 242.
[322] B. Furie, A. N. Schechter, D. H. Sachs, and C. B. Anfinsen, *J. Mol. Biol.*, 1975, **92**, 497.
[323] J. G. R. Hurrell, J. A. Smith, and S. J. Leach, *Biochemistry*, 1977, **16**, 175.
[324] J. G. Beeley, *Biochem. J.*, 1976, **155**, 345.
[325] E. M. Press and R. R. Porter, *Biochem. J.*, 1962, **83**, 172; R. G. Reed, R. C. Feldhoff, and T. Peters, *Biochemistry*, 1976, **15**, 5394.
[326] A. B. Edmundson, K. R. Ely, E. E. Abola, M. Schiffer, and N. Panagiotopoulos, *Biochemistry*, 1975, **14**, 3953.

primary sequence has been shown to have 95% of the immunogenicity of the intact protein,[327] demonstrating not only that the molecule is made up of three globular domains, but also that the conformation in each domain is similar enough for surface antigenic characteristics to be repeated almost exactly in each. Antibodies to restricted regions of serum albumin were used to demonstrate that the domains which, when isolated, can fold independently to a native conformation influence the folding of other domains within the protein.[328] The regeneration of the native antigenic sites was used as an index of the refolding of serum albumin in the presence of a disulphide interchange enzyme.[329]

4 Circular Dichroism

Contributed by T. Brittain

Circular dichroism (c.d.) continues to be an area of active study, and has now almost totally replaced optical rotatory dispersion (o.r.d.). In the past year there has been a large increase in the reported use of magnetic circular dichroism (m.c.d.) as a means of investigating proteins. In this review the c.d. and m.c.d. literature will be reported separately, even though the techniques are similar, as the two methods find their application in somewhat different areas.

The literature shows, in the main, use of c.d. as a probe of structure and conformational changes. However, new techniques have been published which deal with its use under experimentally difficult conditions, particularly in the presence of scattering and fluorescence.

General.—*Instrumental.* Descriptions of a number of new instruments have appeared including one for simultaneous measurement of absorption, o.r.d., and c.d.,[330] and a universal ellipsometer, which automatically records o.r.d. and c.d. spectra as well as performing ellipsometry.[331] A review of optical activity in the vacuum-u.v. region has been presented, which discusses the c.d. spectrometers employed together with the results obtained with these machines.[332] Systems applicable under difficult experimental conditions including a pressure cell designed for use up to 300 MPascal,[333] and a cryostat suitable for use down to 77 K, incorporating a flexible window seating which is not affected by birefringence, have been designed.[334] A variable temperature chamber, which can be fitted to a Cary 60, and is capable of cooling a sample to 90 K in <5 min has also been described.[335]

A method has been given for the accurate calibration of spectrometers which makes allowances for induced phase lag, static birefringence of the Pockel cell, and the geometry of the system. This method has shown that many commonly used systems require quite large corrections.[336]

[327] M. Z. Atassi, A. F. S. A. Habeeb, and C. L. Lee, *Immunochemistry*, 1976, **13**, 547; A. F. S. A. Habeeb and M. Z. Atassi, *J. Biol. Chem.*, 1976, **251**, 4616.
[328] J. M. Teale and D. C. Benjamin, *J. Biol. Chem.*, 1976, **251**, 4609.
[329] J. M. Teale and D. C. Benjamin, *J. Biol. Chem.*, 1976, **251**, 4603.
[330] D. V. Amato, *Diss. Abs. Internat.* (B), 1976, **36**, 4420.
[331] D. N. Henty and H. G. Jerrard, *Surface Sci.*, 1976, **56**, 170.
[332] E. S. Pysh, *Ann. Rev. Biophys. Bioeng.*, 1976, **5**, 63.
[333] S. Claesson and L. D. Hayward, *Chem. Scripta*, 1976, **9**, 18.
[334] B. Norden, *Spectrochim. Acta*, 1976, **32**, 441.
[335] A. W. Burgstahler and L. O. Weigel, *Lab. Practice*, 1975, **24**, 812.
[336] A. Davidsson and B. Norden, *Spectrochim. Acta*, 1976, **32**, 717.

The use of c.d. as a parameter for following rapid reactions in stopped-flow and temperature jump apparatus has been discussed and examples of its use in following the reactions of alcohol dehydrogenase, carbonic anhydrase, and nickel-histidine given.[337]

Theory and Analysis. The application of exciton chirality methods to the study of amine derivatives has been presented and the general conformation rules obtained from transient moments and c.d. are reviewed.[338] Linear response theory of configurationally induced optical rotation has been formulated in terms of polarizability for helical biopolymers.[339] A discussion of two-photon c.d. due to dissymmetric chromophores has appeared, which utilizes quantum electrodynamics to determine the different rates of absorption associated with electronic transitions, which are dipole-forbidden for one photon. Complex field formulations allow effective interaction operators to be derived and for non-equivalent chromophores a coupling mechanism allows for the necessary chiral discrimination.[340]

The problem of Jahn–Teller distortion-induced optical activity in molecules of D_{2h} symmetry has been analysed.[341]

The theory of dispersion-induced circular dichroism (DICD) has been extended to include vibronic terms, and symmetry rules have been deduced for DICD active states.[342]

The relationship between fluorescence-detected circular dichroism (FDCD) and the standard method of c.d. has been obtained. The effect of concentration on the signal intensity and signal-to-noise ratio has been discussed. As the concentration approaches zero it appears that the signal intensity approaches the Kuhn asymmetry factor and the signal-to-noise ratio approaches a maximum. Methods for the interpretation of FDCD in terms of molecular structure are useful and a comparison of FDCD and c.d. of flexible molecules is particularly interesting.[343] In viscous solution the FDCD spectrum may not be the same as the transmission spectrum in fluid solution, if Brownian rotation is restricted during the lifetime of the excited state.[344]

C.d. studies of nitrosamine amino-acids show that the previously held rules of Snatzke are not operative and support is found for the more recent Potonski and Prajer sector rules for the prediction of c.d. from conformations.[345]

The theory of the angular dependence of the difference in scatter between left and right polarized light by optically active spheres has been given. It is shown that instrument geometry can affect the observed c.d. spectrum.[346] The importance of instrument geometry has been further detailed for small angle scattering from a range of particles (0.1—5 μm), and an interpretation of grossly distorted

[337] P. M. Bayley, S. R. Martin, and M. Anson, *Stud. Biophys.*, 1976, **57**, 53.
[338] Y. Inouye and Y. Yamomoto, *Kagaku*, 1975, **30**, 991.
[339] H. Ito, T. Eri, and Y. J. Ihaya, *Chem. Phys. Letters*, 1976, **39**, 150.
[340] D. L. Andrews, *Chem. Phys.*, 1976, **16**, 419.
[341] D. P. Craig and P. J. Stiles, *Chem. Phys. Letters*, 1976, **41**, 225.
[342] P. E. Schipper, *Chem. Phys.*, 1976, **12**, 15.
[343] I. Tinoco and D. H. Turner, *J. Amer. Chem. Soc.*, 1976, **98**, 6453.
[344] B. Ehrenber and I. Z. Steinberg, *J. Amer. Chem. Soc.*, 1976, **98**, 1293.
[345] B. Liberek, J. Ciarkowski, K. Plucinska, and K. Stachowiak, *Tetrahedron Letters*, 1976, **17**, 1407.
[346] C. F. Bohren, *Chem. Phys. Letters*, 1976, **40**, 391.

spectra of haemoglobin in red blood cells made.[347] An experimental method has been presented to correct for absorption flattening and scattering from red blood cells using highly refractive media to match the refractive index of the cells; the method finds application for many macromolecules in their native environments.[348]

New methods employing integration over all data and calculation of α,β, and random coil contents of proteins have appeared. Curve fitting has been shown to be often misleading. A new method is claimed to possess advantages over other methods available for the analysis of the spectra of myoglobin, lysozyme, lactate dehydrogenase, papain, ribonuclease, and histones.[349]

A dipole–dipole coupling calculation of the tyrosine c.d. of pig insulin, based on the conformation observed in rhombohedral crystals, has been made. The predicted enhancement in the near-u.v. c.d. signal on going to the dimer and hexamer due to the new coupling is qualitatively in agreement with experimental observations.[350]

The Kuhn–Kirkwood dynamic coupling mechanism has been applied to the interaction of trinitrophenyl hapten and the tryptophan residue of its antibody. The theory successfully predicts the observed behaviour when two groups lie close to each other but not necessarily parallel.[351]

Small Molecules, Model Compounds, and Synthetic Polymers.—*Amino-acids and Derivatives.* Reports of c.d. studies on mixed ligand Cu^{2+} complexes with amino-acids have been made. Ternary complexes with two L-α-amino-acids or one L-α-amino-acid and iminodiacetate (ida), 2,2′-bipyridyl (bipy), 2-pyridine carboxylate (pca), or 2,6-pyridine dicarboxylate have been prepared. The c.d. spectra of Cu(ida)(L-Arg), Cu(ida)(L-Orn), and Cu(L-Glu)(L-Lys) show enhancement when compared to the binary systems and a further enhancement when examined in 50% ethanol. These findings were taken to support the view that pairs of ligands in ternary complexes are fixed about the Cu^{2+} by electrostatic ligand–ligand interactions.[352] Ternary complexes with two optically active amino-acids with charged side-chains, when compared to the binary complexes, show an enhancement for all Cu(L-A)(L-Z) (A = Asp or Glu; Z = Arg, Lys, or Orn) except when one ligand is L-Ala or L-Val. This was taken as evidence for the presence of intramolecular electrostatic bonding between oppositely charged groups in the side-chains of the ligands, and is considered to be the driving force responsible for the production of the ternary complexes.[353]

The formation of cobalt complexes of amino-acids has been followed using c.d., and distinctive spectra arising from a 1 : 1 His-Co^{2+} complex have been recorded. However, when an attempt was made to produce mixed bis chelates with amino-acids only mixtures of Co^{3+} isomeric bis chelates were obtained, which were clearly identifiable from their c.d. spectra.[354] The c.d. spectra of

[347] A. Gitter-Amir, A. S. Schneider, and K. Rosenheck, *Biochemistry*, 1976, **15**, 3138.
[348] A. S. Schneider and D. Harmatz, *Biochemistry*, 1976, **15**, 4158.
[349] C. C. Baker and I. Isenberg, *Biochemistry*, 1976, **15**, 629.
[350] E. H. Strickland and D. Marcola, *Biochemistry*, 1976, **15**, 3875.
[351] G. B. Orin, R. C. Davis, R. M. Freed, and J. H. Rockey, *Immunochem.*, 1976, **13**, 517.
[352] T. Sakurai, O. Yamauchi, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1976, **49**, 1579.
[353] T. Sakurai, O. Yamauchi, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1976, **49**, 169.
[354] L. I. Katzin, *Inorg. Nuclear Chem.*, 1976, 219.

three possible isomers of (D-aspartato)(L-2,4-diaminobutyrato)Co^{3+} complexes have also been reported.[355]

A comparison between the c.d. spectra of selenium-containing amino-acids and the corresponding sulphur and methylene analogues has been made. In the region 190—250 nm the observed Cotton effects for the carboxy and Se(S) forms correlate with L (*S*) or L,L (*S,S*) absolute configuration. The results suggested that C—Se—C has three optically active transitions at *ca.* 225, 210, and 195—200 nm and that the absolute configuration of (+)-selenocystathionine is L,L (*S,S*), which is in agreement with previous enzymic results.[356]

A very useful technique for the determination of amino-acid configuration *in situ* has been reported. The chiroptical properties of the pyrrolinone chromophores derived from the reaction of fluorescamine compounds with α-amino-acids *in situ* have been established and as such provide a simple test-tube procedure for configurational determination.[357]

The interactions of di-iodo-L-Tyr (DIT) and tri-iodophenol (TIP) with bovine serum albumin (BSA) have been studied as models for the binding of thyroid hormones by carrier proteins. With DIT-BSA and TIP-BSA c.d. activity was induced at 320 nm. The c.d. signal was measured at various ratios of BSA to ligand in order to find the binding constants. For a single molecule of ligand the affinity constants were 1.3×10^5 l mol^{-1} for DIT and 1.6×10^9 l mol^{-1} for TIP. These results were interpreted in terms of TIP being bound in a cleft in BSA not suited to the binding of DIT.[358]

Dipeptides and Oligopeptides. Vacuum-u.v. c.d. has been carried out on β-sheet forming oligopeptides of the form $Me_3CO_2C(X)_nOMe$ (X = Ala, Val, or NorVal; n = 2—7) down to 140 nm. Large amounts of β-structure were found for Ala_3, $NorVal_6$, and Val_7, whilst Ala_7 and Val_7 gave antiparallel and parallel sheets, respectively. $NorVal_7$ gave antiparallel and parallel sheets in equal amounts.[359]

The importance of coulombic interactions for the induction of β-sheet formation in lysine oligomers (Lys_n, n = 3—5) by sodium dodecyl sulphate (SDS) has been investigated. The anionic detergent head and cationic Lys,NH_3^+ group seem to be essential for the formation of β-structure observed when n = 5 at concentrations of SDS > 3.5×10^{-4} mol l^{-1}.[360]

Liquid phase synthesis, on poly(ethylene glycol), of peptides utilizing *C*-terminal protecting groups allows solubilization of the peptide in many solvents yielding a system transparent down to 190 nm. Secondary structure has been observed to arise during synthesis. The octapeptide of myoglobin (66—73) in trifluoroethanol, when bound to the glycol support, showed more helical structure than when in the free state. Nona- and deca-Ala with free NH end-groups in trifluoroethanol and water have β-structure, but the corresponding *N*-t-butoxycarbonyl derivatives show the presence of helical structures.[361]

[355] M. Watabe, S. Kawaai, and S. Yoshikawa, *Bull. Chem. Soc. Japan*, 1976, **49**, 1845.
[356] C. Cymerman, C. S. Y. Lee, G. Zdansky, and A. Frega, *J. Amer. Chem. Soc.*, 1976, **98**, 6456.
[357] V. Toome, B. Wegrzynski, and G. Reymond, *Biochem. Biophys. Res. Comm.*, 1976, **69**, 206.
[358] N. Okabe, N. Manabe, R. Tokuoka, and K. Tomita, *J. Biochem.*, 1976, **80**, 455.
[359] J. S. Balcerski, E. S. Pysh, G. M. Bonora, and C. Toniolo, *J. Amer. Chem. Soc.*, 1976, **98**, 3470.
[360] W. L. Mattice and W. H. Harrison, *Biopolymers*, 1976, **15**, 559.
[361] M. Mutter, H. Mutter, R. Uhmann, and E. Bayer, *Biopolymers*, 1976, **15**, 917.

A series of glycine-containing heterodetic cyclic cystinyl tripeptides have c.d. spectra dominated by the disulphide group. Strong electronic disulphide–amide interactions are evident at 300 nm and are found to be strongly affected by the solvent.[362]

A review on the determination of configuration of H-Gly-X-$(Gly)_n$-Trp-Gly-OH (X = Trp or Phe; n = 0—2) discusses the effect of variable distance between the two aromatic residues on their configuration.[363]

Homogeneous (n = 1—10, 12) and polydisperse (n = 14, 5—50, 400) H-$(Glu)_n$-OH systems occur in configurations which may be considered to be derived from two limiting configurations, α-helix and random, random and extended, and β-sheet and random. The value of n is found to have a profound effect on the prevalent configuration.[364]

Enantiomeric *N*-t-butyloxycarbonyl $(Ala)_n$ methyl ester oligomers show unordered structure up to the hexamer but β-sheet structures are present in the heptamer and higher polymers.[365]

Cyclic peptides of proline (L-Pro-D-Tyr and L-Pro-D-Phe) in both polar and apolar solvents have been reported to have the same conformation in which the aromatic rings are folded over the diketopiperazine ring. *Cyclo*-Pro-Tyr and *cyclo*-Pro-Phe, however, have a novel folded configuration in water.[366]

Linear $(Ser\text{-}Glu)_3$ and $(Ser\text{-}Orn)_3$ containing two Cu^{2+} ions have been shown to form five-membered rings, whilst the cyclic forms of the oligomers contain seven-membered rings, which possess lower stability.[367]

Bradykinin possesses an ordered conformation in polar solution which is stabilized by intramolecular hydrogen-bonding involving the amino-group of Phe-8.[368]

Polypeptides. A desmosine cross-linked peptide shows a hitherto undescribed elastin-type c.d. spectrum characterized by negative maxima at 230—235 and 190 nm and a positive maximum at 215 nm, all of which are sensitive to pH and temperature changes. Low temperatures and low pH have been found to increase the rotational strength, and from the spectral shape and observed temperature effects it was concluded that the molecule possesses extensive α-helical structure.[369]

Observations at 228 nm give evidence of proton and calcium binding to poly-L-Pro. The pK for proton binding was established as 2 and that for calcium was found to be 1.5, from titration with hydrogen chloride and calcium chloride.[370] The structures of collagen-like homo- and hetero-polypeptides containing proline of the form $(Pro\text{-}Glu\text{-}Gly\text{-}Pro\text{-}Ala\text{-}Gly)_n$ (I), $(Gly\text{-}Pro\text{-}Glu\text{-}Gly\text{-}Pro\text{-}Pro)_n$ (II), and $(Pro\text{-}Glu\text{-}Gly\text{-}Pro\text{-}Glu\text{-}Gly)_n$ (n = 2—6) have been studied. The form

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[368] M. P. Filatova, T. O. Reutova, V. S. Pashkov, S. L. Portanova, E. S. Efremov, and V. T. Ivanov, *Tezisy Doklady-Vses. Simp. Khim. Pept. Belkov 3rd*, 1974, 153.
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[370] J. T. Lo and W. L. Mattice, *Biopolymers*, 1976, **15**, 15.

of the spectra and temperature dependence of the signals obtained suggest collagen-like structures for (I) and (II) in solution.[371] The state of aggregation of poly-L-Pro and its relation to optical activity have been investigated. Helical association of the polymer leads to differential scattering of circularly polarized light, and in solvents which favour aggregation anomalous c.d. signals appear.[372]

Chromatin models based upon polypeptide–DNA complexes have shown that the state of aggregation of poly-L-His and DNA depends upon the salt concentration and the mode of preparation of the complex. These phenomena were attributed to large-scale molecular organization of aggregated particles.[373] Experiments performed on Lys-Leu polypeptide–DNA complexes indicate both random Lys_xLeu_y and block co-polymers which have a sharp ionic strength dependence with regard to complex formation with DNA. The c.d. spectra at optimum sodium chloride concentration resembled the Ψ spectra of DNA. From these findings it was concluded that the formation of complexes may reflect DNA condensation into higher order asymmetrical structures. The distribution of hydrophobic residues also seems important in the condensation process, and these data were discussed in relation to histone–DNA complexes in chromatin.[374]

Isotactic poly(methylacrylic acid) (III) has the ability to induce α helical structures in poly-L-Lys, whilst other stereoisomers do not. The order in which other stereoisomers disturb α-helix formation is iso- $\ll$ syndio- $<$ conventional $\ll$ atactic-, which parallels the degree of atacticity.[375] Polyacrylic acid (III) also reacts with poly-L-Lys to form complexes, independent of the molecular weight of (III). The stoicheiometric reaction yields α helical structures. C.d. has shown that (III), which is capable of forming a left super helix, binds to a core composed of right α-helical poly-L-Lys.[376]

Co-operative reactions have been demonstrated between α-helical poly-L-Lys haem complexes and oxygen, carbon monoxide, and cyanide.[377]

Potentiometric titrations have been used to study the change from helical to random coil forms of L-Glu-2-nitrobenzyl-L-Glu co-polymers in aqueous solution. The free energy for the process was found to be temperature dependent. From these studies it was found that nitrobenzyl-L-Glu stabilizes helical structure in aqueous solution and that the entropy for the conformational change is positive, making this the first example of a non-ionic co-polymer with this property.[378]

C.d. and absorption studies on acridine orange binding to poly-L-Glu show that at pH < 7.0 and a ratio of polypeptide to dye of 25 to 1 optical activity is induced in the acridine spectrum. It is suggested that the acridine orange dimers are bound to the α-helix with the dye molecules perpendicular to the

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[377] E. Tsuchida, E. Hasegawa, and K. Honda, *Biochim. Biophys. Acta*, 1976, **427**, 520.
[378] J. Estevez and M. H. Loucheux-Lefebvre, *F.E.B.S. Letters*, 1976, **71**, 157.

major axis of the helix. A different c.d. spectrum is observed at lower ratios of polypeptide to protein or at pH 7.0 or higher.[379]

Poly(*S*-benzyl-L-Cys) (IV), poly(*S*-benzoxycarbonyl-L-Cys) (V), and poly-(*O*-benzoxycarbonyl-L-Ser) (VI) have c.d. activity in the u.v. region in non-aqueous solvents. Compounds (IV) and (V) exhibit similar phenyl group c.d. and β-structure in both aqueous solution and films.[380]

The kinetics of the disordered to β-transition in poly-L-Tyr following pH jump has been measured by observation of the c.d. signal at 227 nm. Two sequential processes were observed which exhibited sharp pH dependence; the maximum rate was seen at pH 11.29 but fell by a factor of 20 within 0.25 pH units. This is in sharp contrast to the data for poly-Lys. A transition in initial states was identified within the pH domain of the coil to β-transition whose existence was previously unknown; the sharp pH effect was consistent with a high degree of co-operativity.[381]

A variety of synthetic polypeptides poly-L-Pro, poly(γ-OH-L-Pro), ionized poly-L-Glu, poly-L-Lys, and (N^5-ω-hydroxyethyl-L-Glu) exhibit similar changes in c.d. on addition of high concentrations of certain salts. This general behaviour is also seen in blocked L-Ala and L-Lys oligomers which possess minima at 192—195 nm and a maximum in water at 215—217 nm, which disappears on addition of salts. The results show a similar conformation to be present independent of the side-chains present. However, the addition of sodium dodecyl sulphate produces side-chain-dependent changes in c.d. Calculations have been successful in explaining the effects of the detergent on the polymers.[382]

Proteins.—This section is divided such that papers concerned with c.d. arising from specific chromophores are grouped together as follows: (i) aromatic and disulphide chromophores which reflect changes in localized environments; (ii) peptide chromophores which indicate the backbone conformation and which are much used to determine α-helix content; (iii) chromophoric proteins such as haemoproteins; (iv) added extrinsic chromophores, and (v) protein–DNA complexes.

Aromatic and Disulphide Chromophores. The origins of the c.d. of Bowman–Birk soybean trypsin inhibitor has been investigated by comparison with tyrosyl models. The spectrum was analysed in terms of contributions from tyrosyl and disulphide groups. *O*-Acetylation of tyrosine showed that the c.d. signal at 225 nm arises from the disulphide group present and the sharp positive maximum at 231 nm from tyrosyl residues.[383]

The spectrum of soybean leghaemoglobin when compared with that of the *N*-bromosuccinimide-modified protein indidates that Trp-128 is the site of modification and is responsible for the observed band at 290 nm.[384]

Interactions between tyrosine residues on different subunits of tropomyosin have been found to be responsible for the anomalous near-u.v. c.d. signals obtained.[385]

[379] T. Imae and S. Ikeda, *Biopolymers*, 1976, **15**, 1655.
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[382] W. L. Mattice, *Polymer Prepr. Amer. Chem. Soc., Div. Polymer Chem.*, 1975, **16**, 2.
[383] E. Kay, *J. Biol. Chem.*, 1976, **251**, 3411.
[384] G. Sievers and N. Ellfolk, *F.E.B.S. Letters*, 1976, **61**, 154.
[385] B. Bullard, D. A. Mercola, and W. F. Mommaerts, *Biochim. Biophys. Acta*, 1976, **434**, 90.

Comparative studies have appeared of iron-free and saturated human serotransferrin and lactotransferrin in the region 180—800 nm. The binding of two iron atoms does not seem to alter the protein structure markedly but it does appear that tryptophan is involved in the binding site.[386]

Carboxypeptidase A specifically modified to yield the arsanilazo Tyr-248 has been used to quantify the binding constants of competitive inhibitors.[387]

The trypsin inhibitor of Adzuke bean (*Phaseolus angularis*) gives a negative signal at 280 nm and a positive shoulder at 245 nm. Since the inhibitor has no tyrosine or tryptophan residues these signals were attributed to the six disulphide groups and this is supported by the disappearance of these signals on reduction and their convergence to a single negative at 270 nm when modified with glutathione. It is also suggested the disulphides are constrained with respect to their dihedral angles.[388]

Tryptophan c.d. of insect (*Chironomus thummi thummi*) monomeric haemoglobin III and IV and dimeric haemoglobin shows the presence of different conformations in variously liganded states. Fine structure at 292 nm evident in unliganded haemoglobins was reduced in intensity in the oxy form whereas the CO form was intermediate in character. These findings correlate well with the observed corresponding Bohr effects.[389]

The spectra of glycogen phosphorylase (VII) of rabbit muscle in either the *a* or *b* form in the region 250—310 nm were found to be identical. Binding of AMP or borohydride reduction produces large changes such that the difference spectrum of (VIIb) − (VII-AMP) yields a positive maximum at 266 nm and a negative at 289 nm. Rotational strength increases when AMP affinity is raised by such means as addition of glucose-1-phosphate or bivalent metal ions. The reporters suggest that the interaction of the nucleotide with (VII) involves the stacking of the base with the aromatic amino-acids.[390]

On complex formation between histone H3 and H4 an increase in helical content has been reported. At low pH or low ionic strength the helical content is lowered and undergoes time-dependent irreversible changes due to intramolecular oxidation of the cysteine residues of H3.[391]

'*Non-chromophoric*' *Proteins.* A number of papers concerned with glucosamine binding to lysozyme have been published. The interactions of 3-deoxy- and 6-deoxy-derivatives of *N*-acetyl-D-glucosamine and GlcNAc with hen lysozyme at various pH have been followed at 295 nm. These derivatives were found to bind at subsite C of lysozyme and compete with GlcNAc. The pH dependence of the binding of 6-deoxy-derivatives was found to be the same as GlcNAc, but the 3-deoxy-form was found to bind less tightly by a factor of three to ten in the pH range 3—9. *X*-Ray data suggested that O-6 and O-3 of GlcNAc bind at subsite C and are hydrogen-bonded to the imino-groups of Trp-62 and Trp-63, but the c.d studies suggest Trp-63 and not Trp-62 to be important for binding of GlcNAc.[392]

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[390] S. Shimomura and T. Fukui, *Biochemistry*, 1976, **15**, 4438.
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A similar study of the binding of α- and β-*N*-acetyl-D-glucosamines to hen and turkey lysozymes revealed pH dependences of β-GlcNAc binding to be identical. The α- and β-derivatives had the same affinity patterns below pH 3.5 but varied above this pH, showing the participation of different amino-acids in the binding of the two forms of the GlcNAc derivatives.[393] β-MeGlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$ binding to lysozyme in which there is an ester link between Glu-35 and Trp-108 has been followed by c.d. The binding constant of MeGlcNAc and $(GlcNAc)_2$ to the 108 ester is the same as that for the natural protein in the pH range 1—5, above which the binding to the ester derivative is pH-independent unlike the native protein. These data were taken as evidence of the involvement of Glu-35 in the binding of saccharides and the higher affinity of $(GlcNAc)_3$ was interpreted as binding to a different subsite.[394]

Collagens from cod, pike, and rat show quantitative and qualitative similarity in spectra, although they contain different imino-acids, suggesting the triple helix conformation is not dependent on the exact imino-acid content of the proteins.[395] The spectrum of rat skin collagen in solution and in thin film in the range 185—200 nm has been resolved into three components. Based on these studies collagen is found to be composed of *ca.* 40% α- and β-structure.[396] Vacuum-u.v. c.d. of gelatin shows it to be more closely related to a charged polypeptide than is suggested by near-u.v. studies. Gelatin possesses a band at 172 nm, similar to that observed in poly-L-Glu and also in helical poly-L-Pro, which is assigned to an n–σ^* transition not seen in collagen.[397] Alkali- and acid-solubilized collagen exhibit closely similar spectra dominated by a negative peak at 197—198 nm.[398]

U.v. c.d. at 220 and 208 nm has shown tropomyosin to be sensitive to the presence of cetyl trimethylammonium bromide (CTAB), losing 60% of its ellipticity at a CTAB concentration of 24 mmol l^{-1}. Troponin showed less sensitivity to the presence of CTAB.[399] The conformations of some muscle proteins have been re-examined and have indicated the presence of slightly higher helical contents than previously reported; myosin (78%), heavy meromyosin (70%), SFI (60%), G actin (45%), and tropomyosin (90%).[400]

Chen–Yang–Chan [401] analysis of the u.v. spectrum of aqueous staphylococcal enterotoxin B gave 9% α-helix, 38% β- and 53% random structure. In 0.2% sodium dodecyl sulphate the α-helical content is increased to 36%, which is typical behaviour for globular proteins with a low helical content. However, Chou–Fasman [402] analysis suggests the protein should contain 11% α-helix and 34% β-structure between residues 81—148 and 184—217. The presence of regions of highly co-operative antiparallel β-sheet accounts for the slow unfolding observed in guanidine hydrochloride.[403]

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The β-structure of α-neurotoxin from *Naja nigricollis* is maintained in organic solvents but erabutoxin b from *Laticauda semifasciata* changes from predominantly β- in water to α-helix in trifluoroethanol. Trifluoroethanol produces similar structural changes in both toxins around Trp-29, which is found in all known toxins, and also in the vicinity of Trp-25, suggesting that the environment around these two residues changes independently of the rest of the molecule and that these residues probably have a biological function.[404]

Human prostatic acid phosphatase I is a glycoprotein containing 38—41 carbohydrate residues. It has been reported that removal of sialic acid by neuraminidase increases the α-helical content above the native value of 30% suggesting specific interactions between the protein and sialic acid.[405]

The histidine decarboxylase of *Micrococcus sp. n.* is sensitive to pH and guanidine hydrochloride but not to 1% sodium dodecyl sulphate. The native protein (pH 4—8) possesses 20% α-helix and 40% β-structure, while the helical content is reduced to 5% at low or high pH and is completely eliminated by guanidine hydrochloride.[406]

The relative helical and β-structure contents of apo- and holo-D-amino-acid oxidase from pig kidney have been reported.[407]

An increase in the α-helical content of α_1-acid glycoprotein, Bence-Jones protein, carbonic anhydrase B, deoxyribonuclease, pepsinogen, and plasminogen is observed in the range 0.0005—0.05 M sodium dodecyl sulphate in the pH range 2.1—4.4.[408]

Studies of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (phe) from *E. coli* K12 show the native enzyme to contain 26% α-helix. The addition of an inhibitor induces a conformational change involving small alterations of secondary structure and large changes in the interactions of some aromatic residues.[409]

The spectra of lactate dehydrogenase and some other proteins in solution and as thin films have shown that the same conformations are present in both states. A method for finding the concentration and optical path for films was discussed.[410]

The helical content of cytochrome *c* oxidase has been found to increase by 20% at the cost of β-structure on the addition of soybean phospholipids or cardiolipids.[411]

'*Chromophoric*' *Proteins.* The pH dependence of several properties of human fetal (HbF) and adult haemoglobin (HbA) has been investigated to determine the relative stability of high and low affinity (*R* and *T*) conformations. In the presence of inositol hexaphosphate aquomet-HbF undergoes changes in its c.d. spectrum consistent with an *R–T* transition at pH < 6.8; in HbA this

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[409] R. J. Simpson and B. E. Davidson, *European J. Biochem.*, 1976, **70**, 509.
[410] A. G. Sukhomudrenko, V. L. Shnynov, and V. M. Lobachev, *Terzioy Dokl.-Vses. Konf. Spectrosk. Biopolim.*, 1974, 98.
[411] A. Spisni, A. M. Sechi, and L. Masotti, *Boll. Soc. Ital. Biol. Sper.*, 1976, **52**, 480.

transition occurs at pH < 7.2. Even in the absence of phosphate at low pH, HbF shows the presence of some *T* form. The results are consistent with an HbF in which the phosphate site is functionally weakened by replacement of a residue involved in ionic interactions, in which the *T* form is intrinsically more stable than in HbA. These findings thus resolve some of the problems associated with molecular oxygen acquisition by HbF.[412] In analogous studies it was found that inositol hexaphosphate induces an *R–T* transition in nitrosyl HbA.[413]

The meso-haem IX derivative of leghaemoglobin in the region 200—440 nm has a c.d. spectrum only slightly different from the native protein, but the bands at 277 and 352 nm present in the haem spectrum are not observed in the meso-porphyrin IX derivative and may reflect the binding of histidine to the haem iron.[414]

A review of the c.d. of haemoglobin A, its derivatives, and some abnormal haemoglobins has appeared.[415]

At 287 nm the change in c.d. on deoxygenation of cobalt haemoglobin (CoHb) is only approximately half that observed for normal haemoglobin, but in the Soret region the changes are the same. Inositol hexaphosphate has little effect on the spectrum of CoHb in the 287 nm region but a marked effect in the Soret region, suggesting a polyphosphate modification of the tertiary structure of CoHb.[416]

Horse heart cytochrome *c* and baker's yeast iso-cytochrome *c* have similar c.d. spectra in the region 250—600 nm, in the reduced state. In the oxidized form horse heart cytochrome *c* possesses *ca.* 30% α-helix, whilst the yeast iso-cytochrome contains 27%. Both are more stable than methylated or non-methylated iso-DL-cytochrome *c* (21% α-helix) and horse cytochrome *c* is more stable than yeast cytochrome *c*. A correlation between α-helix content and stability is proposed.[417]

The complex between cardiac cytochrome c_1 and *c* shows a c.d. spectrum markedly different from the sum of the spectra of the individual cytochromes, indicating dramatic conformation changes on complex formation.[418]

Mushroom tyrosinase in the resting state has optically active bands at 755 and 653 nm and when treated with hydroxylamine or hydrogen peroxide shows oxygen-sensitive bands at 350 nm similar to those observed on regeneration of aged haemocyanin. The active site of tyrosinase treated with hydroxylamine or hydrogen peroxide is proposed to be structurally similar to that of haemocyanin.[419]

Co-operative binding of oxygen and the associated conformational changes of earthworm erythrocruorin have been followed. The double Soret peak observed is taken as indicative of a distinct distribution of aromatic side-chains interacting with the haem group.[420]

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Both the biliproteins phycoerythrin and phycocyanin possess two chromophores absorbing in the ranges 300—400 nm and 500—650 nm; the positions of the absorption bands are concentration dependent, showing the existence of aggregation.[421]

Rhodopsin and porphyropsin both have positive peaks between 320 and 700 nm, the α band being more intense for rhodopsin than porphyropsin but, for the β band at 355 nm, the converse is true.[422] The addition of Ca^{2+} ions to rhodopsin causes a decrease in the rotational strength of the negative band at 220 nm and the positive bands at 340 and 490 nm, but sodium and magnesium have no such effect. The author supposes that specific changes occur in the photo-receptor on binding of Ca^{2+}.[423]

Synthetic flavinyl peptides, related to the active site of mitochondrial monoamine oxidase, have been shown to have identical c.d. spectra to the naturally occurring peptides.[424]

Added Extrinsic Chromophores. The binding of fluorescein mercuriacetate (VIII) to glyceraldehyde-3-phosphate dehydrogenase produces a rapid induction of a Cotton effect in the region of the absorption of (VIII). On binding, a decrease at 513 nm from 1.1×10^5 to 5×10^4 deg cm^2 dmol is observed for the holoenzyme, whilst at 524 nm an increase in rotational strength is seen from 1.5×10^4 to 6.6×10^4 deg cm^2 dmol; the rate of change depends strongly on the temperature. It has been proposed that the binding of (VIII) induces conformational changes in the protein, but secondary structure apparently did not change as judged by the c.d. of the peptide region.[425]

Anisotropy factors associated with electronic transitions in the *p*-aminobenzene sulphonic acid and heterocyclic groups of four long-acting sulphonamides indicate that both moieties are bound to albumin surfaces. However, different interactions occur depending on whether the serum albumin is from bovine or human sources.[426]

Suramin forms complexes with bovine and human serum albumins, influencing the micro-organization of the proteins. In both cases a high concentration of suramin leads to unfolding of the proteins.[427]

The effect of pH in the range 6.6—8.2 on the Cotton effect observed when benzodiazepine binds to bovine serum albumin indicates the presence of several binding sites, compared with the single binding site on human serum albumin.[428]

It has been established that azo dyes bind to bovine myelin basic protein mainly by charge–charge interactions.[429]

Protein–Nucleic Acid Complexes. Poly-L-His forms two types of complex with DNA depending on pH, one at pH 5.9 and another at pH > 6.5, which show very different c.d. spectra. In the lower pH complex, protonated poly-L-His

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reacts with the A–T base pairs whereas in the higher pH complex weakly protonated poly-L-His interacts with the G–C base pairs.[430]

Histones H1, H2A, and H2B show isosbestic points in their c.d. spectra in the range 3—85 °C, consistent with conformational changes between two states. The band position and form of the c.d. spectra suggest the presence of poly-Pro type II like structures at low temperatures and α-helical structures at higher temperatures.[431]

The use of the c.d. spectrum of chromatin as a standard has led to the conclusion that production of complexes is best performed by mixing at low ionic strength. It has also become apparent that arginine-rich histones play a central role in stabilizing chromatin conformation.[432]

Hormones.—The interaction of insulin with sulphonylureas has been examined and the formation of five derivatives followed. All showed a decrease in ellipticity at 208 nm, whilst at 222 nm the ellipticity remained unchanged. The results have been interpreted as showing that drug effects arise out of induced changes in the insulin conformation.[433] C.d. has disclosed that a particular conformation is critical for zinc–insulin and insulin–receptor complex formation. The binding site of insulin appears to be composed of hydrophobic residues and the complexes show the presence of antiparallel sheet structures, as is found in insulin dimers.[434]

Curve resolution has allowed band assignment of peptide, aromatic, disulphide, and *N*-acetylated amino sugar contributions to the c.d. of human pituitary luteinizing hormone and its glycopeptides. A Gaussian fit of u.v. data leads to an estimate of 28% β structure and no helical content and shows that dissociation into subunits has very little effect on the conformation of the protein.[435] A new radio-labelling technique for luteinizing hormone and luteinizing hormone releasing factor has allowed a characterization of some of their physicochemical properties by the use of c.d.[436] Reoxidation of the disulphide bridges of the α-subunit of bovine luteinizing hormone, both in the presence and absence of denaturing agents, yields products indistinguishable from the native protein with the exception of a slight decrease in optical activity at 233 nm. The intact hormone and that produced by recombination of the α- and β- or reoxidized α- and β-subunits show identical spectra.[437]

The far-u.v. c.d. of sheep β-lipotropic hormone (βLPH) as a function of temperature, pH, ionic strength, and solvent composition show that βLPH is stable to strong acid and base up to 50 °C, but high ionic strength or dioxan leads to an increase in α structure. The method of Yang was used to compute the percentage α structure and results were compared to that predicted by Fasman analysis; both agree well favourably with the observed c.d.[438] C.d. has shown the polypeptide of adrenocorticotropic hormone to have a left-hand

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[435] D. Puett, A. Nureddin, and L. A. Holladay, *Internat. J. Peptide Protein Res.*, 1976, **8**, 183.
[436] P. Marche, *Inis. Atomindex*, 1976, 7.
[437] L. C. Guidice and J. G. Pierce, *J. Biol. Chem.*, 1976, **251**, 6392.
[438] S. St. Pierre, C. Gilardeau, and M. Chretien, *Canad. J. Biochem.*, 1976, **54**, 992.

helical conformation, analogous to that of poly-L-Pro type II in aqueous solution.[439]

Mild alkali induces a new band at 250 nm in ovine lutropin hormone, without any detectable changes in secondary structure. These changes were correlated with the ionization of two to three tyrosine residues the nitration of which lowers the pK of ionizations as expected. Removal of residues α92 and α93 Tyr results in a decrease in intensity at 235 nm. Therefore it was concluded the 250 nm band arises from the ionization-induced red-shifted bands of Tyr α21, α92, and α93.[440] The effect of pH on the structure of human transcortin correlates with the known affinity of the protein and state of polymerization; the affinity decreases as the α-helical structure is destroyed.[441]

The natural opiate metenkephalin forms complexes with sodium and potassium ions, which induce changes in the protein conformation.[442] The solution conformations of the smooth-muscle hypotensive bradykinin and D-Pro^2- and D-Pro^3-bradykinin have been determined, and it was found that the activity at 221 nm is due to the presence of two chromophores; the 217 nm band is due to phenylalanine and the 223 nm band arises from the *N*-terminal sequence Arg-Pro-Pro.[443] Investigations on truncated and other analogues of angiotensin II have led to a number of conclusions concerning these hormones, *viz.* β structure is present in the protein; folding occurs at both the *C*- and *N*-terminals; the *N*- and *C*-terminals interact leading to an increase in stability.[444]

Immunoglobulins and Antibody–Hapten Complexes.—*Immunoglobulins* (*Ig*). The activity at 290 nm of a group of myeloma immunoglobulins is unaffected by the subclass specificity of the immunoglobulin. The position and molar mean residue ellipticities are reported for IgG_1, IgG_2, and IgG_4.[445] A similar investigation has that shown the molecular conformations of subclasses IgA do not correlate with the observed c.d. spectra.[446]

Exposure of Fc fragments derived from IgG_1 myeloma protein to acid pH was found to make them susceptible to trypsin cleavage yielding Cγ2 and Cγ3 fragments. The c.d. of these fragments could not be fully accounted for on the basis of spectra obtained for the isolated domains, suggesting strong interdomain interactions.[447]

The binding of phosphorylcholine to an IgM Waldenstroem heavy chain has been followed.[448]

Antibody–Hapten Complexes. Studies of hapten–antibody complexes suggest that tryptophan is involved in the binding site of MOPC-315 mouse IgA myeloma protein, and also in the reconstituted protein and its Fv fragments.[449]

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[440] M. Ascoli, D. N. Ward, and B. Jirgensons, *European J. Biochem.*, 1977, **72**, 157.
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[442] J. H. Poupaert, P. S. Portoghese, and V. Garsky, *J. Medicin. Chem.*, 1976, **19**, 1354.
[443] J. R. Cann, J. M. Stewart, R. E. London, and N. Matwiyoff, *Biochemistry*, 1976, **15**, 498.
[444] D. Greff, S. Fermandjian, P. Fromageot, M. L. Khosla, R. R. Smeby, and M. F. Burnpus, *European J. Biochem.*, 1976, **61**, 297.
[445] G. Amend, W. Bonnekoh, and O. Wetter, *Clin. Chim. Acta*, 1976, **71**, 339.
[446] W. F. Riesen, H. Huser, and F. Skvaril, *F.E.B.S. Letters*, 1976, **61**, 243.
[447] J. R. Ellerson, D. Yasmeen, R. H. Painter, and K. J. Dorrington, *J. Immunol.*, 1976, **116**, 510.
[448] W. F. Riesen, J. Schlessinger, and J. C. Jaton, *Biochemistry*, 1976, **15**, 3391.
[449] R. M. Freed, J. H. Rockey, and R. C. Davis, *Immunochemistry*, 1976, **13**, 509.

Three different anti-fluorescein combining sites have been investigated by following the induced c.d. of the hapten bound to autologous and heterologous recombinants. It was concluded that hydroxyxanthenone sites do not exist in isolated chains and the environment of subsites is due to the heavy chains.[450]

Calculations have been carried out on the interactions of trinitrophenyl hapten with tryptophan as a model for study of hapten–antibody complex formation.[351]

Lipoproteins.—The conformational stability of the polypeptide components of human high-density serum lipoprotein towards guanidine hydrochloride has been investigated by following changes in c.d. at 222 nm. It was found that the lipid-free protein denatures at low concentrations of guanidine hydrochloride but possesses a much higher stability when incorporated into membranes.[451] A number of low-density lipoproteins and human low-density lipoproteins have been compared. Apo low-density L and apo low-density L_2 were found to be indistinguishable, whereas pig apo low-density had similar c.d. to human apo low-density L_1.[452]

Protein–lipid complex formation between bovine serum albumin and phosphatidylcholine has been characterized.[453]

5 Magnetic Circular Dichroism

Contributed by T. Brittain

As magnetic circular dichroism (m.c.d.) is sensitive to the chemical constitution of the molecules under study but is relatively insensitive to conformational variations of the surrounding protein, it finds its application in different fields from 'conventional' c.d., even though both techniques measure the interaction between polarized light and asymmetric centres. The main growth area in this field during the past year has been the study of haemoproteins. The spectral region explored has been extended to 180 nm, and variable temperature measurements at <10 K have been made.

Theory and Analysis.—Theoretical discussions have appeared of the phenomena responsible for the production of m.c.d. spectra, which treat the subject in varying depths. A basic review has appeared [454] together with a fuller treatise which presents dispersion calculations, moments, and vibrationally induced transitions.[455] A series of theoretical and technical papers has been reported.[456–458] The relationship between multiple Hamiltonians and m.c.d. has been investigated. Power–Zienau–Wooley transformations in molecular quantized electrodynamics for molecules in the presence of strong fields are given. The Einstein B coefficient for the absorption of left and right circularly polarized light was obtained.

[450] J. R. Gollogly and R. E. Cathou, *J. Immunol.*, 1976, **117**, 180.
[451] J. A. Reynolds, *J. Biol. Chem.*, 1976, **251**, 6013.
[452] R. L. Jackson, O. D. Taunton, R. Segura, J. G. Gallagher, H. F. Hoff, and A. M. Antonio, *Comp. Biochem. Physiol.* (B), 1976, **53**, 245.
[453] A. Jones, *Biochim. Biophys. Acta*, 1976, **427**, 325.
[454] W. Haberditzel and M. Von Loewis, *Ber. Bunsengesellschaft Phys. Chem.*, 1976, **80**, 191.
[455] P. J. Stephens, *Adv. Chem. Phys.*, 1976, **35**, 197.
[456] P. J. Stephens, *N.A.T.O. Adv. Study Inst. Ser. C*, 1975, 95.
[457] B. Briat, *N.A.T.O. Adv. Study Inst. Ser. C*, 1975, 141.
[458] R. G. Denning, *N.A.T.O. Adv. Study Inst. Ser. C*, 1975, 157.

Calculations were extended beyond the electric dipole approximation so as to include the contribution of natural dichroism, and the use of the appropriate Kramers–Kronig dispersion relationships was discussed.[459]

A comprehensive parameterization and computational program for the analysis of m.c.d. and absorption data has been published.[460] The mechanisms whereby spin–orbit coupling may arise in excited states have been studied. Experimental data from the Soret region of ferricytochrome *c* and deoxyhaemoglobin in the range 80—300 K have led to the suggestion that both spectra arise from the sum of two close-lying *C* terms of opposite sign. However, it would seem that a different mechanism of spin–orbit coupling is operative in each case.[461]

The fluorescence detected m.c.d. (FDMCD) of tryptophan has been analysed and, with the aid of added molecules, so has that of ferricytochrome *c*. Both spectra agree with transmission data. This mode of analysis has potential for use with optically dense material or macromolecules in their native environments.[462]

Models and Proteins.—*Models.* The vibrational fine structure seen in the m.c.d. of porphyrins and metalloporphyrins in the region of the vibrational satellites of the *β*- and *B*-absorption bands is sensitive to axial ligands, the substitution pattern of the porphyrins, and the oxidation state of the metal present.[463] The m.c.d. of both high- and low-spin ferriporphyrins has been analysed and these may form a good theoretical background for studies on haem proteins.[464, 465] Haemin exhibits similar spectra in the *Q* and Soret regions in both ethylene glycol and alkaline aqueous solution, showing that no fundamental differences exist between monomeric and dimeric high-spin forms. Addition of pyridine to haemin in aqueous solution yields a high-spin complex whereas in ethylene glycol this treatment produces a high to low spin transition. Haem in ethylene glycol shows a spectrum similar to that of deoxymyoglobin. Haem–haem interactions have been identified in aggregates or dimeric high-spin forms.[466]

Model studies have been performed in order to investigate the nature of the axial ligands of cytochromes P450, P440, and P420. The results obtained for haem isocyanide complexes in aqueous media were not consistent with a ferro-ethyl isocyanide model for P440.[467]

Ferrous porphyrin complexes with sodium methyl mercaptide and carbon monoxide in benzene yield spectra identical to P450, whilst models with propyl mercaptan or *N*-methylimidazole are identical with P420.[468] This, together with other model studies, has led to the suggestion that the fifth ligand of P450 is probably cysteine.

[459] W. P. Healy, *J. Chem. Phys.*, 1976, **64**, 3111.
[460] D. J. Caldwell and H. Eyring, *J. Chem. Phys.*, 1976, **64**, 915.
[461] M. A. Livshits, A. M. Arutyunyan, and Y. A. Sharonov, *J. Chem. Phys.*, 1976, **64**, 1276.
[462] J. C. Sutherland, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 276.
[463] Y. A. Sharonov and A. M. Arutyunyan, *Tezisy. Dokl.-Vses. Konf. Spectrosk. Biopolim.*, 1974, 109.
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[465] H. Kobayashi, T. Higuchi, and K. Eguchi, *Bull. Chem. Soc. Japan*, 1976, **49**, 457.
[466] T. Shimizu, T. Nozawa, and M. Hatano, *Bioinorganic Chem.*, 1976, **6**, 119.
[467] T. Shimizu, T. Nozawa, and M. Hatano, *Bioinorganic Chem.*, 1976, **6**, 1.
[468] J. P. Collman, T. N. Sorrell, J. H. Dawson, J. R. Trudell, E. Bunnenberg, and C. Djerassi, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 6.

Investigations of the m.c.d. of Co^{3+}-mixed amino-acid complexes of the form $Co(ox)_x(Gly)_y(en)_z$ have shown the signal intensity to be dependent upon the co-ordination pattern prevalent.[469] The spectra of Co^{2+} and Ni^{2+} complexes of concanavalin A confirm the presence of six tryptophans per dimer of the protein and lead to the conclusion that the co-ordination sphere relative to the fixation site of the metal cations is octahedral.[470]

Proteins. Evidence to support the proposition that chloroperoxidase has a similar haem to P450 has been reported. In fact chloroperoxidase has an identical spectrum to P450 in the high-spin oxidized and reduced CO states.[471] The ferri haem-cyanide and ferro haem-CO complexes of horseradish peroxidase have very similar spectra to the corresponding myoglobin derivatives. However, since the alkaline form spectrum is completely different from that of myoglobin-OH it was concluded that the m.c.d. is sensitive to the spin state of the haem iron rather than the structure of the porphyrin moiety.[472]

At room temperature oxidized cytochrome *c* has a Soret spectrum typical of haemichromes such as low spin ferri myoglobin. At extreme pH values the m.c.d. is consistent with a change of spin state. The visible region is dependent on co-ordination but not on the specific ligand present. The reduced cytochromes *c* and b_5 show similar visible region m.c.d., unlike the Soret regions. Variable-temperature studies reveal the presence of *C* terms in the oxidized spectrum.[473]

A study of oxidized and substrate-bound P450 in association with model compounds has suggested the presence of a cysteine ligand. In the light of this suggestion the ability of the protein to transfer electrons to oxygen which allows its hydroxylation activity has been discussed.[474]

Various reports on myoglobin and its derivatives have appeared during the past year. Ethyl, n-propyl, and isopropyl mercaptan complexes of met-myoglobin have similar spectra to P450. Cysteine and cystine ligate the protein at pH 9.18 but not at pH 6.86 or pH 11.65, to give similar spectra to P450. Addition of 2-mercaptoethanol at pH 6.86 reduces myoglobin and sodium sulphide yields on addition a typical thiol spectrum at pH 6.86, but the protein is gradually reduced.[475] Two similar studies on myoglobin complexes, designed to characterize haem spin states, have appeared. The former studied the complexes in the region 300—650 nm down to −196 °C, and for all paramagnetic derivatives *C* terms were observed. For ferric derivatives (CN, imidazole, N_3, OH, H_2O, F) the intensity of the Soret band m.c.d. was found to correlate with the percentage low spin present. In the visible region the ferric derivatives showed the presence of weak *A* and *C* terms associated with porphyrin π–π^* transitions and porphyrin-to-metal charge-transfer transitions, the shape of the bands being

[469] N. Matsuka and Y. Shimura, *Bull. Chem. Soc. Japan*, 1976, **49**, 2118.
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[471] J. H. Dawson, J. R. Trudell, G. Barth, R. E. Linder, E. Bunnenberg, C. Djerassi, R. Chiang, and L. P. Hager, *J. Amer. Chem. Soc.*, 1976, **98**, 3709.
[472] T. Nozawa, N. Kobayashi, and M. Hatano, *Biochim. Biophys. Acta*, 1976, **427**, 652.
[473] L. E. Vickery, T. Nozawa, and K. Sauer, *J. Amer. Chem. Soc.*, 1976, **98**, 351.
[474] J. H. Dawson, R. H. Holm, J. R. Trudell, G. Barth, R. E. Linder, E. Bunnenberg, C. Djerassi, and S. C. Tang, *J. Amer. Chem. Soc.*, 1976, **98**, 3707.
[475] T. Shimizu, T. Nozawa, and M. Hatano, *Biochim. Biophys. Acta*, 1976, **434**, 126.

dependent on the sixth ligand. Previously unresolved charge-transfer bands were detected in the region 440—500 nm. For ferro high-spin, hydrated, and deoxy forms, *C* terms were evident in the Soret region and *A* and *C* terms in the visible region, whilst only low-spin diamagnetic derivatives showed *A* terms typical of other metalloporphyrins.[476] The second study, which extended these results down to 20 K, showed that at <100 K the oxy form of myoglobin contains between 10 and 20% ferrous high-spin haem.[477] Ferric high-spin myoglobin, when examined out to 1800 nm, shows the presence of positive *A* terms assigned to charge transfer to degenerate excited states $[e_g(d_\pi)]$. The *A* term is estimated at 80% for the fluoride derivative and 35% for the hydrated derivative, with the consequent suggestion that the symmetry of the fluoride derivative is D_{4h}, whilst the hydrated derivative possesses a lower symmetry. Ferric low-spin (CN, N_3, imidazole) complexes show positive *B* terms arising from charge transfer from porphyrin π to iron *d*. Deoxymyoglobin and ferrous high-spin forms exhibit positive terms at 760 nm and small bands in the range 800—1800 nm.[478]

For the systems of high- and low-spin ferro- and ferri-haemoglobin with non-zero spin the m.c.d. contains *Q* and *B* absorption bands which exhibit *C*-type temperature dependence. As the ground states of these proteins are non-degenerate the *C* terms must arise from spin–orbit coupling.[479] The m.c.d. of deoxy- and met-haemoglobin is found to be dependent on quaternary structure with isolated α,β monomeric haemoglobin and haemoglobin in the *R* states showing the presence of two equal intensity minima. The tetramer shows these peaks with equal intensity. Using these data the equilibrium processes in deoxy-desArg-*N*-ethylsuccinimide haemoglobin have been probed, but it was found that no such correlations exist for the polymeric states of met-haemoglobin.[480]

Mammalian cytochrome *c* oxidase has been investigated with respect to the haem spin states under different conditions. Cytochrome oxidase in the oxidized form was found to show *C* terms arising from low-spin haem, whereas the reduced protein showed the presence of both low- and high-spin haems.[481] A model for the active centre of the oxidase has been proposed in which high-spin haem a_3 is antiferromagnetically coupled to Cu^{2+}. Reduction is viewed as being accompanied by a conformational change which makes a_3 accessible to ligands.[482] In a second study measurements were made at temperatures down to 15 K with the conclusion that in the reduced enzyme at least one haem is high spin, whilst in the oxidized state a_3 is predominantly high spin and *a* is low spin. This study has also highlighted the possible pitfalls in the use of model compounds to spin states.[483] Variable-temperature studies on the mixed valence carbon monoxide complex of cytochrome oxidase indicate at room temperature a low-spin ferrous haem and a low-spin ferric haem. Below 100 K it appears that carbon monoxide

[476] L. E. Vickery, T. Nozawa, and K. Sauer, *J. Amer. Chem. Soc.*, 1976, **98**, 343.
[477] J. Springall, M. J. Stillman, and A. J. Thomson, *Biochim. Biophys. Acta*, 1976, **453**, 494.
[478] T. Nozawa, T. Yamamato, and M. Hatano, *Biochim. Biophys. Acta*, 1976, **427**, 28.
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[480] Y. Sharanov and B. Atanasov, *Biochim. Biophys. Acta*, 1976, **434**, 440.
[481] G. T. Babcock, L. E. Vickery, and G. Palmer, *Biophys. J.*, 1976, **16**, 87.
[482] G. Palmer, G. T. Babcock, and L. E. Vickery, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2206.
[483] A. J. Thomson, T. Brittain, C. Greenwood, and J. Springall, *F.E.B.S. Letters*, 1976, **67**, 94.

may be photolysed irreversibly from the protein to give both haems in a high-spin form, hence the suggestion of haem–haem interactions even at very low temperatures.[484]

6 Fluorescence

Contributed by J. G. Hoggett

This section deals chiefly with the literature published during 1976; the gap since the subject was last reviewed in 1974 [485] is in part covered by sections of related reviews on fluorescence correlation spectroscopy,[486] fluorescent nucleosides and nucleotides,[487] fluorometric and phosphorometric analysis,[488] fluorescence lifetimes of biomolecules,[489] excited states of biomolecules,[490] and triplet states and photosynthesis,[491] which in general cover the period to the end of 1975. Coverage of immunofluorescence and membranes has been very selective, concentrating either on areas where protein structure is fairly well defined, or where techniques have been used which might find wider application in the study of protein structure. Articles or reports of meetings on related areas of fluorescence outside the scope of this review deal with membranes,[492] fluorometric enzyme assays,[493] and fluorescence-activated cell sorting.[494]

Books and Reviews—Two volumes of a book on biochemical fluorescence, contains many useful articles.[495] A more general two-volume set on fluorescence spectroscopy,[496] and books on molecular energy transfer,[497] excited states of biomolecules,[498] and exciplexes [499] have appeared. Nanosecond pulse fluorometry has been authoritatively reviewed.[500] Several articles have appeared on fluorescence correlation spectroscopy covering basic principles,[501] rotational diffusion of macromolecules,[502] and membranes;[503] de Maeyer has pointed out the danger that in fluorescence (but not light scattering) correlation spectroscopy

[484] T. Brittain, J. Springall, C. Greenwood, and A. J. Thomson, *Biochem. J.*, 1976, **159**, 811.
[485] G. R. Penzer, in this series, 1976, Vol. 7, p. 204.
[486] E. L. Elson, and W. W. Webb, *Ann. Rev. Biophys. Bioeng.*, 1975, **4**, 311.
[487] N. J. Leonard and G. L. Tolman, *Ann. New York Acad. Sci.*, 1975, **255**, 43.
[488] C. M. O'Donnell and T. N. Solie, *Analyt. Chem.*, 1976, **48**, 175R.
[489] L. F. Forster, *Photochem. and Photobiol.*, 1976, **23**, 445.
[490] R. D. Fugate and P. S. Song, *Photochem. and Photobiol.*, 1976, **24**, 629.
[491] J. R. Norris, *Photochem. and Photobiol.*, 1976, **23**, 449.
[492] A. Azzi, *Quart. Rev. Biophys.*, 1975, **8**, 237; P. A. G. Fortes, in 'Mitochondria, Bioenergetics, Biogenesis, and Membrane Structure', ed. L. Packer and A. Gomez-Puyon, Academic Press, New York, 1976, p. 327; A. Waggoner, in 'The Enzymes of Biological Membranes', ed. A. Martonosi, Plenum, New York and London, 1976, vol. 1.
[493] D. H. Leaback, *F.E.B.S. Letters*, 1976, **66**, 1.
[494] L. A. Herzenberg, R. G. Sweet, and L. A. Herzenberg, *Sci. American*, 1976, **234**, 108; E. Simpson, *Trends in Biol. Sci.*, 1976, **1**, N30.
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[496] E. L. Wehry, 'Modern Fluorescence Spectroscopy', Vol. 1 and 2, Plenum, New York, 1976.
[497] R. Levine and J. Jortner, 'Molecular Energy Transfer', Halstead press, 1976.
[498] J. B. Birks, 'Excited States of Biological Molecules', Wiley, London, 1976.
[499] M. Gordon and W. R. Ware, 'Proceedings of International Exciplex Conference 1974', Academic Press, London, 1975.
[500] P. Wahl, in 'New Techniques in Biophysics and Cell Biology', ed. R. H. Pain and B. J. Smith, Vol. 2, Wiley, London, p. 233.
[501] D. Magde, *Quart. Rev. Biophys.*, 1976, **9**, 35.
[502] M. Ehrenberg and R. Rigler, *Quart. Rev. Biophys.*, 1976, **9**, 69.
[503] W. W. Webb, *Quart. Rev. Biophys.*, 1976, **9**, 49.

the occurrence of irreversible dissipation processes such as heat conduction, convection, and photodecomposition might destroy the condition that the open system examined should remain at thermodynamic equilibrium.[504] In the same collection of articles, Eisinger discusses energy transfer and dynamic structure,[505] emphasizing the virtues of polarization spectroscopy for eliminating some of the uncertainties surrounding the value of the orientation factor $\langle\kappa^2\rangle$ adopted in calculations of intramolecular energy transfer. An account of fluorescence relaxation spectroscopy in the analysis of macromolecular structure and function concentrates on the example of ethidium bromide and $tRNA^{Phe}$.[506] Reviews on the acid–base properties of the electronically excited states of organic molecules,[507] ATP analogues (including a section on fluorescent analogues),[508] and double-beam fluorescence spectroscopy[509] have appeared.

Theory, Methods, and Techniques.—*Technical Developments.* The difficulty in having universally accepted values of the quantum yields of fluorescence standards such as quinine bisulphate was mentioned in an earlier review.[485] A similar uncertainty about the fluorescence properties of 9,10-diphenylanthracene (DPA), another commonly used emission quantum yield standard, appears to have been due to the effects of high concentration, and the fact that the correction factor used to allow for differences in the refractive index depends strongly on the geometry of the sample compartment. Quantum yields and lifetimes of DPA at 293 K in ethanol, 3-methylpentane, cyclohexane, and benzene have been redetermined.[510]

A new versatile instrument is capable of a variety of measurements useful in biophysical spectroscopy such as fluorescence detected c.d., linear dichroism, linearly polarized fluorescence, m.c.d., fluorescence-detected m.c.d. (FDMCD), and magnetic circular emission.[511] A differential spectrofluorimeter utilizing a common source, monochromator and photomultiplier has been described,[512] as have two simple methods for increasing the sensitivity of standard analogue fluorimeters involving voltage to frequency conversion and event counting,[513] and the use of concave retromirrors and rotation of the slit image of the monochromators by 90° to permit viewing along the length of the excitation slit image.[514] The use of repetitive scanning on a photon-counting instrument interfaced to a computer enables measurements to be made in the picomolar range,[515] whereas the use of pulsed lasers extends the range to the part per trillion level.[516]

A simple device incorporating a bifurcated fibre optic system is particularly suited to rapid routine or automated measurements.[517] Microspectro-

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[505] J. Eisinger, *Quart. Rev. Biophys.*, 1976, **9**, 21.
[506] R. Rigler and M. Ehrenberg, *Quart. Rev. Biophys.*, 1976, **9**, 1.
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[513] V. Glushko, R. Caley, and C. Karp, *Analyt. Chem.*, 1976, **48**, 2077.
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[517] D. G. Mitchell, J. S. Garden, and K. M. Aldous, *Analyt. Chem.*, 1976, **48**, 2275.

fluorimeters have been developed which are capable of quantitative measurements on nanolitre samples,[518] and simultaneous detection of the whole fluorescence spectrum in single cells using a multichannel analyser.[519] Measurement of the fluorescence polarization of phospholipid dispersions,[520] and the accurate and rapid (20 ms) measurements of fluorescence polarization using a single photomultiplier and 'chopped' optics have been described.[521] The use of a laser light source and fluorescence detection in the analytical ultracentrifuge results in a marked improvement of sensitivity and the ability to discriminate between species on the basis of their fluorescence characteristics.[522] Instrumental modifications for fluorescence lifetime measurements,[523] and the extension of the range to 90 ps [524] and 100 ps [525] have been reported. Picosecond laser pulses have been used to study the decay kinetics of chlorophyll [526] and the purple membrane protein of *Halobacterium halobium*.[527]

Proteins, nucleic acids, and fluorescein-conjugated antibodies have been identified *in situ* using the fluorescence excited by the focused electron beam of a scanning electron microscope.[528] Resolutions of 1500 and 900 Å for protein and nucleic acid, respectively, have been obtained using relatively low sensitivity; in the case of nucleic acid, expected improvements in sensitivity should increase this resolution to 60 Å. An account has appeared of the theoretical basis and some practical guidelines for using fluorescence photobleaching recovery to monitor the lateral mobility of fluorescent particles on the cell surface.[529] Microspectrofluorimetric techniques have been used to study glycolysis in single cells,[530] and turnover in enzyme systems immobilized or encapsulated in single Sepharose beads, which have certain analogies with intracellular reactions.[531]

The fluorescence resulting from the reaction of *o*-phthalaldehyde with protein and amino acids can be used for the detection of nanogram quantities of protein,[532] and for amino acid analysis [533] and peptide maps [534] with microgram quantities of protein. A method has been described in which the binding of a ligand to protein was studied using a non-fluorescent ligand–fluorphore conjugate (a fluorogenic conjugate), in which the ligand-fluorophore bond was susceptible to cleavage by another enzyme in the mix to yield free ligand and

[518] G. Rutili, K.-E. Arfors, and H. R. Ulfendahl, *Analyt. Biochem.*, 1976, **72**, 539.
[519] M. M. Jotz, J. E. Gill, and D. T. Davis, *J. Histochem. Cytochem.*, 1976, **24**, 91.
[520] C. L. Bashford, C. G. Morgan, and G. K. Radda, *Biochim. Biophys. Acta*, 1976, **426**, 157.
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fluorophore (now fluorescent).[535] The method depends on the bound fluorogenic reagent being protected from enzymatic cleavage. Although the fluorescence spectra of 1,N^6etheno-ATP, -ADP, and -AMP are very similar, their fluorescence intensities in the presence of bivalent metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} (but not Mg^{2+}) are different. Since these ions bind more strongly to the triphosphate than to the di- or mono-phosphate, the resulting quenching can be used in particularly simple assays of enzymes which accept the etheno-modified adenosine residues.[536]

Newer Methods. The detection of natural c.d. by fluorescence (FDCD) was reported two years ago.[485] Detailed accounts have appeared of the theory of FDCD and comparison with c.d.[537] Only transitions which after absorption of light lead to fluorescence, either directly or by energy transfer to a fluorophore, contribute to the FDCD. Comparison of the c.d., which is the average over all conformations, and FDCD, which is the average over fluorescent conformations, should be particularly valuable in studying the conformation of non-rigid molecules. It should be noted that if a chiral species is non-fluorescent, then addition of a non-chiral and non-interacting fluorophore detects the c.d. of the sample by acting simply as a reporter molecule. The method has been extended to FDMCD.[538] Whereas c.d. is sensitive to the conformation and environment of the absorbing molecule, m.c.d. is characteristic of the nature of the molecule and is relatively insensitive to environment. FDMCD of both fluorescent and non-fluorescent molecules (in the latter case examined in the presence of a non-interacting 'reporter' fluorophore) should prove useful in examining optically dense material, biological material in its natural environment, and systems showing inter- or intra-molecular energy transfer. The first example has been reported of circularly polarized emission (CPE) induced by an optically active solvent (α-phenylethylamine) with an achiral fluorescent molecule (fluorescein);[539] it was postulated that solute–solvent interactions preferentially stabilized one enantiomeric form of the fluorescein, yielding partial resolution.

The occurrence of triplet–singlet energy transfer in the α-chymotrypsin–proflavin complex has been demonstrated by optical detection of magnetic resonance (ODMR).[540] Although the rate constants for triplet–singlet transfer yield the same information about the spatial relationships of chromophores as those of singlet–singlet transfer, the principal advantage stems from the longer time-scale of the triplet process, making them simpler experimentally and potentially more accurate. However, the use of liquid helium temperatures in the ODMR measurements is an obstacle to the technique becoming widespread in biochemical investigations. The technique of paramagnetic fluorescence quenching has been used to study lipid–protein interactions in artificial and natural membranes;[541] the method depends on quenching of Trp or Tyr

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fluorescence by nitroxide-labelled lipids. It is easier to determine the difference in the lifetimes of two polarized components by direct measurement of their phase difference than by independent measurement of each. Differential polarized phase fluorometry, the technique which exploits this fact, has been used to study the motion of a fluorophore bound to a cell;[542] a further paper on the general theory and its application to small fluorophores in homogeneous solution has been promised.[542] Although double-stranded DNA is not fluorescent at room temperature, binding of polylysine to DNA induces a strong fluorescence (λ_{ex} 250 nm; λ_{em} 300 nm, shoulder 360 nm) which is proportional to the ratio of polylysine to DNA.[543] This observation opens interesting possibilities in the study of protein–nucleic acid interactions.

Fluorescence Lifetimes. It has long been recognized that nanosecond pulse fluorometry has many advantages over steady-state measurements in investigating excited-state reactions. The fluorescence of 2-hydroxy-1-naphthalene acetic acid involves two species in the ground state but, as a result of proton transfer, at least three excited state species.[544] The difficulty with fluorescence lifetime experiments is that very accurate data are required especially if decay does not follow a simple mono-exponential time course. The problem caused by the wavelength dependence of the instrumental response-function in deconvoluting the data[545] has received renewed attention.[546] The use of the Laplace transformation[547] and moment index displacement[548] to analyse fluorescence decay curves, and a non-linear regression algorithm to obtain deconvoluted time-resolved emission spectra[549] have been discussed. Fluorescence lifetime standards have been used to determine the lower limit of measurable lifetimes[550] and as a means of characterizing systematic errors in the instrumental system.[551] A very readable account of the use of the time-correlated single-photon technique in determining fluorescence lifetimes has appeared.[552]

Energy Transfer. The difficulties in the way of a proper analysis of intramolecular fluorescence energy transfer to obtain the separation (R) of a donor (D) and acceptor (A) using the Förster theory (discussed in an earlier report[485]) have been further expounded by Dale and Eisinger.[505, 553] They point out that in the dynamic limit, in which the motions of D and A are fast with respect to the rate of energy transfer, the transfer efficiency for each molecule i is the same

$$\langle T_i \rangle_d = \langle T \rangle_d = \frac{\langle \kappa_i^2 \rangle}{C^{-1}R^6 + \langle \kappa_i^2 \rangle}$$

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and that if limits can be set to the orientation factor $\langle\kappa_i^2\rangle$ (for example, by the use of polarized emission spectroscopy)[505] values of R can be calculated (within the limits set for $\langle\kappa_i^2\rangle$). However, in the static case in which D and A do not re-orient during energy transfer, the transfer efficiency varies from molecule to molecule. For the whole ensemble of molecules the averaged transfer is given by

$$\langle T_i\rangle_s = \left\langle \frac{\kappa_i^2}{C^{-1}R^6 + \kappa_i^2} \right\rangle$$

and it is not possible to obtain $\langle T_i\rangle_s$ directly in terms of $\langle\kappa_i^2\rangle$ and R. It is emphasized that the static limit does not lend itself readily to determinations of distance in intramolecular systems except in the limit of low transfer efficiencies, and that cases intermediate between the static and dynamic limits are virtually impossible to analyse. Theoretical calculations of the conformation of a segment of the polypeptide chain with the sequence Tyr-Tyr based on semi-empirical potential functions suggest that for Tyr and all luminophores having a comparable number of degrees of freedom (*e.g.* Trp) in a long polypeptide chain in the randomly coiled state, the average orientation factor will be close to $\frac{2}{3}$.[554] However, in molecules with a rigid backbone the average value of κ^2 and its distribution will depend on the conformation. An alternative approach to the problem of the orientation factor $\langle\kappa^2\rangle$ has been provided by Hillel and Wu, who described a statistical method for systems consisting of a single acceptor and single donor, or a single acceptor and multi-donor.[555] The method enables calculation of the probability that a specified distance range corresponds to the actual distance of separation, based on the assumption that all independent parameters which describe the geometry of the system have equal probabilities of assuming any particular value.

Circularly polarized emission of Tb^{III} and energy transfer from Trp (and to a lesser extent from Tyr and Phe) to Tb^{III} have been observed in a series of 40 proteins in which Tb^{III} was used to replace calcium.[556] Radiationless energy transfer between two metal ions in a protein has been reported for the first time.[557] The procedure was tested using thermolysin in which zinc was replaced by Co^{II} and a single calcium by Tb^{III}; the value of R_0 for this donor–acceptor pair was found to be 19.6 Å, and the separation of the two probes to be 13.7 Å, identical with the value determined crystallographically. The main advantages of this type of distance probe over organic donor–acceptor pairs are that the metal ions are held rigidly in specific enzyme sites, and that since both the donor and acceptor energy levels possess degeneracies or near degeneracies a value of $\frac{2}{3}$ can be used for the orientation factor $\langle\kappa^2\rangle$. The possibility of extending this approach to systems containing natively only one metal site by means of covalent modification of specific amino-acids with suitable edta derivatives is also mentioned.[557] The use of triplet–triplet energy transfer to measure intramolecular distances in enzyme inhibitor complexes (at 77 K) has been described.[558]

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Fluorescence Probes.—*Covalent Labels.* A selection of some of the applications of covalent probes is collected in Table 1. The synthesis or use of several new probes, some of them variations on an old theme, has been reported. Dansyl fluoride is more selective in labelling serine proteases than is the chloride, attack

Table 1 *Covalent probes and their applications**

Probe	*Application*	*Ref.*
1-Anilinonaphthyl-4-maleimide	actin, troponin, tropomyosin (A)	*a*
	membrane proteins (P)	*b*
	pyruvate dehydrogenase complex	*c*
8-Azido-1,N^6-ethenoadenosine 3′,5′-cyclic monophosphate	photosensitive label (N)	560
4-Chloro-7-nitrobenzofuran	mitochondrial ATPase	*d*
N-Dansylaziridine	membrane proteins	*b*
Dansyl chloride	F-actin (P)	569
	aspartate aminotransferase and glutamate dehydrogenase (A,P)	*e*
	α-chymotrypsin and trypsin inhibitor (A,E)	575, 578—580
	lysozyme, lactalbumin (P)	*f*
	pepsin	*g*
	porcine lutotrophin	*h*
Dansyl fluoride	serine proteases (N)	559
Dansylcysteine-Hg	thiol reagent	*i*
9-(4-Diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine (quinacrine)	new probe	565
Eosin isothiocyanate	sucrase-isomaltase complex (P,T)	574
Fluorescamine	antibody labelling	563
	lysozyme, lactalbumin (P)	*f*
	malate dehydrogenase (A,P)	577
	membrane proteins	*j*
Fluorescein	antigen–antibody assay (A,E)	576
	aspartate aminotransferase and glutamate dehydrogenase (A,P)	*k*
	α-chymotrypsin and trypsin with inhibitor (A,E)	575
	malate dehydrogenase (A,E,P)	577
Guanosine analogues	photosensitive labels (N)	561
5-[2-(Iodoacetamido) ethylamino]-1-naphthalene and its 6-isomer	α-chymotrypsin and trypsin inhibitor (A,E)	578—580
	myosin subfragment 1 and actin (A,P)	572
	phosphorylase B (L)	548
	RNA polymerase (E,L)	568
4-Iodoacetamidosalicylic acid	phosphorylases A and B (L)	566
S-Mercuric-*N*-dansylcysteine	troponin (A,L)	*l*
2-Methoxy-2,4-diphenyl-3(2*H*)-furanone	gel electrophoresis	*m*
	antibody labelling	563
Methyl-3-(5-dimethylamino-1-naphthalenesulphamido) propionimidate hydrochloride	α-chymotrypsin and trypsin inhibitor (A,E)	578—580
Substituted *N*-naphthylmaleimides	new probes	*n*

Table 1 (*cont.*)

Probe	*Application*	*Ref.*
N-(1-Pyrene)maleimide	fluorescence cross-linking probe	562
	RNA polymerase (A)	*o*
	σ-factor of RNA polymerase (A)	*p*
N-(3-Pyrene)maleimide	F-actin (P)	570
	actin, troponin, tropomyosin (A)	*a*
	ribosomes (*E. coli*) (P,T)	573
Pyridoxal derivatives	aspartate transaminase (A,E)	581
Rhodamine B	antigen–antibody assay (A,E)	576
	malate dehydrogenase (A,E)	577

* Some applications are characterized into the following classes: (A) protein association, (E) energy transfer, (L) ligand binding, (N) new probe, (P) polarization methods, (T) triplet probe.

(*a*) T. Ohyashiki, Y. Kanaoka, and T. Sekine, *Biochim. Biophys. Acta*, 1976, **420**, 27. (*b*) H. Borochov and M. Shinitzky, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4526. (*c*) H. J. Grande, H. J. van Telgen, and C. Veeger, *European J. Biochem.*, 1976, **71**, 509. (*d*) S. J. Ferguson, W. J. Lloyd, G. K. Radda, and E. C. Slater, *Biochim. Biophys. Acta*, 1976, **430**, 189. (*e*) J. E. Churchich and Y. H. Lee, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 409. (*f*) L. H. Tang, Y. Kubola, and R. F. Steiner, *Biophys. Chem.*, 1976, **4**, 203. (*g*) B. O. Glotov, L. V. Kozlov, and L. L. Zavado, *Mol. Biol.* (*Russ.*), 1976, **10**, 231. (*h*) Y. Combarnous and G. Henne, *Internat. J. Peptide Protein Res.*, 1976, **8**, 491. (*i*) W. E. Harris and W. L. Stahl, *Biochim. Biophys. Acta*, 1976, **426**, 325. (*j*) S. P. Hawkes, T. D. Meehan, and M. J. Bessel, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 1226. (*k*) C. Salerno, J. E. Churchich, and P. M. Fasella, *Ital. J. Biochem.*, 1975, **24**, 351. (*l*) J. D. Potter, J. C. Seidel, P. Leavis, S. S. Lehrer, and J. Gergely, *J. Biol. Chem.*, 1976, **251**, 7551. (*m*) B. O. Barger, F. C. White, J. L. Pace, D. L. Kemper, and W. L. Ragland, *Analyt. Biochem.*, 1976, **70**, 327. (*n*) M. Machida, M. Bando, Y. Migita, M. I. Machida, and Y. Kanaoka, *Chem. and Pharm. Bull.* (*Japan*), 1976, **24**, 3045. (*o*) Z. Hillel and C. W. Wu, *Biophys. J.*, 1976, **16**, 15a. (*p*) F. Y. H. Wu, L. R. Yarborough, and C. W. Wu, *Biochemistry*, 1976, **15**, 3254; *Biophys. J.*, 1976, **16**, 15a.

being almost exclusively confined to the active serine residue.[559] The synthesis of 8-azido-1,N^6-ethenoadenosine 3′,5′-cyclic monophosphate, which combines the photosensitivity of the azido group with the fluorescent properties of the etheno-adenosine residue, has been described,[560] as have been the syntheses of a series of fluorescent analogues of guanosine containing a tetrazole ring, some of which are in equilibrium with their azido forms rendering them also susceptible to photolysis.[561] Conjugation of *N*-(1-pyrene)maleimide to thiol groups occurs rapidly and is accompanied by an increase in fluorescence intensity; if primary amino-groups are in the vicinity of the thiol group a further slow red-shift in the fluorescence occurs due to aminolysis of the succinimido ring of the first formed adduct.[562] This reagent should prove useful as a spectroscopic cross-linking probe of the spatial proximity of thiol and amino-groups. Fluorescamine (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione) and 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF) offer certain advantages in antibody labelling over more commonly used groups.[563] Sodium dodecylsulphate (SDS) strongly inhibits the labelling of proteins by fluorescamine, suggesting that the fluorophore cannot penetrate the highly charged envelopes of the SDS–protein complexes.[564]

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Proteins are readily labelled with quinacrine [9-(4-diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine] yielding conjugates with useful fluorescence properties, but with the disadvantage (shared with fluorescein isothiocyanate) of having an ionizable group in the neutral pH range leading to a non-uniform fluorophore population.[565]

Several studies of the binding of nucleotides to covalently labelled phosphorylase have been reported;[566, 567] in the binding of AMP to phosphorylase B,[567] comparison of the binding function with the conformational state function (revealed by fluorescence) suggests that the observed positive co-operativity follows a mechanism intermediate between that of the concerted and sequential mechanisms. Energy-transfer experiments (analysed statistically *q.v.*) indicate that the rifampicin binding site in *E. coli* RNA polymerase is between 42 and 85 Å (with a $\leqslant 5\%$ probability that $R > 72$ Å) distant from a single 1,5-IAENS group attached to the σ-subunit, implying that rifampicin does not inhibit initiation of transcription by direct interaction with the σ-subunit.[568]

The use of fluorescence polarization to investigate rotational diffusion and molecular mobility is a standard method, tending in the past few years very much towards transient rather than steady-state techniques. Nanosecond pulse fluorometry in polarized light of F-actin with dansyl [569] or N-(3-pyrene) maleimide [570] conjugated to the unique Cys-373 adjacent to the *C*-terminal end of the actin monomer yields correlation times in good agreement with one another, but much smaller than had previously been observed with $1,N^6$-etheno-ADP bound to F-actin.[571] The suggestion is that this is due to the flexibility of the *C*-terminal end of the chain which is on or near the surface of actin. The decay of fluorescence anisotropy has been used to study the association of actin and myosin sub-fragment I (S-I) labelled with *N*-iodoacetyl-*N'*-1-sulpho-5-naphthyl)ethylenediamine (1,5-IAEDANS);[572] the method depends on the large difference in rotational mobility of S-I (mol. wt. 10^5) and its complex with actin (mol. wt. 10^6—10^7). The long correlation times of very large macromolecules, used to such advantage in the previous example, effectively prevents the use of fluorescence methods in studying the motion of large particles such as viruses, or smaller proteins in a relatively viscous environment such as a membrane. The use of triplet-state probes extends measurable correlation times into the micro- to milli-second range. The decay of the triplet–triplet absorption dichroism of *E. coli* ribosomes labelled with *N*-(3-pyrene) maleimide appears to be mono-exponential with a time constant of 4.4 μs, compatible with the rotation of a spherical particle of radius 147 Å;[573] unlike fluorescence, decay of triplet–triplet dichroism cannot easily be followed for long enough to reveal deviations

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from simple exponential behaviour. The preparation and characterization of the triplet probe eosin thiocyanate have been described, and applied to a study of the sucrase–isomaltase complex from the small intestine.[574]

Singlet–singlet energy transfer has been used in a variety of ways to study interactions between proteins (Table 1).[575–580] Applications include a simple immunochemical assay of antigen–antibody interactions,[576] attempts to map the topography of the complexes formed between trypsin and chymotrypsin with black-eyed pea inhibitor,[575] and studies of the kinetics and binding constants of association–dissociation reactions;[578–580] the particular virtue of the last method is that protein concentrations as low as 10^{-9} mol dm^{-3} can be used, allowing direct measurement of strong binding processes. Pyridoxal fluorescence has been used in a novel study of the dimeric enzyme aspartate transaminase.[581] Energy transfer was measured within a hybrid enzyme, in which one of the subunits was linked covalently to a pyridoxal residue at the active site, and the other was similarly linked to a reduced pyridoxal. Adopting a value for $\langle\kappa^2\rangle$ of 0.475, considered to be the average value for an ensemble of random and fixed orientations, yielded a separation of 18 Å, indicating that the two active sites were not in contact.

Non-covalent Probes. Table 2 summarizes some of the studies involving non-covalent probes; for discussion they can usefully be divided according to whether or not they show some biological specificity.

Table 2 *Non-covalent fluorescent probes and their applications*

Probe	*Application*	*Ref.*
7-Substituted 1,N^2-(allylidene) guanines	synthesis	591
ANS	chymotrypsinogen and chymotrypsin	*a*
	crystal structure	601
	elastin	605
	fluorescence and c.d. probe	603
	insulin des-pentapeptide (B26—30)	*b*
	lactoferrin and transferrin	*c*
	lysozyme, α-lactalbumin	*d*
	monoamine oxidase	*e*
	phosphoenolpyruvate carboxylase	*f*
	pyruvate dehydrogenase	*g*
	ribosome association	*h*
	ribulose-1,5-bisphosphate carboxylase	*i*
	yeast enolase	*j*
ANS and other probes	chloroplast coupling factor 1	*k*
Anthroyloubain	$(Na^+ + K^+)$ ATPase	595

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Table 2 (*cont.*)

Probe	*Application*	*Ref.*
N-Arylaminonaphthalene sulphonates	time-resolved emission spectroscopy	603
Auramine O	liver alcohol dehydrogenase	*l*
lin-Benzadenosine derivatives	synthesis, and application to a variety of kinases	590
2-*p*-Bromo- and 2-*p*-chloro-anilino-naphthalene sulphonates	membranes	*m*
Chlorpromazin	monoamine oxidase	*n*
CTP analogues	synthesis	*o*
Dansylacylcholines	acetylcholine receptor	599
Dansylcadaverine	membranes	600
Dansyl-Gly-Gly-L-Phe-L-Phe-3-(4-pyridyl)propyl ester	swine pepsin	*q*
ε-Dansyllysine	antibody–hapten binding	592, 593
Dansylphosphatidylethanolamine	protein–lipid interactions	*p*
Dansylsulphonamide	carbonic anhydrase	*r*
1,N^6-ethenoATP	G-actin	*s*
1,N^6-ethenoATP (Mn^{II})	actin	585
1,N^6-ethenoADP	F-actin	*t*
	myosin subfragment 1, heavy meromyosin	*u*
1,N^6-etheno-AMP and -2′-dAMP	phosphorylase B	*v*
1,N^6-etheno-3′,5′-cyclic AMP	protein kinase	584
1,N^6-etheno-CoA	pyruvate dehydrogenase	587
1,N^6-etheno-NADP and -NADPH	dihydrofolate reductase	*w*
3,N^4-ethenocytosine	acid–base properties	583
	crystal structure	582
Ethidium bromide	acetylcholine receptor	597
FAD	pyruvate dehydrogenase	*g*
	NADPH/cytochrome *c* reductase	*x*
Flavinyl peptides	monoamine oxidase	*y*
FMN	apoflavodoxin	*z*
	NADPH/cytochrome *c* reductase	*x*
Fluorescein	circularly polarized emission	539
Fluorescein-labelled hyaluronic acid	synthesis and characterization	*aa*
Formycin TP	(Na^+ + K^+)ATPase	589
Harmaline, harmine	(Na^+ + K^+)ATPase	596
2-Hydroxy-1-naphthalene acetic acid	acid–base properties	544
N-(Iodoacetylaminoethyl)-5-naphthylamine-8-sulphonic acid linked to S^6-GTP (S^6-GTP-AEDANS)	glutamate dehydrogenase and RNA polymerase	*bb*
6-(*N*-Methylanilino)-2-naphthalene-sulphonyl peptides	papain	*cc*
6-(*N*-Methylanilino)-2-naphthalene-sulphonyl BSA	antibody–hapten binding	594
NADH	liver alcohol dehydrogenase	*dd*
NADPH	NADPH-cytochrome *c* reductase	*x*
2′(3′)-*O*-L-Phenylalanyl-1′,N^6-etheno-ATP and -CTP	ribosomes	586
Quinacridine	acetylcholine receptor	*ee*
Retinol and related compounds	retinol binding proteins	*ff*
Terbium(III)	acetylcholine receptor	598
	thermolysin	557
2′,4′,5′,7′-Tetraiodofluorescein	aspartate transcarbamylase	*gg*

Table 2 (*cont.*)

Probe	*Application*	*Ref.*
Tetramethylrhodamine labelled α-bungarotoxin	acetylcholine receptor	*hh*
TNS	cyclodextrins	607
2′ (or 3′)-*O*-(2,4,6-trinitrophenyl)-adenosine derivatives	heavy meromyosin ATPase	*ii*

(*a*) J. D. Johnson and M. A. El-Bayoumi, *Biophys. J.*, 1976, **16**, 203a. (*b*) The Conformational Study Group, *Scientia Sinica*, 1976, **19**, 486. (*c*) N. Bilić, M. Casey, and B. Blanc, *Biochem. J.*, 1976, **157**, 233. (*d*) V. Versee and A. O. Barel, *Bull. Soc. chim. belges*, 1976, **85**, 585. (*e*) J. B. Massey and J. E. Churchich, *Biophys. J.*, 1976, **16**, 165a. (*f*) T. Yoshinaya, *Biochim. Biophys. Acta*, 1976, **452**, 566. (*g*) G. B. Shepherd and G. G. Hammes, *Biochemistry*, 1976, **15**, 311. (*h*) V. Favaudon and F. Pochon, *ibid.*, p. 3903. (*i*) G. F. Wildner, *Z. Naturforsch.*, 1976, **31C**, 267. (*j*) J. M. Brewer, *European J. Biochem.*, 1976, **71**, 425. (*k*) L. C. Cantley and G. C. Hammes, *Biochemistry*, 1976, **15**, 1; *ibid.*, p. 9. (*l*) P. M. Bronskill and J. T.-F. Wong, *Canad. J. Biochem.*, 1976, **54**, 287. (*m*) I. Tasaki, A. Warashima, and H. Pant, *Biophys. Chem.*, 1976, **4**, 1. (*n*) J. B. Massey and J. B. Churchich, *Biochim. Biophys. Acta*, 1977, **480**, 70. (*o*)N. K. Kochetkov, V. N. Shibaer, A. A. Kost, A. P. Razjivin, and A. Y. Borisov, *Nucleic Acids Res.*, 1976, **5**, 1341. (*p*) J. F. Faucon, J. Dufourcq, C. Lussan, and R. Bernon, *Biochim. Biophys. Acta*, 1976, **436**, 283. (*q*) P. G. Richmann and J. S. Fruton, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3915. (*r*) P. Johnson and R. P. McIntosh, *Biochim. Biophys. Acta*, 1976, **453**, 521. (*s*) S. C. Harvey and H. C. Cheung, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 865; F. Waechter and J. Engel, *Experientia*, 1976, **32**, 814. (*t*) M. Miki, T. Kouyama, and K. Mihashi, *F.E.B.S. Letters*, 1976, **66**, 98. (*u*) F. Garland and H. C. Cheung, *ibid.*, p. 198; *Biophys. J.*, 1976, **16**, 46a. (*v*) B. Vandenbunder, M. Morange, and H. Buc, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2696. (*w*) V. G. Neef and F. M. Huennekens, *Biochemistry*, 1976, **15**, 4042. (*x*) G. E. Trout, *European J. Biochem.*, 1976, **71**, 533. (*y*) M. C. Falk and D. B. McCormick, *Biochemistry*, 1976, **15**, 646. (*z*) R. Gast, B. E. Valk, F. Müller, S. G. Mayhew, and C. Veeger, *Biochim. Biophys. Acta*, 1976, **446**, 463. (*aa*) A. N. deBelder and K. O. Wik, *Carbohydrate Res.*, 1975, **44**, 251. (*bb*) P. Faerber and W. Vizethum, *F.E.B.S. Letters*, 1976, **61**, 267; *J. Carbohydrate Nucleosides Nucleotides*, 1976, **3**, 15. (*cc*) J. A. Mattis and J. S. Fruton, *Biochemistry*, 1976, **15**, 2191. (*dd*) A. Gafni and L. Brand, *ibid.*, p. 3165. (*ee*) H. H. Grünhagen and J. P. Changeux, *J. Mol. Biol.*, 1976, **106**, 497. (*ff*) U. Cogan, M. Kopelman, S. Mokady, and M. Shinitzky, *European J. Biochem.*, 1976, **65**, 71; M. Kopelmann, U. Cogan, S. Mokady, and M. Shinitzky, *Biochim. Biophys. Acta*, 1976, **439**, 449. (*gg*) P. C. Keck, T. M. Schuster, and C. H. McMurray, *Biophys. J.*, 1976, **16**, 203a. (*hh*) D. Axelrod, P. Ravdin, D. E. Koppel, J. Schlessinger, W. W. Webb, E. L. Elson, and T. R. Podleski, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4594. (*ii*) T. Hiratsuka, *Biochim. Biophys. Acta*, 1976, **453**, 293.

Specific probes. Of the large selection of probes based on nucleic acids, derivatives of $1,N^6$-ethenoadenosine and $3,N^4$-ethenocytidine continue to be the most popular. The crystal structure of $3,N^4$-ethenocytidine hydrochloride has been reported.[582] The $3,N^4$-ethenocytidine group is only fluorescent in its protonated form; alkylation of N^1 prevents reversion to the non-fluorescent form and the resulting compounds are fluorescent over a wide pH range.[583] Further spectroscopic data on $1,N^6$-ethenoadenosine 3′,5′-cyclic monophosphate and its application to the protein kinase system of beef adrenal glands have been reported.[584] In a combined fluorescence and e.s.r. study, it was found that Mn^{II} (substituted for Mg) quenches the fluorescence of $1,N^6$-etheno-ATP equally effectively in the binary metal–nucleotide complex as in the ternary complex with G-actin.[585] Although it is tempting to suggest that the conformations of the metal–nucleotide complex are similar in the free and actin-bound forms, it is

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pointed out that there is a lack of an adequate quantitative theory of the effect of paramagnetic ions on fluorescence to support this view.[586] 2′(3′)-*O*-L-Phenylalanyl derivatives of 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine (both synthesized for the first time) are accepted as substrates in ribosomal peptidyltransferase reactions and should prove useful in ribosome studies.[587] Energy transfer between 1,N^6-etheno-coenzyme A, which binds to the dihydrolipoyl transacetylase enzyme of the pyruvate dehydrogenase complex, and the flavin adenine dinucleotide group indicates a separation of >50 Å (with the assumption of rapid and random orientation of the groups).[587]

The use of fluorescent (and chromophoric) analogues of ATP in the study of ATPase mechanisms has been discussed.[588] Formycin di- and tri-phosphates bind unusually strongly to the ($Na^+ + K^+$) ATPase, which facilitates kinetic measurements.[589]

New fluorescent probes continue to be added to the selection available for biochemical investigations (Table 2). Perhaps potentially the most useful are a series of 'stretched-out' adenosine analogues, termed '*lin*-benzoadenosines' (*lin* for linear) in which the interposition of a benzene ring extends the adenine moiety by 2.4 Å.[590] The derivatives, which will be generally applicable in probing the steric requirements of enzyme active sites, have been used in preliminary studies with a series of kinases; in addition to their fluorescence, they combine the useful properties of strong binding with reduced rates of reaction. A range of 7-substituted tricyclic 1,N^2-(allylidene)guanine derivatives (analogues of the naturally occurring Y bases found in transfer RNA) has been prepared by reaction with substituted malondialdehydes; the 7-(*p*-methoxyphenyl) derivative shows characteristics which make it a suitable replacement for guanosine.[591]

Fluorescent derivatives not normally considered as specific probes do act as such on binding to antibodies elicited specifically for them. This approach to antibody–hapten interactions has been used in studies with ε-dansyllysine [592, 593] and *N*-methyl-2-anilinonaphthalene-6-sulphonyl bovine serum albumin.[594] The cardiac glycoside binding site of ($Na^+ + K^+$) ATPase has been investigated using the newly synthesized conjugate anthroyl-oubain.[595] Some of the difficulties which can arise in interpreting the results of studies with non-covalent probes are illustrated in another study of ($Na^+ + K^+$) ATPase.[596] Harmine and harmaline, two linear tricyclic alkaloids, are strongly fluorescent and could apparently be useful site-specific probes to investigate sodium-dependent membrane transport processes. However, their usefulness is limited by the observation that they form strong 1 : 1 complexes with ATP, and the fact that addition of Mg^{2+} further affects this binding. The binding of ethidium bromide,[597]

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Tb^{III},[598] and the synthesis and binding of a series of dansylated acylcholines[599] to acetylcholine receptor sites have been reported. Dansyl cadaverine [*N*-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulphonamide] has a very low affinity for proteins but binds strongly to membrane anionic sites; energy transfer between Trp and the membrane-bound dansyl group has been used to study protein–lipid interactions.[600]

Non-specific probes. The crystal structure of 8-anilino-1-naphthalene sulphonic acid (ANS) shows the existence of two distinct conformers.[601] Both conformations are non-planar, but one is appreciably more planar than the other and this may possibly be the species observed in ethanol solution which was thought to be planar.[602] These observations raise the possibility that coplanarity of the two rings is not essential for fluorescence. Nanosecond time-resolved emission spectroscopy (TRES) has been used to investigate the interactions of *N*-arylaminonaphthaelne sulphonates with glycerol and binding sites in egg lecithin vesicles.[603] The wavelength dependence of the decay kinetics is consistent with the occurrence of a dynamic interaction associated with a significant change in energy of emission in the nanosecond range. The use of ANS as a c.d. as well as a fluorescent probe of protein structure has been reported.[604] The decrease in the fluorescence intensity of ANS-labelled elastin upon stretching has been shown to be precisely that expected from a simple one-phase model of randomly cross-linked chains.[605] The results do not warrant postulating a more complex two-phase 'liquid-drop' model in which protein chains are segregated into globular domains joined by cross-links at their surfaces.[606] 6-*p*-Toluidinylnaphthalene-2-sulphonate (TNS) shows pronounced fluorescence enhancement on binding α-, β-, or γ-cyclodextrins.[607] The strong enhancement seems to be due to formation of inclusion complexes of TNS in the hydrophobic region within the rings. This preferential interaction with cyclodextrins has been exploited to develop a simple assay of Taka amylase A in cyclodextrin hydrolysis.

Intrinsic Fluorescence.—Studies of isolated amino-acids and simple model compounds can be of value in understanding their behaviour in proteins. Thus, although Trp buried within a protein molecule is usually associated with an emission maximum at short wavelength,[608] the spectra of Trp and a range of Trp-containing peptides exhibit a red shift when transferred from an aqueous to an organic phase using tri-octylmethylammonium chloride as the transporting agent.[609] The reversed micelle so formed is considered to resemble the environment of Trp buried inside a protein out of contact with water, but in close

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proximity to charged groups. The fluorescence of indole [610] and its quenching by carbonyl compounds [611] have been discussed in relation to the behaviour of Trp and proteins. Yet another photodecomposition product of Trp, formed on excessive exposure to u.v. radiation or solar light, has been characterized (λ_{max} for emission *ca.* 380 nm), and tentatively identified as a C_3-hydroxylated derivative,[612] a further warning if needed to those using protein fluorescence. Acrylamide has certain advantages over more commonly used species as a quencher of Trp fluorescence;[613] ionic quenchers such as I^-, NO_3^-, and Cs^+ are solvated and are susceptible to specific charge effects, and oxygen tends to permeate the interior of the protein fairly readily and hence is indiscriminate. Collisional and static components of quenching by acrylamide have been distinguished and used to give a fuller description of the dynamic structure of a protein in solution. Modified or more sensitive procedures for determining the number of Trp residues in a protein have been described.[614]

The fluorescence of Tyr residues is less sensitive to the environment than that of Trp. However, 3-aminotyrosine residues, obtained by mild nitration followed by reduction, are sensitive to solvent polarity and shift to lower wavelength in a buried environment;[615] the application of such modified groups to ribonuclease A has been discussed.[616]

Protein Conformation. Table 3 summarizes some of the structural and related studies of intrinsic fluorescence.

Table 3 *Structure studied by intrinsic protein fluorescence*

Protein	*Application*	*Ref.*
F-Actin	effect of perturbants on structure	*a*
L-Asparaginase (*E. coli*)	general structure	*b*
(*Erwinia carotovora*)	Tyr quenching	639
Aldolase	refolding and reactivation	*c*
Apo-azurin and other enzymes	Trp environment	617
Bence–Jones proteins	association–dissociation	*d*
Bovine serum albumin	quenching mechanisms	*e*
α-Chymotrypsin	covalently immobilized conformation	631
Gonadotrophin releasing hormone	general structure	633
Gramicidin C	Trp–dansyl energy exchange	626
Haemoglobin and apo-form	Trp–haem energy exchange	628
Haemopexin	Trp-quenching	*f*
Human and bovine growth hormones	quenching experiments	621
Human chorionic gonadotrophin	Tyr fluorescence	640
Human serum albumin	Trp environment	618
Lactalbumins and lysozyme	Trp environment	622
β-Lactoglobulin	effect of perturbants on structure	*g*
	effect of temperature on structure	629

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[616] R. L. Seagle and R. W. Cowgill, *Biochim. Biophys. Acta*, 1976, **439**, 470.

Table 3 (*cont.*)

Protein	*Application*	*Ref.*
Luliberin	Tyr–Trp energy exchange	634—636
Lysozyme	renaturation	*h*
$(Na^+ + K^+)$ATPase	Trp environment	*i*
Neurotoxins	Trp environment	625
Papain	active-site structure	*j*
Phosphofructokinase	reassembly	*k*
3-Phosphoglycerate kinase	Tyr fluorescence	638
Rhodanese	Trp environment	623
Riboflavin binding protein	effect of pressure on structure	630
Ribonuclease	modified Tyr residues	615, 616
	Tyr quenching	639
Ribosomes	conformation	*l*
	subunit structure	*m*
Subtilisin inhibitor	Tyr fluorescence	637
Thymidylate synthetase	Trp–dansyl energy exchange	*n*
Viral coat protein (potato virus X)	conformation of isolated and assembled subunits	*o*
Wheatgerm agglutin	Trp and Tyr environments	619, 620 *p*

(*a*) M. Taniguchi, *Biochim. Biophys. Acta*, 1976, **427**, 126. (*b*) R. B. Homer and S. R. Allsop, *ibid.*, 1976, **434**, 100. (*c*) M. Engelhard, R. Rudolph, and R. Jaenicke, *European J. Biochem.*, 1976, **67**, 447, 455. (*d*) H. Maeda, J. Engel, and H. J. Schramm, *ibid.*, 1976, **69**, 133; *Experientia*, 1976, **32**, 803. (*e*) I. Feldmann, D. Young, and R. McGuire, *Biopolymers*, 1975, **14**, 335. (*f*) W. T. Morgan, R. P. Sutor, and U. Müller-Eberhard, *Biochim. Biophys. Acta*, 1976, **434**, 311. (*g*) R. I. Kaplanas, T. G. Bukolovo, and E. A. Burstein, *Mol. Biol.* (*Russ.*), 1976, **9**, 635. (*h*) A. S. Acharya and H. Taniuchi, *J. Biol. Chem.*, 1976, **251**, 6934. (*i*) N. M. Mirsalikhova, E. E. Gussakovsky, S. Burkhanov, R. A. Ziyamukhamedov, and B. A. Tashmukhamedov, *Biofizika*, 1975, **20**, 980. (*j*) M. R. Bendall and G. Lowe, *European J. Biochem.*, 1976, **65**, 481, 493. (*k*) G. R. Parr and G. G. Hammes, *Biochemistry*, 1976, **15**, 857. (*l*) J. C. Brochon, P. Wahl, P. Vachette, and M. P. Daune, *European J. Biochem.*, 1976, **65**, 35; P. Spitnik-Elson, N. Schechter, R. Abramovitz, and D. Elson, *Biochemistry*, 1976, **15**, 5246. (*m*) D. Gerard. G. Lemieux, and G. Lanstrist, *Photochem. and Photobiol.*, 1975, **22**, 89. (*n*) L. A. May, H. C. Cheung, J. L. Aull, and J. D. Clickson, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 653. (*o*) R. B. Homer and D. I. Dalton, *Biochim. Biophys. Acta*, 1976, **446**, 542. (*p*) J. P. Privat and M. Monsigny, *European J. Biochem.*, 1976, **60**, 555.

The power of fluorescence decay measurements in probing the surroundings of a fluorophore is evident in several studies of proteins containing either only one or a few Trp groups per molecule.[617–620] Of all the proteins investigated, it seems that multi-exponential decay of the fluorescence of single Trp is the rule (apo-azurin being the exception[617]); of the various possible explanations discussed the preferred one is that this behaviour reflects variability in the micro-environment of the Trp groups. Further evidence of the sensitivity of Trp fluorescence to small differences in environment is provided by comparison of human and bovine growth hormones,[621] and a series of lysozymes and α-lactal-albumins containing extensive sequence homologies.[622] The care needed in the

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[620] J. P. Privat, P. Wahl, M. Monsigny, and J. C. Auchet, *European J. Biochem.*, 1976, **68**, 573.
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structural interpretation of fluorescence data is illustrated by the case of rhodanese. The Trp emission spectrum has a relatively narrow half bandwidth with a maximum at 330 nm, implying that all of the Trp are inaccessible to solvent; however, chemical modification showed the presence of a structurally important Trp exposed at the active site. This discrepancy has been resolved by Cs^+ quenching experiments, which revealed the presence of exposed residues with an emission maximum at 340 nm, and buried residues with a maximum at 323 nm; the abnormally low wavelength of the latter was attributed to close contact with apolar side-chains which shield the Trp from the peptide backbone.[623] The emissions of neurotoxins from the venom of the Middle Asian cobra, which had suggested the presence of a single Trp group freely accessible to water,[624] have been re-interpreted in the light of the newly available primary structures which show that two Trp are involved.[625]

Fluorescence energy transfer from Trp of gramicidin A to a modified *O*-dansyltyrosine at position 11 supports the idea that the gramicidin isolated species are aggregates.[626] It has been known for many years that fluorescence quantum yields of haem proteins are very small ($Q \leqslant 0.002$) but increase dramatically upon removal of the haem group.[627] This was thought to be due to Förster-type radiation transfer which would result in very short lifetimes. Recent results on haem proteins excited by synchrotron radiation confirm the 100-fold increase in quantum yield on going from haemoglobin to its apo-form, but indicate that the lifetimes are very little changed.[628] These results imply strong static quenching arising from the formation of a non-fluorescent complex between Trp and the haem group; the residual fluorescence in haemoglobin could be due to statistical fluctuations in the structure resulting in a small fraction emitting its normal fluorescence.

The marked fluorescence change which accompanies gross denaturation of proteins has been used to study the mechanisms of unfolding resulting from increases of temperature[629] and pressure,[630] and covalent attachment to soluble carriers.[631] The difference between the spectra of native and denatured actin is the basis of a simple method for determining the proportion of denatured form in preparations.[632] The conformation of the decapeptide gonadotrophin-releasing hormone in solution appears to be fully random coil, devoid of any intrachain interactions between residues.[633]

[623] K. Guido, R. D. Baillie, and P. M. Horowitz, *Biochim. Biophys. Acta*, 1976, **427**, 600.
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[633] S. Mabrey and I. M. Klotz, *Biochemistry*, 1976, **15**, 234.

Energy transfer between Trp and Tyr in the fluorescence[634, 635] and luminescence[636] spectra of luliberin (which contains only one residue of each type) has been observed. The separation of the two groups calculated from the fluorescence results are in reasonable agreement considering the different assumptions which can be used to interpret the data, and the difficulty in assessing the overlap integrals for the two electronic transitions in the first absorption band of Trp. Streptomyces subtilisin inhibitor joins a small group of proteins (including subtilisin *Carlsberg*, azurin, and yeast 3-phosphoglycerate kinase) which show an emission maximum at around 308 nm upon irradiation at 280 nm;[637] this is in general due not only to a relatively high Tyr content, but also to the quantum yield of Trp being abnormally low. The emission maximum of the streptomyces inhibitor shifts to 340 nm below pH 4, presumably due to the Trp fluorescence being released by some structural change in the protein.[637] Yeast 3-phosphoglycerate kinase has also been investigated;[638] Tyr → Trp energy transfer is very weak, possibly indicating partial separation of the residue in different lobes of the protein.

Although the intrinsic fluorescence of Trp has been widely used as a probe of its environment and the effect on this of a variety of perturbing factors, the relatively small amount of work on proteins containing Tyr but not Trp has seemed to show (as commented on earlier) that Tyr groups are fairly insensitive to similar perturbations. Electrostatic and steric contributions to the accessibility of Tyr groups in ribonuclease and L-asparaginase have been distinguished by quenching studies with phosphate, Cs^+, and I^-.[639] The idea, based on results from ribonuclease, that Tyr located in the interior of a protein fluoresces very weakly is supported by the results of studies on human chorionic gonadotrophin, which also show that its isolated subunits possess little tertiary structure beyond that stabilized by disulphide bonds.[640] The accessibility of Tyr groups can also be investigated using oxidative phenol coupling to yield cross-linked dityrosine residues which have an intense blue fluorescence.[641]

Ligand Binding. The changes in the intrinsic fluorescence of a protein on binding a ligand can be interpreted in a variety of ways, depending chiefly on the degree to which the protein structure is known; results can be used to give detailed information about alterations of conformation and in particular of Trp environment associated with the process or, more commonly, to monitor the rates, binding constants, and sequence of the elementary steps involved. A selection of the very large number of studies reported is collected in Table 4; the list is not intended to be comprehensive, and only a few of the studies are commented on below.

[634] M. Shinitzky and M. Fridkin, *Biochim. Biophys. Acta*, 1976, **434**, 137.
[635] P. Marche, T. Montenay-Garestier, C. Hélène, and P. Fromageot, *Biochemistry*, 1976, **15**, 5730.
[636] P. Marche, T. Montenay-Garestier, C. Hélène, and P. Fromageot, *Biochemistry*, 1976, **15**, 5738.
[637] Y. Uehara, B. Tonomura, K. Hiromi, S. Sato, and S. Murao, *Biochim. Biophys. Acta*, 1976, **453**, 513.
[638] H. Nojima, A. Ikai, and H. Noda, *Biochim. Biophys. Acta*, 1976, **427**, 20.
[639] R. B. Homer and S. R. Allsop, *Biochim. Biophys. Acta*, 1976, **434**, 297.
[640] K. C. Ingham, C. Tylenda, and H. Edelhoch, *Arch. Biochem. Biophys.*, 1976, **173**, 680.
[641] R. Aeschback, R. Amado, and H. Neukom, *Biochim. Biophys. Acta*, 1976, **439**, 292.

Table 4 *Binding studied by intrinsic protein fluorescence*

Protein	*Ligand*	*Ref.*
Acetylcholine receptor	acetylcholine and carbamylcholine	*a*
Actin, myosin subfragment 1	ATP and others	648
Aminoacyl-tRNA synthetases	tRNA's	643
Apoferritin	Fe^{2+}	*b*
ATPase	ATP	*c*
	dinitrophenylated dipalmitoyl phosphatidylethanolamine	*d*
Bovine serum albumin	di-iodo-L-Tyr and tri-iodophenol	*e*
	steroids	*f*
Citrate synthase	NADH	*g*
Concanavalin A	α-methyl-D-mannoside	649
FV fragment from myeloma protein MOPC315	Tb^{III}, haptens	*h*
Glutamate dehydrogenase	L-glutamate, GTP, NADPH	*i*
Glutamine synthetase	ADP	*j*
Glyceraldehyde 3-phosphate dehydrogenase	NAD^+	*k*
Heart lactate dehydrogenase	nucleotides, pyruvate	*l*
Hexokinase	glucose	*m*
Histidine decarboxylase	methylhistidine	*n*
Histidyl-tRNA synthetase	histidine, ATP, and analogues	*o*
Isocitrate dehydrogenase	NADPH	*p*
lac-repressor	isopropyl-β-D-thiogalactoside	644
Liver alcohol dehydrogenase	oxidized and reduced coenzymes	*q*
Myosin subfragment 1	MgATP	647
Maltose binding protein	Maltose, maltotriose	*r*
Phage T_4 gene 32 protein	nucleotides	642
Sarcoplasmic reticulum calcium pump	Ca^{2+}	*s*
Thymidylate synthetase	methylene tetrahydrofolate 5-fluorodeoxyuridylate	*t*
Yeast alcohol dehydrogenase	NAD^+, NADH	*u*

(*a*) R. Bonner, F. J. Barrantes, and T. M. Jovin, *Nature*, 1976, **263**, 429. (*b*) S. Stefani, E. Chiancone, and E. Antonini, *F.E.B.S. Letters*, 1976, **69**, 90. (*c*) R. S. Taylor and A. G. Weeds, *Biochem. J.* 1976, **159**, 301. (*d*) P. M. D. Hardwicke, *European J. Biochem.*, 1976, **62**, 431. (*e*) N. Okabe, N. Manabe, R. Tokuoka, and K. Tomita, *J. Biochem.*, 1976, **80**, 455. (*f*) A. M. Romeu, E. E. Martino, and A. O. M. Stoppani, *Biochim. Biophys. Acta*, 1976, **439**, 175. (*g*) H. W. Duckworth and E. K. Tong, *Biochemistry*, 1976, **15**, 108. (*h*) R. A. Dwek, D. Givol, R. Jones, A. C. McLaughlin, S. Wain-Hobson, A. I. White, and C. Wright, *Biochem. J.*, 1976, **155**, 37. (*i*) J. C. Brochon, P. Wahl, J. M. Jallon, and M. Iwatsabo, *Biochemistry*, 1976, **15**, 3259. (*j*) S. G. Rhee and P. B. Chock, *Biochemistry*, 1976, **15**, 1755. (*k*) G. Allen and J. I. Harris, *Biochem. J.*, 1975, **151**, 747. (*l*) M. J. Boland and H. Gutfreund, *ibid.*, p. 715. (*m*) J. G. Hoggett and G. L. Kellett, *European J. Biochem.*, 1976, **66**, 65; **68**, 347. (*n*) S. R. Mardashev, L. A. Semina, L. A. Gonchar, I. D. Mitrofanova, and S. S. Aleksandrova, *Biochemistry* (*Russ.*), 1976, **40**, 673. (*o*) P. DiNatale, A. N. Schechter, G. L. Castranuovo, and F. DeLorenzo, *European J. Biochem.*, 1976, **62**, 293. (*p*) M. F. Carlier and D. Pantaloni, *Biochemistry*, 1976, **15**, 4703. (*q*) I. Iweibo, *Biochim. Biophys. Acta*, 1976, **446**, 192. (*r*) S. Szmelcmann, M. Schwartz, T. J. Silhavy, and W. Boos, *European J. Biochem.*, 1976, **65**, 13. (*s*) Y. Dupont, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 544. (*t*) H. Donato, J. L. Aull, J. A. Lyon, J. W. Reinsch, and R. B. Dunlap, *J. Biol. Chem.*, 1976, **251**, 1303. (*u*) D. Karlović, P. Amiguet, F. J. Bonner, and P. L. Luisi, *European J. Biochem.*, 1976, **66**, 277.

Trp fluorescence of bacteriophage T_4 coded gene 32 protein is quenched on binding nucleotides, longer nucleotides being more effective than shorter ones.[642] Quantitative studies of binding combined with iodide quenching experiments have been used to map the involvement of Trp at the binding site, and provide an explanation of the co-operative binding of the protein to long-chain nucleic acids. The mechanisms of specific and non-specific interaction of tRNA with aminoacyl-tRNA synthetases have been investigated using intrinsic protein fluorescence and that of the Y base of $tRNA^{Phe}$.[643] The picture emerging from these studies is that specific interactions occur in a two-step mechanism in which an almost diffusion-controlled association is followed by a rapid conformational change, whereas non-specific interactions occur in a single-step process; it is argued that the conformational transition is an essential part of the recognition process. Several studies have been published of the binding of inducer to *lac*-repressor.[644–646] The effect of binding on the fluorescence of Trp-190 and Trp-209 residues of the *lac*-repressor has been investigated by comparing its behaviour with that of two modified repressors obtained from characterized mutants in which each of the Trp was replaced by a non-fluorescent amino-acid; Trp-190 is buried in the core of the protein, whereas Trp-209 is partially exposed to solvent and although its environment is changed upon binding it does not appear to come into direct contact with the inducer.[644] Fluorescence temperature-jump methods reveal the presence of two conformations of the repressor, even in the absence of inducer.[645] The kinetics of the binding of inducer were the same when protein fluorescence or the change in absorption of the inducer or of a chromophoric group linked to the protein was used to follow the process, suggesting that the conformational changes are translated rapidly through the protein.[646]

The similarity between the fluorescence bathochromic shifts which occur when the two thiol groups of the myosin head (SF1) are covalently linked using a bifunctional reagent, and when MgATP is added to unmodified SF1 point to structural similarities between the steady-state intermediate (M**,MgADP,Pi) and covalently bridged SF1.[647] The advantages which can accrue when different techniques are combined are evident in a study using fluorescence and light-scattering stopped-flow of a single cycle of ATP hydrolysis by a complex of actin and myosin subfragment 1.[648] The major conformational changes which are associated with specific sugar binding (α-methyl-D-mannoside) to concanavalin A do not affect either the number or the accessibility of the exposed Trp groups.[649]

[642] R. C. Kelly and P. H. von Hippel, *J. Biol. Chem.*, 1976, **251**, 7229; R. C. Kelly, D. E. Jensen, and P. H. von Hippel, *ibid.*, p. 7240.
[643] D. Riesner, A. Pingoud, D. Boehme, F. Peters, and G. Maass, *European J. Biochem.*, 1976, **68**, 71; G. Krauss, D. Riesner, and G. Maass, *ibid.*, p. 81.
[644] H. Sommer, P. Lu, and J. H. Miller, *J. Biol. Chem.*, 1976, **251**, 3774.
[645] F. Y. H. Wu, P. Bandyopadhyay, and C. W. Wu, *J. Mol. Biol.*, 1976, **100**, 459.
[646] B. E. Friedmann, J. S. Olson, and K. S. Matthews, *J. Biol. Chem.*, 1976, **251**, 1171; A. P. Butler, A. Revzin, and P. H. von Hippel, *Biophys. J.*, 1976, **16**, 90a.
[647] M. Burke, E. Reisler, and W. F. Harrington, *Biochemistry*, 1976, **15**, 1923.
[648] S. P. Chock, P. B. Chock, and E. Eisenberg, *Biochemistry*, 1976, **15**, 3244; S. P. Chock, E. Eisenberg, and P. B. Chock, *Biophys. J.*, 1976, **16**, 45a.
[649] R. Pelley and P. Horowitz, *Biochim. Biophys. Acta*, 1976, **427**, 359.

7 Infrared Spectroscopy

Contributed by R. M. Stephens

Model Compounds.—The interaction between the peptide group and carboxylic acids is one of either protonation or hydrogen bonding. Both interactions will affect the vibrational frequency of the peptide group, resulting in changes in the characteristic amide vibrations. I.r. spectroscopy has been used to examine the changes in the amide I and II absorption bands of the model compound *N*-methylacetamide (NMA) when dissolved in deuteriochloroform and various deuteriochloroform–carboxylic acids, such as acetic, formic, dichloroacetic, and trifluoroacetic acid (TFA). The results indicate that the interaction is one of hydrogen bonding except when NMA is dissolved in the strongest acid used (TFA). The interactions between TFA and the peptide groups of poly-(L-alanine), various poly-(DL-alanines), and poly-(γ-benzyl-L-glutamate) show that hydrogen-bond interaction occurs with little evidence of protonation taking place. In poly-(γ-benzyl-L-glutamate) it was found that the TFA interacted both with the peptide group and the side-chain carboxy-group.[650] Investigations of the secondary structure of $Me_3C\cdot CO\cdot O(L\text{-}Ala)_nOMe$ and $Me_3C\cdot CO\cdot O(D\text{-}Ala)_nOMe$ (n = 2—7) in the solid state revealed predominantly intermolecular anti-parallel β structure in pentamers and higher oligomers, but dimers were predominantly unordered and trimers and tetramers consisted of a mixture of unordered and β structures. C.d. studies in trifluoroethanol solutions showed unordered structures through to the hexamer with the β structure appearing in heptamers and higher oligomers.[651] The i.r. spectra of chemically monodisperse and optically pure linear homo-oligopeptides $Me_3C\cdot CO\cdot O(X)_nOMe$ (X = Ala, Nval, Val, Leu, Ile, Phe, Met, or Cys(Me); n = 2—7) in the solid state and in solution indicated that all pentamers and higher oligomers assume a β form, whereas the trimers and tetramers exist in a mixture of β and unordered forms with the dimers existing predominantly in the unordered structure. Conformational analysis showed a high content of intramolecular hydrogen-bonded forms for all homo-tetramers in solution, with the exception of those derived from β-branched amino-acid residues. The effect of a sulphur atom or an aromatic ring in the amino-acid side-chain on the stability of the folded forms was studied. The unusual intermolecular β parallel chain conformation was suggested for $Cl^-H^+(X)_7OMe$ with X = Val, Ile, or Phe.[652] The conformational states of several model compounds in dilute carbon tetrachloride solution have been studied. Besides the common C_7 structure a second conformer has been identified which is stabilized by an intramolecular interaction between the N—H proton donor site and the π electrons of the tertiary amide link. A theoretical analysis of the data supports this idea.[653] The far-i.r. transmission spectra of nine amino-acids were recorded using TPX (poly-4-methylpentene) sheets at 300 and 80 K. The spectra of glycine, cystine, lysine,HCl, lysine,2HCl, and histidine,HCl were basically unaffected by lowering the temperature, but those of norvaline, methionine, tryptophan, and arginine,HCl were better

[650] R. M. Stephens and E. M. Bradbury, *Polymer*, 1976, **17**, 563.
[651] C. Toniolo and G. M. Bondra, *J. Polym. Sci., Polymer Chem.*, 1976, **14**, 515.
[652] M. Palumbo, S. Da Rin, G. M. Bondra, and C. Toniolo, *Makromol. Chem.*, 1976, **117**, 1477.
[653] M. Marraud, J. Neel, and B. Maigret, *J. Chim. phys.*, 1975, **72**, 1173.

resolved at 80 K. All the amino-acids showed characteristic bands suitable for analysis at 80 K. Polyamino-acids and DNA showed only a generalized absorption that could not be resolved at 4 K.[654] Using the frequency *versus* phase relationship for a finite chain with free ends, the extended phonon dispersion curves for polyglycine, poly-L-alanine and poly-L-proline were used to interpret the vibrational spectra of glycine, alanine, and proline oligomers, respectively. The oligoglycines and alanines up to the hexamer approach a linear zig-zag conformation, whereas the proline oligomers beyond the tetramer fold into a helical structure with a three-fold screw axis like poly-L-proline II. Dodecaglycine also assumes a three-fold helical structure nearer to polyglycine II.[655] The low-frequency far-i.r. and Raman spectra of $H(Ala)_nOH$ (n = 1—6) have been interpreted in terms of intermolecular vibrations between oriented molecules in the unit cell of crystals. When $n > 3$ the spectra become increasingly similar to those of polyalanine and the vibrations are more adequately described in terms of skeletal modes.[656] Differences in the carbonyl and NH stretching vibrations from α- and γ-glutamyl dipeptides have been correlated with n.m.r. shifts in acidic, basic, and neutral solutions.[657] Frequency shifts of characteristic amide bands of related amide compounds have also been analysed.[658]

Polypeptides.—Broadening of the amide A, I, and II absorption bands of α-helical polypeptides has been observed for thermodynamically unstable α-helices. This can be explained by the geometrical distortions of the backbone of the helical structure. Two models for distorted helices which include regular or irregular distortions of the angles of internal rotation of the main polypeptide chain have been considered.[659] Theoretical treatment of resonance interactions of amide I vibrations has been carried out using a dipole–dipole approximation on the basis of perturbation theory. A single infinite antiparallel chain pleated sheet, as well as different kinds of its finite fragments, have been considered. Good agreement has been obtained between calculated spectral parameters of the amide I vibration from the infinite sheet and the observed ones from the i.r. and Raman spectra of fibrous proteins. A theoretical dependence of the resonance frequency shift of the main components and frequency splitting of two components active in the i.r. spectra of a number of polypeptide chains in the finite sheet have been found.[660] The Raman and i.r. spectra of poly-(L-lysine) and poly-(DL-lysine) in various salt solutions have been investigated. α-Helix formation in solution is induced by specific salts, and the spectral data support the hypothesis of regions of local order for poly-(L-lysine) in aqueous solutions of low ionic strength.[661] The hyperchromic and hypochromic changes in the intensity of the amide I and amide III lines of polypeptides and certain ring vibrations of the bases of polynucleotides have been related to similar changes in the lower energy u.v. absorption bands. For polypeptides and polynucleotides there is a large hypochromic intensity change in the first $\pi \rightarrow \pi^*$ exciton band

[654] X. Gergaux and A. Hadni, *Compt. rend.*, **282**, *B*, 181.
[655] M. K. Gupta and V. D. Gupta, *Indian J. Biochem. Biophys.*, 1976, **13**, 1.
[656] E. Loh, *Commentat. Phys.-Math. Soc. Sci. Fenn.*, 1976, **46**, 37.
[657] T. Kasai and S. Sakamura, *J. Fac. Agric. Hokkaido Univ.*, 1975, **58**, 283.
[658] C. G. Cannon, *J. Phys. Chem.*, 1976, **80**, 1247.
[659] N. Yu. Chirgadze, E. V. Brazhnikov, and N. A. Nevskaya, *J. Mol. Biol.*, 1976, **102**, 781.
[660] N. Yu. Chirgadze and N. A. Nevskaya, *Biopolymers*, 1976, **15**, 607, 627, 637.
[661] P. C. Painter and J. L. Koenig, *Biopolymers*, 1976, **15**, 229.

associated with coil-to-helix transitions. Through the selection rules certain conformationally sensitive Raman lines derive their intensity predominantly from this band, and hence also display hypochromism. Again through the application of selection rules certain Raman lines can be demonstrated to depend for their intensity upon the $n \rightarrow \pi^*$ transition and exhibit the opposite hyperchromic intensity change upon the same conformational transition.[662] Laser-excited Raman spectra of complexes of polylysine with DNA reveal the frequencies and intensities of Raman scattering from both the polypeptide and nucleic acid components. Most sensitive to the complex formation are characteristic vibrational frequencies of the DNA bases. The results show that the DNA–polylysine complex is formed with conservation of the backbone structure normally occurring in aqueous solutions of DNA, the so-called B-DNA structure. However, interactions between bases in the DNA double helix are significantly altered by polylysine binding. The same results were obtained for DNA–polylysine complexes formed in either high or low ionic strength conditions and by either direct mixing or annealing of the constituent biopolymers. The results are consistent with a model in which the extended polylysine chain is bound by electrostatic interactions between positively charged lysyl chains and negatively charged DNA phosphate groups. Laser Raman spectra of the complex of polyadenylic acid (polyA) and polylysine showed, on the other hand, that both the geometry of the backbone and the mode of interaction between stacked bases of polyA are altered by complex formation. In this complex the extended polylysine chain may be bound to polyA by electrostatic interactions between lysyl and phosphate groups. However, *gauche*$^+$–*gauche*$^+$ conformations normally occurring in the phosphodiester linkages of aqueous polyA are distorted as a consequence of polylysine binding.[663] Changes in the amide II absorption in the i.r. spectra have been determined for poly-(γ-benzyl-L-glutamate) in chloroform (100% α-helix conformation), haemoglobin and globin in β-chloroethanol and sodium dodecyl sulphate ($\simeq$70% α-helix), haemoglobin and globin in dimethyl sulphoxide (0% α-helix), native haemoglobin in D_2O ($\simeq$70% α-helix) and poly(lysylalanine) at pH 2.0, during changes in the degree of deuteriation of the peptides. For the peptides containing no α-helical structure increasing deuteriation had no effect on the form or position of the amide II peak. For the α helical peptides the position of the amide II peak shifted linearly towards lower frequencies with increasing deuteriation, reaching an extrapolated value of 1536—1540 cm^{-1} at 100% deuteriation. This value corresponds to the unperturbed frequency for the amide II band. Analysis of the i.r. spectra for poly(lysylalanine) and the very slight shift of its amide II peak with increasing deuteriation indicated that this peptide manifests a β-conformation with no intermolecular hydrogen bonds.[664] The structures of polypeptides containing glycine, proline, hydroxyproline, analine, and leucine were analysed using i.r. spectroscopy and hydrogen–deuterium reactions. Structures were correlated with chain length, degree of chain order stabilized by hydrogen-bonding, amino-acid composition, and amino-acid order in tripeptide units.[665] The i.r. dichroic

[662] P. C. Painter and J. L. Koenig, *Biopolymers*, 1976, **15**, 241.
[663] B. Prescott, C. H. Chou, and G. J. Thomas, *J. Phys. Chem.*, 1976, **80**, 1164.
[664] S. V. Sychev, *Biol. Nauchno-Tekh. Prog. Tezisy Dokl.-Vses. Konf. Molodykh Uch. Spets.*, 1974, 120.
[665] A. Yu Lazarev, N. G. Esipova, A. V. Laxareva, and U. A. Shibnev, *Tezisy Dokl.-Vses. Simp. Khim. Pept. Belkov.*, 1974, **82**.

ratios from liquid crystalline solutions of poly-(γ-benzyl-L-glutamate) and poly-(γ-benzyl-D-glutamate) have been measured in static electric fields and transition moment directions compared.[666]

Proteins.—The (β-strand)–(α-helix)–(β-strand) unit consists of two parallel but not necessarily adjacent β-strands which lie in a β-pleated sheet and are not connected by more thanone α-helix. This unit, which occurs in 17 functionally different globular proteins, may adopt a right- or left-handed conformation. An analysis of the distribution showed that 57 out of the 58 units are right handed. If the unit had no right-handed preference, the probability of observing such a distribution by chance is 10^{-16}. This may be explained in terms of the twist of the β-sheet which favours a right-handed unit as otherwise steric hindrance would occur in the loop regions. The right-handed (β-strand)–(α-helix)–(β-strand) unit determines the sense of the super-secondary structure in dehydrogenases and of related folds in other structures. The evolutionary relationship between proteins containing this unit was re-evaluated in terms of this preference. The high probability that the unit will fold with a right-handed conformation has implications for the prediction of tertiary structure.[667] Conformational studies of various membrane proteins have shown that the amide I peak for protein from a mitochondrial membrane occurred at 1652 cm^{-1} and from a microsomal fraction at 1655 cm^{-1}, indicating the presence of proteins with α-helical conformations and/or unordered structure in both samples. The amide II vibrations from both samples were almost the same and occurred at *ca.* 1542 cm^{-1}. The presence of shoulders on both amide peaks in both samples indicated the presence of proteins with β-conformations. Extraction of the nucleo- and lipo-proteins of the sarcoplasmic reticulum with iso-octane resulted in a decrease in the level of β-conformation in the proteins. Thus the β-conformations present in the samples are apparently stabilized by hydrophobic and lipid–protein interactions. In general, the molecular conformations of the proteins in the mitochondria and the sarcoplasmic reticulum are very similar.[668, 669] The i.r. spectra of skin procollagen from rat, pike, and cod and those of synthetic polytripeptides $(Gly\text{-}Pro\text{-}Pro)_n$, $(Gly\text{-}Pro\text{-}HyPro)_n$, $(Gly\text{-}HyPro\text{-}HyPro)_n$, and $(Gly\text{-}Pro\text{-}Ala)_n$ were analysed during formation and denaturation of triple helical structures in D_2O. The effect of molecular length of the synthetic oligomers on the triple helical structure and the correlation of the amide I band components with the triple helical structure are discussed.[670] Similar studies concerned with formation and denaturation of triple helical structure in rat procollagen caused by temperature changes have been reported by the same authors.[671] Native fibrillar collagen isolated from bovine tendon, collagen dissolved by treatment with an alkaline salt solution, collagen solubilized by enzymic methods, and tropocollagen isolated from calf dermis were analysed by i.r. spectroscopy for different struc-

[666] E. Iizuka, *Polymer J.*, 1975, **7**, 650.
[667] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1976, **105**, 367.
[668] V. P. Verbolovich and E. V. Poletaev, *Biofizika*, 1976, **21**, 757.
[669] V. P. Verbolovich, E. V. Poletaev, and E. D. Ryudiger, *Vestnik Akad. Nauk Kaz. S.S.R.*, 1976, 72.
[670] A. Yu Lazarev, A. I. Pisachenko, B. A. Grishkovskii, T. B. Khromova, and A. G. Sukhomudrenko, *Tezisy Dokl.-Vses. Konf. Spectrosk. Biopolim.*, 1974, 70.
[671] T. B. Khromova, A. Yu Lazarev, V. M. Lobochev, and A. G. Sukhomudrenko, *Tezisy Dokl.-Vses. Konf. Spectrosk. Biopolim.*, 1974, 138.

tures. The i.r. spectra of KBr pellets of all four samples contained amide I bands which were broadened and split into several components, indicating ordered structures. The amide I bands from the enzyme and the alkaline salt-treated collagens were shifted by *ca.* 30 cm^{-1} into the low-frequency region compared to those from the native collagen and tropocollagen. In the region 3000—3600 cm^{-1} which includes the amide A band, the spectra of the native samples were similar whereas the treated samples yielded peaks shifted to higher frequencies and with intensities indicating an increase in hydroxy-groups and in the amount of tightly bound water. The alkaline salt-treated sample differed more markedly from the native samples than the enzyme-treated one. In the i.r. spectra of films of these samples the amide II peak of the alkaline salt-treated collagen was shifted by 40 cm^{-1} into the higher frequency region compared to that from enzyme-treated samples. Also the addition of poly(vinyl alcohol) to the films had no effect on the spectra of the alkaline salt-treated collagen, but caused a high-frequency shift of 25—30 cm^{-1} in the amide II band from the enzyme-treated samples indicating an increased number of hydroxy-groups in the latter sample. Thus alkaline salt and enzyme treatment of collagen causes changes in the tertiary structure, resulting in a loosening of the structure and an increase in the amount of tightly bound water, but only slightly affecting the main secondary structure.[672] Improved techniques have been developed for near-i.r. studies of water binding to globular proteins and intact cells or tissues. These include the construction of a cylindrical absorption cell that is of variable path-length and that can be reopened for sample dehydration and reclosed for subsequent scans. A matrix composed or copolymers of methacrylic acid and diethylaminoethyl methacrylateto gether with agarose is used in order to hold globular or non-gelling proteins in the form of uniform transparent films while water is removed. Corrections were made for light scattering by the samples. Results obtained with serum albumin and with intact eye lens and dura mater membranes were presented. The spectra provided sufficient resolution and accuracy for analytical studies and for the detection of differences between bulk and protein-bound forms of water.[673] Overall vibrations and longitudinal acoustical modes of peptides, proteins, and other higher polymers have been studied using i.r. and Raman spectra. The frequencies of these overall vibrations were roughly proportional to the reciprocal dimensions. They were usually small, and hence the amplitudes due to thermal motions were large. These were related to Young's modulus and other elastic constants of the materials.[674]

8 Nuclear Magnetic Resonance

Contributed by H. W. E. Rattle

It is a pleasure to report that 1976 has seen an increase in the quality as well as the quantity of published work on the n.m.r. of peptides and proteins. The ratio of biochemical effort to spectroscopic effort needed for the attainment of

[672] V. M. Gorbalov, O. O. Babloyan, M. A. Borisoua, L. R. Komissarova, and P. M. Golovanova, *Abs. Commun. European Meet. Meat Res. Work*, 1974, 223.

[673] N. Ressler, V. Ziauddin, C. Vygantas, W. Janzen, and K. Karachorlu, *Appl. Spectroscopy*, 1976, **30**, 295.

[674] T. Shimanouchi, in 'Structural Studies of Macromolecules by Spectroscopic Methods', ed. K. J. Ivin, John Wiley, New York, 1976, p. 59.

biologically significant results from n.m.r. is probably at least ten to one, and many laboratories now appear to be investing heavily in the often subtle and difficult biochemical techniques necessary to ensure that what goes into the n.m.r. sample tube is worthy of the sophistication of the technique used in analysing it. It may now be true that n.m.r. is being employed more by biochemists who know some spectroscopy than by erstwhile physicists who have learned some biochemistry, and two books intended to help them make the transition have been published, one a basic introduction to both n.m.r. and e.p.r. in the study of biomolecules [675] and the other a more extensive work devoted entirely to the n.m.r. of peptides and proteins.[676] Among the general reviews published is one on ^{13}C spectroscopy of biopolymers.[677]

With biochemical modifications assuming even greater importance in n.m.r. studies, several papers dealing exclusively with preparative techniques have appeared. One potentially useful probe molecule is leucine specifically deuteriated at the γ-carbon,[678] and others are selectively deuteriated aromatic amino-acids.[679] N.m.r. with stable isotopes in biosynthetic studies is described with an extensive bibliography,[680] and the synthesis of macrocyclic enzyme models [681] and the biological incorporation of ions into macromolecules [682] have also been covered.

As well as guides to preparation, it is always useful to have published data to help in spectral analysis. A major review on the relationship between the conformation of peptide systems and the spin–spin couplings observed in their n.m.r. spectra [683] should be essential reference material here. ^{13}C chemical shifts for amines, carboxylic acids, and amino-acids [684] and preliminary ^{15}N studies on 95% enriched amino-acids [685] add to the store of data on these nuclei, and ^{13}C–^{15}N coupling constants in enriched amino-acids as a function of pH have been correlated with theoretical calculations.[686] The possibility of using tritium n.m.r. to determine the distribution of this radioactive label in amino-acids may interest those whose main interest is in biochemical tracer experiments.[687]

As well as its uses for conformational analysis *via* chemical shifts and spin–spin splittings, the dynamic measurement of T_1 and T_2 is extensively employed for macromolecules, frequently to give a measure of molecular motions. A theoretical paper [688] points out that spin-diffusion in protein molecules tends to equalize the

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proton T_1 values of the various chemical groupings to that of the rotating methyl groups which act as 'relaxation sinks'. The theory was tested on papain and ribonuclease and found to work well. Internal motions in proteins by T_1 and nuclear Overhauser effect measurement in proton-decoupled ^{13}C spectra have been subjected to theoretical analysis.[689] Two papers challenging the validity of some studies on the enhancement of relaxation by paramagnetic ions have appeared, both of them arguing that the assumption that dipolar interactions are the main contributors to line broadening may not be justified. One [690] points out that this invalidates the general applicability of the r^{-6} dependence of linewidth on the distance between the metal ion (Mn^{2+}) and the resonant nucleus for studies of the binding of small ligands to proteins unless the dipolar interaction is proved to dominate, while the other [691] challenges published work on Cu^{II} complexes of amino-acids and peptides on the grounds that it is chemical exchange rather than dipolar effects that make the major contribution to line broadening.

Amino-acids and Small Peptides.—Moving on to results from biomolecular studies, we may restrict our consideration of isolated amino-acids to a study of the complexing of adenosine monophosphate with aromatic amino-acids [692] and to some empirical equations for predicting ^{13}C chemical shifts in various chemical derivatives of glycine.[693] Glycine dipeptides are the subject of a study of pH-induced chemical shift changes in ^{15}N and ^{17}O spectra, revealing an increase in the amide C—N double bond character as the molecule goes from cation to zwitterion and electron density transfers from the N to the O atom.[694] A study which will be of value in the investigation of histidine titration in proteins describes the influence of charged neighbour residues on these titrations in di- and tri-peptides.[695]

Proline is of great interest to all who study protein conformation and a set of general rules for use in determining the *cis–trans* isomerization in peptide chains containing X-Pro by using ^{13}C resonance has been presented.[696] Both ^{1}H and ^{13}C resonance are used in the study of the pH dependence of *cis–trans* isomerism in Pro-7 of angiotensin peptides.[697] Proline-containing cyclic dipeptides have been studied by ^{13}C resonance,[698, 699] and with the assistance of lanthanide shift reagents,[700] followed by a discussion of how cyclic peptides containing proline and glycine may be structurally analysed by a combination of energy calculations and c.d. measurements with ^{1}H and ^{13}C n.m.r.[701] Ring conformations in cyclic dipeptides have been discussed,[702] and some ^{13}C T_1 data for cyclic amino-acids

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and peptides demonstrate anisotropic rotational diffusion and intramolecular motion.[703] A D-analogue of the cyclic pentapeptide tocinamide [704] and a cation-binding analogue *cyclo*-(D-Val-L-Pro-L-Val-D-Pro)$_3$ of valinomycin [705] have been investigated, the latter showing a higher affinity for cations than the normal molecule, although retaining the ability to form the same structure. Other interesting papers on small peptides cover the sequence determination of glycines in tetrapeptides and branched peptides through pH variation of their spectra [706] and report that in tyrosine-containing oligopeptides the tyrosine side-chain orientates preferentially towards the *N*-terminus of the chain.[707] One report on H-(L-Ala)$_n$-L-Pro-OH (n = 1—3) considers that the conformation is determined *first* by electrostatic considerations and then stabilized by hydrogen-bond formation.[708]

The study of small peptides yields much detailed structural information, so it is not surprising that small fragments of proteins receive attention. Rotamer populations in di- and tri-peptides originating in wool, and their pH dependence, come under this heading,[709, 710] as do the *C*-terminal tripeptides of oxytocin and vasopressin.[711] Non-exponential longitudinal proton relaxation for the methionine methyl group in the *C*-terminal tetrapeptide of gastrin has been attributed to cross correlations between the methyl proton spin pairs,[712] and further measurements in dimethyl sulphoxide [713] point up the possibility of obtaining conformational data from such relaxation measurements if a dipolar relaxation mechanism is assumed. A long series of studies on tetrapeptides of elastin has been reviewed,[714] and a type II β-turn is demonstrated for repeating tetra- and penta-peptides, of the form X-Pro-Gly-Y-(Z) of the same protein,[715] and the studies are extended to give complete ^{1}H and ^{13}C assignments of the polyhexapeptides of elastin HCO-Val-(Ala-Pro-Gly-Val-Gly-Val)$_{18}$-OMe,[716] which has a right-hand β-spiral structure of *ca.* 212 repeats per turn, including in each repeat a β-turn and a γ-turn, a seven-atom turn which has been observed also in Boc-Gly-Val-Gly-OMe [717] and is considered an independent structural feature. The same laboratory also reports conformations of tetrapeptides of tropoelastin [718] and some measurements on the internal mobility of elastin itself, measured using ^{13}C resonance.[719] β-Turns as a common structural feature of

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proteins have been subjected to model studies in spin-labelled tetrapeptides[720] and have been found in an opiate-like pentapeptide which adopts a conformation similar to that of morphine.[721]

Synthetic polypeptides as models of proteins in n.m.r. have largely fallen from favour now that high-field Fourier transform spectrometers have made study of real proteins so much more straightforward (or at least less impossible) but still find some specialized application, notably in a study of the structure of poly-proline[722] and of poly-L-valine solubilized in aqueous solution by flanking blocks of poly DL-lysine.[723]

Peptides.—Reports on natural peptides published in 1976 include work on thyrotropin-releasing factor[724] and bradykinin;[725] an interesting 250 MHz study originating in China[726] finds that the conformational changes observed on splitting off the terminal pentapeptide of insulin affect its aggregation behaviour but not, apparently, its activity. Myelin basic protein is shown to bind to the red component of trypan blue (an azo dye) by a metachromatic mechanism,[727] and the same protein shows aggregation, involving part of the chain only, at pH 7 but not at pH 4.[728] Some ring-current shifted resonances observed in the muscle protein troponin appear to depend on the presence of calcium, indicating that calcium binding alters the conformation of the molecule.[729] Collagen molecules have also been investigated[730] by ^{13}C resonance: evidence for rapid anisotropic molecular motion is presented.

Peptide hormones remain a fruitful field for the n.m.r. investigator. A 250 MHz study of oxytocin in H_2O, with 'blind' decoupling under the water resonance to assign NH resonances to individual α-CH resonances,[731] also demonstrated transfer of saturation from solvent protons to NH groups and the aromatic protons of tyrosine, indicating the intimate contact of both with the solvent. Experiments on the interaction of oxytocin and vasopressin with neurophysin, using ^{13}C labels, led to an estimate of the internal mobility of the bound hormone.[732] Hyperactive and hypoactive derivatives of luteinizing hormone-releasing hormone failed to reveal any significant spectral changes, either in flexibility as measured using T_1, or in chemical shift, which might help to account for their abnormal activity.[733] The conformation of methionine enkephalin (Tyr-Gly-Gly-Phe-Met) has been discussed in two papers. Measurements of ^{13}C longitudinal relaxation are reported, and a head-to-tail interaction

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proposed in one,[734] while the other reports free rotation of the phenylalanine and methionine side-chains, but restricted motion of the tyrosine ring which may point to a similar overall conformation.[735] Alamethicin has been shown [736] to be a linear polypeptide of 19 residues, and ^{13}C chemical shift and T_1 measurement for Pro^3,Pro^5-angiotensin-II shows conformational heterogeneity in solution.[737] ^{13}C assignments and biosynthesis studies of aflatoxin B1 and sterigmatocystin have been reported.[738]

Nuclear Overhauser enhancement (NOE) is a relatively little used phenomenon in biological n.m.r. The mechanism of intramolecular NOE has been discussed for peptides and depsipeptides [739] and employed in a study [740] at two frequencies of valinomycin in dimethyl sulphoxide, where the enhancement was found to be positive at 90 MHz and negative at 250 MHz, indicating a predominantly dipolar relaxation mechanism. The analysis was used to evaluate various previously predicted models for the structure, favouring that proposed by Patel and Tonelli.[741]

Non-haem Proteins.—Moving on to larger peptides, one that has received considerable attention is the basic pancreatic trypsin inhibitor (BPTI). Of the 16 protons belonging to the four tyrosines of this molecule, 15 have been assigned and the tyrosine pK_a values evaluated at 9.9 (Tyr-10), 10.6 (Tyr-21), 11.6 (Tyr-23), and 11.0 (Tyr-35).[742] The same paper also reports assignment and titration of nitrated tyrosines, a study which is extended [743] following the discovery that nitrotyrosine residues chelate lanthanides with stability constants of 50—150 l mol^{-1}, and a subsequent assignment of some resonances from buried NH groups following a comparison of lanthanide effects on the spectrum with the interatomic distances determined by *X*-ray crystallography. The aromatic resonances of BPTI were also assigned by another group working at 360 MHz [744] in a study which confirmed that the structure adopted by the molecule in crystal form was largely maintained in solution; such differences as did exist were ascribed to the intermolecular interactions in the crystal lattice.[745]

Relaxation mechanisms have again been a subject for discussion in a two-frequency natural-abundance ^{13}C study of lysozyme.[746] It is concluded that for aliphatic and methine carbons ^{13}C—H dipolar relaxation mechanisms should be dominant at all magnetic field strengths currently available for n.m.r. From the

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same group comes a discussion [747] of the use of ^{13}C longitudinal relaxation times for measuring the rotational correlation times of proteins, with the use of an isotropic rigid model discussed and compared with results from several proteins. The mobility of one of the first proteins ever studied, serum albumin, has been measured under a wide range of solution conditions [748] by spin-echo T_2 experiments. The same protein has also been subjected to binding studies with aliphatic sulphates [749] and, surprisingly, on denaturation.[750]

To quote another reviewer of the n.m.r. of biomolecules,[751] the spectrum of enzymes being investigated is now so wide that the next section of the review must inevitably read something like an enzyme catalogue. There may, of course, be more than one reason for this.

A 68 MHz study of lysozyme,[752] long a favourite enzyme, yields titration curves for carboxyl carbon atoms which may be useful in assignment. Ribonuclease remains another favourite for investigation, although the methods get more subtle. The long-standing debate on the assignment of the resonances of His-119 and His-12 appears at last to be settled by a study [753] of RNase S using ^3H and ^2H exchange and titration to reverse the original assignments. This is followed by studies [754] of the separated S-peptide and S-protein; in the latter, inflexions in the titration curves of His-48 and His-119 disappear, a change ascribed to the absence of Asp-14 in the first case and to a change in overall molecular conformation in the second. Guanidination of the lysine residues of RNase A may be carried out at nine or ten sites, the odd residue being Lys-41. Although the two modified enzymes have the same structure [755] the 10-guanidinated molecule loses its activity, pointing to an involvement of this residue in the activity. Guanidyloribonuclease has been the subject of two other studies.[756, 757] A ^{31}P examination of the interaction 3′-UMP and 2′-, 3′-, and 5′-CMP with RNase A showed pH dependence of phosphorus chemical shift, one part of which is associated with the ionization state of His-12 and points to the close association of this residue with the phosphate.[758] The smaller ribonuclease-T_1 has also been complexed with a monophosphate, 3′-GMP, which is found to complex with His-40, while the N-7 of the purine base probably hydrogen-bonds to His-92, which is also in the active site.[759] Another old favourite, α-chymotrypsin, has been subjected to binding studies with 4-bromomercuricinnamic acid, which provides labels for both ^{79}Br and ^{81}Br resonance

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studies,[760] and another study which shows that the transition-state analogue *trans*-cinnamaldehyde is not covalently bound.[761] Complexes of α-chymotrypsin with phosphorus-containing molecules reveal that di-isopropyl phosphoryl α-chymotrypsin has two slowly exchanging isomers, and that the phosphate ester of catechol cyclic phosphate α-chymotrypsin reacts with the enzyme to open the five-membered phosphate ring.[762] pK_a values of the four histidines of porcine trypsin are reported [763] as 7.21, 6.71, 6.67, and 5.03, the last of these being assigned to the active site His-57.

Enzyme–substrate interactions continue to justify their earlier promise as a subject for n.m.r. Creatine kinase, in a manganese-ADP or -ATP substrate–enzyme complex, showed an alignment of the transferable phosphate with the acceptor moiety, with the bivalent metal probably liganded to the α and β phosphates of the nucleotide.[764] Intermolecular NOE, apparently involving an arginyl residue, has also been demonstrated for the ADP–creatine kinase complex.[765] An impressive trio of papers [766–768] describes in great detail the experimental evidence leading up to a model of the Mn-ATP–pyruvate kinase substrate complex. It seems that these complexes lend themselves to the most subtle and advanced n.m.r. investigations. Another is of yeast phosphoglycerate kinase, using difference spectra and the information that the lanthanide-ATP inhibitors of the enzyme bind identically to the manganese-ATP substrate. The lanthanide-ATP conformation was unchanged on binding to the enzyme, while the binding of 3-phospho-D-glycerate altered the conformation of the enzyme but not the general enzyme–metal–nucleotide arrangement.[769] The ^{31}P spectra of the Mg-ATP–arginine kinase–arginine and Mg-ADP–arginine kinase–phosphoarginine complexes gave separate resonances for the phosphates of ATP, ADP, and phosphoarginine, thus enabling evaluation of the reaction rates (192 s^{-1} in the forward and 154 s^{-1} in the reverse direction).[770] Two papers [771, 772] describe studies of the binding and structure of the propionyl-coenzyme A–transcarboxylase complex. The use of simultaneous equations from ^1H and ^{31}P n.m.r. using Co^{II}, Cu^{II}, and Zn^{II} ions, coupled with a search among the possible structures, gave a unique conformation for the propionyl-coenzyme A.[771] The proximity and spatial arrangement of a spin-labelled ester of coenzyme A and pyruvate on transcarboxylase were further established using a combination of n.m.r. and e.p.r. measurements.[772] E.p.r. was also combined with n.m.r. in studies of Mn^{II}-succinyl-coenzyme A synthetase from *E. coli*.[773] Perhaps because of their complexity and the united effort needed for their

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pursuit, enzyme–substrate studies often seem to appear as pairs of papers – another such pair [774, 775] describes the binding of fluorinated and ^{13}C-labelled carbamoyl phosphate to aspartate transcarbomoylase and its catalytic subunits, and the structural changes consequent on adding succinate to form a ternary complex. The authors point out, from comparisons with other C_4 dicarboxylic acids, the importance of an unhindered carbonyl group with trigonal symmetry to the operation of the enzyme. Glutamate alanine transcarbamylase is the subject of a report [776] on the deuteriation of the α and β carbons of alanine, which it catalyses. The same exchange reaction in alanine is also described in another paper, where glutamic pyruvic and glutamic oxaloacetic transaminases were used to catalyse in addition the deuterium exchange of the β-H atoms of pyruvate and fluoropyruvate, and of only *one* of the α-H atoms of glycine.[777] The coenzyme phosphopyridoxal trifluoroethylamine, bound to aspartate transaminase, exhibits a fluorine resonance which has a titration shift at a pK_a of 8.4, possibly due to the titration of a lysine at the binding site.[778]

Several studies of alkaline phosphatase have been published; it has been studied in the form of Zn and Co derivatives of apoenzyme [779] and by ^{31}P resonance of the binding of inorganic phosphates.[780] Another ^{31}P study [781] leads to a proposal for the binding of phosphates and phosphonates to four different alkaline phosphatases from *E. coli*, involving co-ordination of the phosphate by the metal ion and unusual strain in the bond angles of the phosphoserine intermediate. The uncomplexed alkaline phosphatase molecule has been modified by *in vivo* enrichment of γ-positions of histidine residues to yield a useful probe,[782] and fluorination of tryptophan and tyrosine residues to give another, with separate resonances for the fluorines of different tryptophans.[783] The possibility of using ^{113}Cd resonance as a probe into the number or nature of co-ordinating ligands has been explored, for alkaline phosphatase and for bovine and human carbonic anhydrase B.[784] Another probe into carbonic anhydrase structure has been the ^{13}C monocarboxymethylation at His-200 of human carbonic anhydrase B. The probe revealed titration shifts with pK_a's of 6.0 and 9.2, identified as His-200 and a water ligand of the zinc ion; binding of inhibitors appeared to affect the ^{13}C chemical shift at pH 7.9.[785]

A study of the complex between 3-*O*-methyl pyridoxal 5′-phosphate and rabbit skeletal muscle glycogen phosphorylase *b*, using ^{31}P and ^{1}H resonance, resulted in the conclusion that there is no proton shuttle between the 3-OH and

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[785] D. J. Strader and R. G. Khalifah, *J. Amer. Chem. Soc.*, 1976, **98**, 5043.

the pyridine nitrogen.[786] The stereochemistry of the activity of sheep liver threonine dehydratase has been studied,[787] as has the binding of ^{13}C-labelled pyridoxal 5′-phosphate to D-serine dehydratase and to L-glutamate decarboxylase.[788] Chemical modifications to the labelled molecule cause ^{13}C shift changes greater than 100 p.p.m., making it a sensitive indicator for enzyme-binding studies. Labelling as a probe into enzyme binding also featured[789] when a complex was formed between an active-site peptide of thymidylate synthetase, produced by enzymatic degradation, covalently linked to 5-fluoro-2′-deoxyuridylate and to 5,10-methylenetetrahydrofolate. Another synthetase, this time for glutamine, has been studied in its Mn^{2+}–glutamate complex by paramagnetic relaxation enhancement, using methionine sulphoxime as a competitive inhibitor; there appear to be two binding sites for manganese ions, at least 6 Å apart, and e.p.r. measurements confirm that the environment of the metal ions changes on sulphoxime binding.[790, 791] Relaxation enhancement measurements on threonine-sensitive aspartokinase complexed with ^{13}C-enriched threonine yields the distance of 4.4 Å between the metal ion and the carboxy-group of the amino-acid.[792]

An interesting result on triose phosphate isomerase is the assignment of three histidines (residues 95, 115, and 185) which do not titrate in the pH range 5.4—9.[793] Workers whose proteins denature or precipitate readily will perhaps envy a little the technique of unfolding and refolding the protein in D_2O, to exchange slowly-exchanging NH groups, employed in this work. Two studies of carp muscle parvalbumin appeared in 1976 – one covering the mobility of phenylalanyl side-chains[794] and the other also noting rapid motion in these residues, and a Ca^{2+}-controlled conformational transition.[795] Also the subject of two communications is azurin, both in its natural copper-containing form, and as a cobalt derivative in which some proton resonances are shifted by more than 30 p.p.m. It appears that one histidine is close to, and one far from, the metal atom.[796, 797]

An extremely attractive and so far almost untapped field for n.m.r. investigation is that of the protein toxins of snake venoms. These are of almost ideal size (60—70 residues in most cases), contain all the aromatic residues, mostly in ones and twos, and have conformations restricted by up to four disulphide bridges. A large number of sequences are available, and one or two crystal structures; the major disadvantage seems to be that of availability, since their biological activity in the nervous and other systems, the details of which are

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largely unknown, makes them of great biological interest. A paper has appeared on the neurotoxin II of *Naja naja oxiana*[798] in which all the aromatic resonances are assigned, histidines titrated, and chemical modifications effected; it seems certain to be the first of many.

Of other non-iron proteins covered in the 1976 literature, we may mention ^{31}P studies of phosphoglycopeptides from rat brain glycoprotein,[799] evidence for co-operative ionization of Asp-158 and His-159 in papain,[800] and the location of the binding site of α-methyl D-pyranoside on concanavalin A relative to the metal binding site.[801] Fluorine resonance was used to establish the presence, in *E. coli* lac repressor modified with 3-fluorotyrosines, of four or five surface tyrosines, two buried and one ionized or H-bonded for each of the four subunits of the protein.[802] Proton resonance at 360 MHz of porcine pancreatic colipase showed some ring-current-shifted resonances which shifted slightly on association with sodium taurodeoxycholate micelles, while the spectrum as a whole broadened.[803] A complex between Gd^{III} phospholipase A2 and monomer and micellar alkylphosphorylcholines was studied using paramagnetic relaxation enhancement to suggest that the active site is removed from the enzyme/micelle interface.[804] The longitudinal and transverse relaxation times of ^{35}Cl bound to lactate dehydrogenase have been reported.[805]

Iron-containing Proteins.—The n.m.r. of haemoglobin continues to attract considerable attention. A major review of the field was published at the end of 1975.[806] Resonances shifted by the presence of the paramagnetic iron and the huge π-system of the porphyrin ring provide useful probes into the state of the molecule: one such, assigned to the β-deoxy subunit, was found to reflect the oxygenation state accurately, and the linearity of the oxygenation–peak intensity relationship suggested that there was no large difference in oxygen affinity between the α and β subunits. The same paper describes a special tube insert for the optical measurement of oxygenation and methaemoglobin formation.[807] A method of following resonances whose movement through the oxy–deoxy titration of haemoglobin is discontinuous has been presented;[808] it depends on the exchange rate between the states being faster than T_1^{-1} for the resonance.

A commonly used probe for haemoglobin studies is ^{13}CO, although[809] $C_2H_5{}^{13}CN$ provides a more sensitive probe. ^{13}CO has been used in a comparative study of trout haemoglobin I and IV[810] and in studies showing the non-equivalence

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of α- and β-chains[811] and some sensitivity of the haem environment of α-chains to the tertiary structure of β-chains.[812] Variations in solvent proton relaxation have been used to establish, by measurements at various Larmor frequencies, a value of 6.5×10^{-10} s for the rotational correlation time of high-spin Fe^{3+} ($S = \frac{5}{2}$) human fluoromethaemoglobin after correction for the outer-sphere paramagnetic contribution[813] and some details of the haem accessibility in leghaemoglobin of *Lupinus luteus*.[814] Various human haemoglobins have been subjected to ^{35}Cl nuclear quadrupole relaxation measurements on Cl^- ions in solution. A strong linewidth dependence on the Cl^- concentration suggests binding sites, more so with the deoxy than with the oxy form.[815] Relaxation enhancement studies have shown[816] that the haem pocket of a nitroxyl-labelled haemoglobin widens on inositol hexaphosphate (IHP) binding, and that[817] IHP appears to block access to the haem group in isolated sheep methaemoglobin C, but not in the A or B variants. A line of enquiry may be opened up by the downfield shifts exhibited by the ^{31}P resonances of diphosphoglycerate and ATP on binding to haemoglobin.[818] The co-ordinated imidazoles in low-spin ferric haem complexes are naturally shifted and broadened; the visible proton signals from them are assigned to non-equivalent 5-CH_2 protons.[819]

The differences between normal and abnormal haemoglobins can, of course, be very informative. Hb Milwaukee has a mutation (β67 Val → Glu) which inhibits oxygenation of the β subunit, so that changes in the spectrum from β subunit resonances on oxygenation of the molecule may be ascribed to changes in the β-haem environment consequent upon α oxygenation. This is used[820] to decide between different models for the co-operativity. The abnormal Hb Malmo (β97 FG4 His → Gln) has very different shifted resonances from normal in the oxy and carbonmonoxy forms, but shows very little difference in the deoxy form, suggesting that it assumes a normal 'deoxy' quaternary structure when unliganded.[821] In 'leukaemic' haemoglobin labelled with [^{15}N]glycine, two resonances at 81.4 and 90 p.p.m. were assigned to glycine residues hydrogen-bonded to water and to a carbonyl group, respectively.[822] In studies of water in sickle α-erythrocytes, relaxation measurements were used to suggest a hydrophobically driven sickling mechanism[823] and haemoglobin S gelation was detected in agreement with optical methods (birefringence and turbidity).[824]

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Myoglobins have been subjected to further study by ^{13}C resonance both at natural abundance,[825] where tyrosine titration, following peak assignments, enabled recognition of surface and buried tyrosine resonances, and by specific enrichment of methionyl residues[826] enabling assignments of two methionyls (55 and 131) and motional measurements on them.

Like haemoglobins and myoglobins, cytochromes come in many varieties. A comparison between animal and yeast cytochrome *c* has been reported, in which the sixth ligand of the iron, Met-80, is among the resonances assigned.[827, 828] The temperature variations of the tyrosine resonances in several different ferrocytochrome *c*[829] are consistent with hindered rotation and are similar for all the molecules tried, arguing for a similar molecular structure. Some ^{13}C spectra of cytochrome *c* and carboxymethyl derivatives[830] show several carbonyl resonances with complex pH dependence, for which a simple model is proposed, while high-frequency studies of slow-exchanging NH resonances fail to reveal any resistant cluster with particularly slow exchange rates.[831]

In low-spin ferric cytochrome b_5, some contact-shifted resonances have been identified in a series of experiments involving, among other techniques, comparison with molecules containing deuteriated haem IX. Two different molecular species were observed.[832] Cytochrome b_5 has also been studied in complex with phosphatidylcholine vesicles,[833] and similar complexes with phospholipid vesicles have been studied *via* ^{19}F resonance with trifluoracetylated cytochrome *c*.[834] Proton spectra at 360 MHz of cytochrome *c*-551 in the oxidized, reduced, and half-reduced forms revealed an atypical pattern of haem methyl resonances, and also led to the conclusion that Met-61 was co-ordinated to the haem iron in the oxidized and reduced forms, with a histidine being co-ordinated in the reduced form only.[835] Proton relaxation enhancement experiments on cytochrome P450 from rabbit liver suggest that the haem is more accessible in the ferric low-spin state than in either methaemoglobin or myoglobin.[836]

The pH dependence of the spin state of ferric horseradish peroxidase and horse heart myoglobin has been monitored using hyperfine shifted ^1H n.m.r. resonances. The transition midpoint was about pH 11 for the peroxidase and the average of the metmyoglobin acid and alkali forms at pH 9.4, with rapid exchange between the two forms.[837] Following ^{35}Cl and ^1H experiments on

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chloroperoxidase, it was concluded that the principal Cl^- binding site is not at the haem iron.[838] By paramagnetic relaxation enhancement of solvent water for a solution of beef liver catalase, it was shown that formate and acetate ions displace one molecule of water at the haem group.[839]

Histones and Other Nuclear Proteins.—Since histones appear to have no equivalent of an active site or active centre, they present somewhat different problems to the n.m.r. spectroscopist, and the technique is often used somewhat empirically, although there are signs of change. The self-aggregation of histones H1, H3, and H4[840, 841] has been studied by ^1H n.m.r., and that of H2B[842] by ^{13}C n.m.r.; in general, the results agree with those of previous experiments. A study of the ^{13}C longitudinal relaxation of histone complexes relevant to chromatin structure indicates[843] that the complexes have a rigid core with more flexible regions, presumably those which interact with DNA in nucleosome formation. The core of a nucleosome is made up of two each of histones H3, H4, H2A, and H2B, with the strongest complexes being formed as tetramers of H3 and H4 and oligomers of H2A and H2B. These complexes have been studied by 270 MHz ^1H n.m.r., showing evidence in both H3–H4[844] and H2A–H2B[845] of secondary and tertiary structure changes in comparison with the isolated molecules in their structured state. Outside the nucleosome are found the remaining histones, generally classified as very lysine-rich. Each seems to have a well-defined globular region, and studies of H1[846] and H5[847] localize this structure and provide the first steps towards its solution by n.m.r. The $\phi 1$ histone of sea-urchin sperm has been studied in association with DNA and in chromatin.[848] In a study of chromatin by ^{31}P resonance, the conclusion was that only the internucleosomal DNA is flexible enough to give rise to an observable signal.[849]

Two non-histone chromosomal proteins of the high mobility group (HMG) are reported,[850] both in structured form in solution and in association with DNA; the gene 5 protein has been studied in complex with oligodeoxynucleotides, showing intercalation of tyrosyl residues.[851]

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Water.—Some of the studies of water in protein systems published in 1976 are on lysozyme crystals, in which a significant proportion of the water behaves as free liquid,[852] in solution of lysozyme,[853] and in solid hydrated elastin, in which the water may be divided into bulk water, bound water, and true water of hydration.[854]

9 Mössbauer Spectroscopy

Contributed by D. P. E. Dickson

The main sources of literature information for this report are 'The Index of Publications in Mössbauer Spectroscopy of Biological Materials', by L. May, Department of Chemistry, The Catholic University of America, Washington, D.C., and 'The Mössbauer Effect Data Index', by J. G. and V. E. Stevens, University of North Carolina, Asheville.

In 1976 the majority of work reported involved ^{57}Fe Mössbauer spectroscopy on haem proteins and iron–sulphur proteins.

Haem Proteins.—There have been three main areas of interest in recent work on haem proteins: the co-ordination chemistry, symmetry, and electronic structure of the iron site; the investigation of various physical and chemical treatments and pathological conditions on haem proteins, often in a way that relates to their biological functions; and studies on model compounds containing the planar haem group which, it is hoped, will elucidate the properties of the more complex proteins.

Trautwein *et al.*[855] have shown that there are small but definite differences between the Mössbauer parameters of isolated α- and β-chains of deoxygenated human haemoglobin and those of the complete protein, in the range 4.2—100 K. By preferentially enriching one or other of the chains with ^{57}Fe it was possible to compare the parameters of the two subunits within the whole tetramer to the parameters of the fully enriched protein. In these circumstances no significant differences were observed.

Deoxygenated human haemoglobin has been observed in two states (R and T) with different oxygen affinities. Merli *et al.*[856] have obtained Mössbauer spectra of the protein in both states at 78 K. The Mössbauer parameters of the two states are slightly different reflecting changes in the electronic structure of the ferrous ion which cannot be directly related to the different conformational states of the molecule.

Cianchi *et al.*[857] have made a theoretical study of the anomalously large temperature dependence of the quadrupole splitting of the ferrous ion in oxyhaemoglobin and the similar but less marked behaviour in reduced haemoglobin. The effect is explicable in the case of the reduced protein by the mixing into the ground state of low-lying excited states as a result of spin–orbit coupling. This is not possible in the $S = 0$ case of oxyhaemoglobin, and the authors explain

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[857] L. Cianchi, M. Mancini, and G. Spina, *Lett. Nuovo Cimento*, 1976, **16**, 505.

the effect in terms of a model in which the O–O axis is at 60° to the haem plane, with the oxygen molecule free to rotate about an axis normal to the haem plane. This fits the experimental data well while an alternative model, in which the axis of the oxygen molecule lies parallel to the haem plane, leads to no temperature dependence of the quadrupole splitting.

Maeda *et al.*[858] have derived the orientation of the principal axis of the electric field gradient (EFG) tensor from single-crystal Mössbauer measurements on deoxyhaemoglobin. Theoretical estimates for the asymmetry parameter on the EFG from the experimental data lead to the conclusion that the principal axis of the EFG lies within 14° of the haem plane.

Eicher *et al.*[859] have evaluated the term scheme of the ferrous ion in sperm whale myoglobin and human haemoglobin with a Hamiltonian involving coulomb repulsion, ligand-field, and spin–orbit coupling terms. Relating these term schemes to the temperature dependence of the quadrupole splitting yields values for the energy separation of the levels which can be interpreted within crystal field theory to give information about the structural properties of the iron site. The results indicate that in myoglobin the iron lies 40 ± 10 pm above the haem plane, while in haemoglobin the out-of-plane distance is 10 pm greater. The bond length with the imidazole nitrogen is shorter in haemoglobin (200 ± 3 pm) than in myoglobin (212 ± 5 pm), suggesting a stronger interaction with the proximal histidine.

Mössbauer spectroscopy has been used by Sharrock *et al.*[860] to investigate the haem iron in the various states of cytochrome $P450_{cam}$ from the camphor hydroxylating system of *Pseudomonas putida*. The native camphor-free protein contains low-spin Fe^{3+}, part of which (50—70%) is converted into high-spin upon the addition of camphor. The experimental spectra for both spin states have been successfully simulated using a model based on crystal-field theory with simple covalency considerations. The native low-spin ferric state forms a complex with 2-phenylimidazole which leads to small changes in the Mössbauer and e.p.r. parameters that can be accounted for by the crystal-field model. The addition of putidaredoxin to the camphor-complexed oxidized cytochrome decreases the intensity of the high-spin component and changes its quadrupole splitting.

Reduced cytochrome $P450_{cam}$ contains high-spin Fe^{2+} in both the presence and absence of camphor. The complex of reduced cytochrome $P450_{cam}$ with O_2 is diamagnetic and shows a Mössbauer spectrum which is unusual for Fe^{2+} but similar to that of oxyhaemoglobin. These results are compatible with the bound superoxide Fe^{2+}–O_2^- model proposed for oxyhaemoglobin. Computer fits to spectra obtained in applied magnetic fields show that reduced cytochrome $P450_{cam}$ and its oxy- and carboxy-complexes are analogous in some respects to the corresponding haemoglobin complexes.

Nassif *et al.*[861] have obtained Mössbauer spectra from the undecapeptide of ferricytochrome *c* in the form of both solid and solid solution and at pH 1.5, 7,

[858] Y. Maedo, T. Harami, A. Trautwein, and U. Gonser, *Z. Naturforsch.*, 1976, **31b**, 487.
[859] H. Eicher, D. Bade, and F. Parak, *J. Chem. Phys.*, 1976, **64**, 1446.
[860] M. Sharrock, P. G. Debrunner, C. Schulz, J. D. Lipscomb, V. Marshall, and I. C. Gunsalus, *Biochim. Biophys. Acta*, 1976, **420**, 8.
[861] R. Nassif, C. Baumgartner, M. Sellers, and L. May, *Z. Naturforsch.*, 1976, **31c**, 232.

and 10. At low pH the iron is high-spin whereas under neutral and alkaline conditions it is low-spin. The authors suggest that the low pH form corresponds to a monomer and that polymerization occurs at higher pH.

The reaction of cytochrome *c* peroxidase with ethyl peroxide produces a compound ES which has been examined by Mössbauer spectroscopy over a range of temperatures and applied magnetic fields.[862, 863] The Mössbauer spectra have been interpreted in terms of a crystal-field model of the iron site in which the iron is in an Fe^{IV} state with $S = 1$. There is no observable Mössbauer component corresponding to the e.p.r.-visible free radical, implying that it is remote from the iron.

^{57}Fe-enriched oxy- and carboxy-complexes of haemoglobin have been photodissociated at 4.2 K and investigated by Spartalian *et al.*[864] using Mössbauer spectroscopy. The spectra show different chemical shifts and quadrupole splittings from those of the deoxy forms. The haemoglobin complexes exhibit narrow lines indicating a well-defined iron environment, where the myoglobin complexes and the deoxy proteins show broader lines. In the case of a photodissociated NO complex the spectrum indicates the possiblity of a magnetic interaction between the iron atom and the unpaired electron of the nearby NO group.

A medically oriented investigation has used Mössbauer spectroscopy to study the effects of *X*-radiation and heat treatment on oxyhaemoglobin in arterial red cells.[865] Irradiation with *X*-rays leads to the production of a ligand-free deoxy form and a high-spin ferric haemoglobin. With increased dosage the haem group is destroyed and a compound corresponding to a high-spin ferric ion in a rhombic environment results. Mössbauer spectra show that these same compounds, except the ligand-free deoxyhaemoglobin, are formed by the action of heat.

In the blood of persons suffering from thalassaemia there is known to be an excess of haemoglobin α-chains. Ofer *et al.* obtained Mössbauer spectra of diseased blood showing one third less absorption than that observed in healthy blood.[866] In addition to components corresponding to haemoglobin, the spectra of diseased blood provide evidence for the presence of large amounts of haemochrome. This is the first time that haemochrome has been associated with this disease.

Devyatkov *et al.*[867] have reported the effects of millimetre radiation on haemoglobin as observed by Mössbauer spectroscopy. Measurements on lyophilized rat blood haemoglobin showed that on irradiation at 7.1 mm wavelength with a power of 8 mW the spectrum changed from one consistent with the low-spin ferrous ion of oxyhaemoglobin to show a component characteristic of a high-spin ferrous ion.

[862] G. Lang, K. Spartalian, and T. Yonetani, *Biochim. Biophys. Acta*, 1976, **451**, 250.
[863] G. Lang, K. Spartalian, and T. Yonetani, *J. Physique*, 1976 **37** C6-217.
[864] K. Spartalian, G. Lang, and T. Yonetani, *Biochim. Biophys. Acta*, 1976, **428**, 281.
[865] C. Kellershohn, J. N. Rimbert, A. Chevalier, and C. Hubert, *J. Physique*, 1976, **37**, C6-185.
[866] S. Ofer, S. G. Cohen, E. R. Bauminger, and E. A. Rachmilewitz, *J. Physique*, 1976, **37**, C6-199.
[867] N. D. Devyatkov, V. V. Khrapov, R. E. Garibov, V. A. Kudryashova, V. I. Gaiduk, G. F. Bakaushina, A. M. Khrapko, A. A. Levina, and A. P. Andreeva, *Doklady Akad. Nauk S.S.S.R.*, 1975, **225**, 962.

Spartalian and Lang[868] have obtained Mössbauer spectra of an oxyhaem model compound, oxygenated *meso*-tetra-(α,α,α,α-*O*-pivalamidophenyl)-porphyrin between 4.2 K and room temperature. The results for the temperature dependence of quadrupole splitting are interpreted in terms of a conformational excitation corresponding to the relaxation of the oxygen molecule between the two orientations which are shown in *X*-ray crystallographic studies. Similarities with the Mössbauer spectra of oxyhaemoglobin and oxymyoglobin suggest that conformational excitation is also present in those proteins and is responsible for the temperature dependence of the quadrupole splitting.

Papaefthymiou *et al.*[869] have investigated a number of high-spin ferric porphyrin complexes in order to develop experimental criteria to distinguish between the different axial ligation possibilities of O, N, and S co-ordination in oxidized cytochrome P450.S. Their results support the designation of sulphur as the axial ligand in this protein.

Cohen *et al* have studied tetraphenylporphinatoiron(III) fluoride,[870] and observed that, depending on the preparation technique, two different solid phases with different magnetic properties can be obtained. The Mössbauer data show that one phase has $S = \frac{5}{2}$ while, in the other, apparently diamagnetic spectra are observed corresponding to antiferromagnetic coupling between the iron atoms to give a state with net spin of zero.

The synthesis and Mössbauer spectra of ferrous octaethylporphyrin complexes have been studied by Dolphin *et al.*[871] The iron is observed to be in an intermediate $S = 1$ state from Mössbauer spectra taken between 4.2 and 300 K and in applied fields of up to 5 T. The quadrupole splitting is observed to be positive with an axially symmetric EFG tensor. Information was obtained on the σ and π bonding characteristics of the quadridentate ligands, and the relationship to the biological function of the haem proteins was discussed. Fitzsimmons *et al.*[872] have studied the ferric octaethylporphyrin compound, octaethylhaemin, and have computer-fitted the spectra obtained at temperatures between 4.2 and 300 K using a spin-Hamiltonian formalism to yield the hyperfine field at the iron nucleus of 49.5 T and the *D* splitting of the axial ligand field of 8.0 cm^{-1}, with a positive quadrupole splitting of 0.93 mm s^{-1}. The difference between these spectra and those of corresponding haem proteins is interpreted as being due to differences in the spin–spin relaxation times of the ferric ions in the two cases.

Chang *et al.*[873] have used the self-consistent charge-extended Hückel procedure to calculate the chemical isomer shift in haemin. The calculated value of -0.374 mm s^{-1} compares well with the experimental value of -0.392 mm s^{-1}. The contribution of the core electrons to the chemical shift is composed of a number of terms of comparable magnitude and differing sign. It is suggested that variation in these terms may lead to the substantial differences in chemical shifts between the various haem compounds.

[868] K. Spartalian and G. Lang, *J. Physique*, 1976, **37**, C6-195.
[869] G. C. Papaefthymiou, R. B. Frankel, S. Foner, S. C. Tang, S. Koch, and R. H. Holm, *J. Physique*, 1976, **37**, C6-209.
[870] I. A. Cohen, D. A. Summerville, and S. R. Su, *J. Amer. Chem. Soc.*, 1976, **98**, 6813.
[871] D. Dolphin, J. R. Sams, T. B. Tsin, and K. L. Wong, *J. Amer. Chem. Soc.*, 1976, **98**, 6970.
[872] B. W. Fitzsimmons, J. R. Sams, and T. B. Tsin, *Chem. Phys. Letters*, 1976, **38**, 588.
[873] J. C. Chang, Y. M. Kin, T. P. Das, and K. J. Duff, *Theor. Chim. Acta*, 1976, **41**, 37.

Iron–Sulphur Proteins.—The work reported during 1976 pertaining to the iron–sulphur proteins also includes a number of studies on model compounds which simulate the active-site structure of the actual proteins.

The simplest of the iron–sulphur proteins is rubredoxin, which contains a single iron atom tetrahedrally co-ordinated to four cysteine sulphur atoms. The molecule contains no labile sulphur atoms as are found in the ferredoxins. Schulz and Debrunner [874] have carried out a complete analysis of the Mössbauer spectra of both redox states of the protein at low temperatures and in large applied magnetic fields. They computer-fitted the spectra using a spin-Hamiltonian to generate the theoretical Mössbauer spectrum followed by variation of the spin-Hamiltonian and hyperfine parameters to minimize the value of chi-squared between the experimental and theoretical spectra. This procedure yields values of the chemical shift, quadrupole splitting, and crystal field splitting as well as information on the components and relative orientations of the magnetic hyperfine coupling and EFG tensors for the ferric and ferrous atoms in the oxidized and reduced forms of the protein. The results obtained are consistent with those from a more qualitative 'stick-diagram' interpretation of the spectra previously given by Rao *et al.*[875] This confirms the usefulness of interpreting spectra using 'stick-diagrams', certainly as the first stage in a more complete analysis. Knowledge of the Mössbauer parameters of single ferric and ferrous ions in a tetrahedral sulphur environment is very useful when considering the more complex spectra of the iron–sulphur proteins which contain more than one iron atom per molecule.

Many iron–sulphur proteins, including four-iron ferredoxins, eight-iron ferredoxins, and high potential iron–sulphur proteins (HiPIPs), contain active centres with four iron atoms and four labile sulphur atoms, some proteins containing two such centres per molecule. Dickson *et al.*[876] have examined the Mössbauer spectra of four different proteins of this class and have deduced from the common behaviour that the active centre must be closely similar in all cases. By considering the changes in the spectra of proteins in the paramagnetic state (reduced ferredoxin, oxidized HiPIP), they deduced that two of the iron atoms in the centre must have their magnetic moments directed parallel to the applied field and the other two antiparallel. This separation of the iron atoms into two non-equivalent pairs is confirmed by a complete computer analysis of the spectra of the four-iron ferredoxin from *Bacillus stearothermophilus*. The authors conclude that in the oxidized state of the ferredoxin the iron atoms within the centre all have essentially the same valency state, intermediate between ferric and ferrous ('$Fe^{2.5+}$'), with one pair spin-up and the other pair spin-down to give the apparently diamagnetic behaviour. On reduction, the extra electron goes predominantly to one pair of iron atoms which become ferrous, the other pair being substantially unchanged. The centre then has a spin of $\frac{1}{2}$ and component Mössbauer spectra with positive and negative hyperfine fields are observed. In the case of the eight-iron ferredoxins (*e.g. Clostridium pasteurianum*

[874] C. Schulz and P. G. Debrunner, *J. Physique*, 1976, **37**, C6-153.
[875] K. K. Rao, M. C. W. Evans, R. Cammack, D. O. Hall, C. L. Thompson, P. J. Jackson, and C. E. Johnson, *Biochem. J.*, 1972, **129**, 1063.
[876] D. P. E. Dickson, C. E. Johnson, P. Middleton, J. D. Rush, R. Cammack, D. O. Hall, and K. K. Rao, *J. Physique*, 1976, **37**, C6-171.

ferredoxin) there is an additional magnetic interaction between the two spin-$\frac{1}{2}$ centres in the paramagnetic state which gives anomalous (apparently diamagnetic) spectra at low temperatures. In large applied magnetic fields this coupling is broken to give behaviour characteristic of each four-iron centre. Dickson *et al.*[876] have shown that the addition of a chaotropic agent can also decouple the two centres in zero field to give a spectrum like that of the four-iron ferredoxin.

Bogner *et al.*[877] have used a model for the four-iron centres in the eight-iron ferredoxin from *C. pasteurianum* in which all four iron atoms are equivalent. While this model can give an approximate fit to their Mössbauer spectra of the reduced protein in intermediate magnetic fields it is inadequate to explain the high-field spectra. The fit is not improved if a transmission integral is used to obtain the spectrum from the calculated nuclear energy levels rather than using the usual sum of Lorentzian lines approach.

A very interesting investigation of the Mössbauer spectra of iron proteins in the whole bacterial cell has been carried out by Bauminger *et al.*[878] Some of the measurements were obtained under *in vivo* conditions, the bacteria used being *E. coli* and *Halobacterium halobium.* The spectra obtained from frozen cells are very similar to those of isolated two-iron ferredoxins. Spectra were also obtained from unfrozen cells and these exhibited a large motional broadening.

Lane *et al.*[879] and Frankel *et al.*[880] have investigated the monoanion $[Fe\{(SCH_2)_2C_6H_4\}_2]^{1-}$, which exhibits a near-tetrahedral Fe^{III}–S_4 co-ordination with a Fe^{III}/Fe^{II} redox couple, and thus represents a structurally unconstrained model for the rubredoxin Fe–S_4 active centre. They have obtained Mössbauer spectra of single-crystal and frozen-solution samples over a wide range of temperature and applied magnetic fields which have been computer-fitted using a spin-Hamiltonian approach. The parameters obtained are very closely similar to those resulting from a corresponding investigation of rubredoxin.[876] This is interpreted as evidence against the entatic state hypothesis which says that the constraints resulting from the protein conformation give the iron active site unique physical and chemical properties.

Mössbauer investigations of another rubredoxin model compound containing the anion $[Fe(SPh)_4]^{2-}$ have been reported by Kostikas *et al.*[881] and Petrouleas *et al.*[882] Computer fits to the applied field spectra of the model compound yield spin-Hamiltonian and hyperfine parameters very close to those obtained for reduced rubredoxin, confirming that the anion constitutes a good synthetic analogue.

[877] L. Bognor, F. Parak, and K. Gersonde, *J. Physique*, 1976, **37**, C6-177.
[878] R. W. Lane, J. A. Ibers, R. B. Frankel, and R. H. Holm, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **72**, 2868.
[879] R. B. Frankel, G. C. Papaefthymiou, R. W. Lane, and R. H. Holm, *J. Physique*, 1976, **37**, C6-165.
[880] A. Kostikas, V. Petrouleas, A. Simopoulos, D. Coucouvanis, and D. G. Holah, *Chem. Phys. Letters*, 1976, **38**, 582.
[881] V. Petrouleas, A. Simopoulos, A. Kostikas, and D. Coucouvanis, *J. Physique*, 1976, **37**, C6-159.
[882] E. R. Bauminger, S. G. Cohen, I. Nowik, S. Ofer, Y. Yariv, M. M. Werber, and M. Mevarech, *J. Physique*, 1976, **37**, C6-227.

Other Proteins.—Biological iron storage and transport compounds have been reviewed by Oosterhuis and Spartalian.[883] A Mössbauer study of human serum transferrin in high applied magnetic fields and at temperatures down to 0.175 K has been reported by Tsang *et al.*[884] The spectra were fitted with a high-spin ferric spin-Hamiltonian to yield crystal field parameters ($D = 0.3\ cm^{-1}$, $\lambda = 0.126$) that are comparable with those obtained from e.p.r. The authors also conclude that the two iron-binding sites are identical as far as Mössbauer spectroscopy can determine.

Debrunner[885] has reviewed Mössbauer spectroscopic studies of enzyme systems. An investigation of the enzyme protocatechuate-3,4-dioxygenase from *Pseudomonas aeruginosa* has been reported by Que *et al.*[886] and Münck *et al.*[887] The spectra indicate that the iron is high-spin ferric in the native state of the enzyme and high-spin ferrous in the reduced state. The Mössbauer parameters suggest that the iron atom is not in a tetrahedral sulphur environment as found in the iron–sulphur proteins.

The only biological Mössbauer work reported in 1976 that does not use the ^{57}Fe Mössbauer nuclide is an investigation of tobacco mosaic virus (TMV) by Haffner *et al.*[888] using ^{129}I. The spectra of TMV with both Tyr-139 and Cys-27 iodinated were compared with the spectra of di-iodo-tyrosine (DIT). The effect of protonation on these compounds was also studied. The Mössbauer parameters of DIT were found to be sensitive to protonation with the DIT in TMV becoming protonated at pH 8. The relative amounts of iodine in tyrosine and cysteine in TMV were found to be stoicheiometric. The authors interpret the results as proving the existence of a hydrogen-bond involving the hydroxy-group of Tyr-139, and excluding the formation of a cysteine bridge at Cys-27.

10 Dissociation and Association of Proteins

Contributed by E. J. Wood

Analytical Ultracentrifuge.—*Techniques.* A useful collection of articles on analytical ultracentrifugation has appeared as the whole of Volume 5 of *Biophysical Chemistry*, celebrating 'Fifty Years of the Ultracentrifuge' (actually the proceedings of a conference held in Bethesda in February 1975). Sections are devoted to instrumental methods, density gradient centrifugation, the analysis of associating systems and of heterogeneous systems, and sedimentation analysis. Several of the articles deal with scanner-computer systems (see Vol. 7), and most of these systems feature on-line computing facilities. The system described by Williams,[889] for example, makes direct use of the instantaneous photomultiplier

[883] W. T. Oosterhuis and K. Spartalian, in 'Applications of Mössbauer Spectroscopy', ed. R. L. Cohen, Academic Press, New York, 1976, Vol. 7.
[884] C. P. Tsang, L. Bogner, and A. J. F. Boyle, *J. Chem. Phys.*, 1976, **65**, 4584.
[885] P. G. Debrunner, in 'Applications of Mössbauer Spectroscopy', ed. R. L. Cohen, Academic Press, New York, 1976, Vol. 1.
[886] L. Que, jun., J. D. Lipscomb, R. Zimmerman, E. Münck, N. R. Orme-Johnson, and W. H. Orme-Johnson, *Biochim. Biophys. Acta*, 1976, **452**, 320.
[887] E. Münck, R. Zimmermann, L. Que, J. D. Lipscomb, and W. H. Orme-Johnson, *J. Physique*, 1976, **37**, C6-203.
[888] H. Haffner, A. Andl, H. Appel, G. Büche, and K. C. Holmes, *J. Physique*, 1976, **37**, C6-223.
[889] R. C. Williams, *Biophys. Chem.*, 1976, **5**, 19.

output and incorporates a stepping motor to drive the photomultiplier for the scans. An elegant scanner–computer system was also described by Spragg and his co-workers,[890] a television scanner incorporating a commercially available optical multichannel analyser for multiple-cell operation was described by Rockholt *et al.*,[891] and a split-beam scanner which it was claimed had been in use for four years was described by Cohen *et al.*[892] Crepeau *et al.*[893] have tried to increase the sensitivity of centrifuge optical systems by designing a u.v. laser scanning and fluorescence monitoring system, again used with an on-line computer. In this system a laser light source is focused to a narrow beam (at $\lambda = 257$ nm using a standard ultracentrifuge cell, the average spot diameter was *ca.* 50 μm) which scans the cell. The exciting laser line must be blocked so that it does not interfere with the longer wavelength fluorescence emission: this was achieved by using a narrow piece of black tape set in the direction of scanning on a flat glass filter just in front of the photomultiplier tube. In preliminary experiments with this system the sedimentation of bovine serum albumin was followed in a solution of initial concentration 20 μg ml^{-1}. Potentially even lower concentrations could be used if the macromolecules are coupled to fluorescent labels excited in the visible region of the spectrum. Szuchet and Yphantis [894] have extended their work on equilibrium centrifugation in solutions of carboxylic acids (acetic, propionic, butyric). The aim of this work was to devise a general procedure for protein molecular weight determinations of useful accuracy in dissociating conditions which would allow the value of the partial specific volume, $\bar{v}_p$, to be used rather than requiring determination of ϕ'. The common practice of either neglecting or assuming an arbitrary correction factor in order to account for preferential interaction in the presence of, for example, guanidinium chloride, may be adequate for certain proteins but has been shown to lead to gross errors for others. The rationale behind this approach is that the short-chain aliphatic acids are almost neutrally buoyant in aqueous solutions and preferential interaction with these solvent components should not greatly affect the determined apparent molecular weights. Previously aldolase had been studied in acetic acid [895] and in the present work β-lactoglobulin A was examined in aqueous acetic, propionic, and butyric acids in the absence of any other electrolyte. Apparent molecular weights at infinite dilution differed by $<5\%$ from the formula weight of the monomer, confirming the feasibility of the method.

Data Evaluation. A powerful application of the sedimentation equilibrium method is in the analysis of associating systems in rapid reversible equilibrium. In the study of mixed associations, however, which necessarily involve three or more species, a single sedimentation equilibrium experiment will provide insufficient information for a unique analysis. This problem can often be overcome partly if other data (*e.g.* the molecular weights of one or more species) are

[890] S. P. Spragg, W. A. Barnett, J. K. Wilcox, and J. Roche, *Biophys. Chem.*, 1976, **5**, 43.
[891] D. L. Rockholt, C. R. Royce, and E. G. Richards, *Biophys. Chem.*, 1976, **5**, 55.
[892] R. Cohen, J. Cluzel, H. Cohen, P. Male, M. Moignier, and C. Soulié, *Biophys. Chem.*, 1976, **5**, 77.
[893] R. H. Crepeau, R. H. Conrad, and S. J. Edelstein, *Biophys. Chem.*, 1976, **5**, 27.
[894] S. Szuchet, and D. A. Yphantis, *Arch. Biochem. Biophys.*, 1976, **173**, 495.
[895] S. Szuchet and D. A. Yphantis, *Biochemistry*, 1973, **12**, 5115.

available. Roark [896] has described the use of sedimentation equilibrium experiments at different speeds during a single analysis to reduce the lack of uniqueness. Linear least-squares multi-speed fits are used to discriminate between association models in which molecular weights are assumed. Further discrimination is achieved by performing in addition a series of experiments at different initial concentrations.

Other contributions deal with the analysis of heterogeneous systems, including the determination of molecular weights and molecular weight distributions of non-ideal solutions.[897]

Nichol *et al.*[898] have considered the sedimentation equilibrium of heterogeneously associating systems and mixtures of non-interacting solutes. At equilibrium the distribution of total solute concentration as a function of radial distance is analysed to give the activity of the unbound ligand, A, which binds to a macromolecular acceptor B thus:

$$BA_{i-1} + A \rightleftharpoons BA_i \quad (i = 1, 2, ..., p)$$

as a function of total weight concentration. (A may of course be a macromolecule.) This information is used to evaluate the equilibrium constant(s) relevant to the system, and the advantage of the procedure is that it involves neither solution of simultaneous equations which are sums of exponentials nor differentiation of experimental data to obtain apparent weight-average molecular weights.

Examples of Associating Systems Examined. The self-association of β-lactoglobulin C was studied over a range of temperatures and in two different buffers.[899] Several models were used to test the self-association and a monomer–dimer association with $K_2 = 2.10 \times 10^3$ dl g^{-1} gave a good description of the curve of $M_1/\bar{M}_w$ *versus* concentration. The association was independent of temperature and ionic strength over the range tested, and in contrast to the behaviour of β-lactoglobulin A, self-association did not appear to proceed beyond dimer. Isozyme P-II of yeast hexokinase also exists in monomer–dimer equilibrium,[900] and the association constants for the monomer–dimer reaction decrease with increasing pH, ionic strength, and concentration of glucose. Glucose binding experiments under different experimental conditions indicated that the two glucose binding sites were equivalent and that dimer formation resulted from the homologous association of two identical subunits. The polymerization pattern of insulin at pH 7.0, which is of interest in relation to considerations of what is the biologically active species under physiological conditions, was reinvestigated by Jeffrey *et al.*[901] Up to a concentration of 0.8 mg ml^{-1} the results were consistent with several different polymerization patterns, and the analytical procedure was based on closed solutions formed by summing infinite series yielding a set of equilibrium constants for each pattern. Distinction between the patterns was

[896] D. E. Roark, *Biophys. Chem.*, 1976, **5**, 185.
[897] P. J. Wan and E. T. Adams, *Biophys. Chem.*, 1976, **5**, 207.
[898] L. W. Nichol, P. D. Jeffrey, and B. K. Milthorpe, *Biophys. Chem.* 1976, **4**, 259.
[899] J. L. Sarquis and E. T. Adams, *Biophys. Chem.*, 1976, **4**, 181.
[900] J. G. Hoggett and G. L. Kellett, *European J. Biochem.*, 1976, **66**, 65.
[901] P. D. Jeffrey, B. K. Milthorpe, and L. W. Nichol, *Biochemistry*, 1976, **15**, 4660.

achieved by analysing sedimentation equilibrium data obtained at a higher concentration range (up to 4 mg ml^{-1}) and taking into account the compositional dependence of activity coefficients. The favoured pattern was a dimerization (dimerization constant 11 × 10^{-4} l mol^{-1}) with isodesmic indefinite self-association of the dimer (association constant 1.7 × 10^{4} l mol^{-1}). Detergent-extracted cytochrome b_5 in aqueous solution appeared to exist as a mixture of monomer and octamer in gel filtration and sedimentation equilibrium studies,[902] but the monomer and octamer did not appear to be in rapid equilibrium.

Many studies on serum high-density apolipoproteins have shown that they have a tendency to self-associate. Data on the self-association of Apo-A-1 from human serum (monomer mol. wt. 28 000), for example, were best fitted by a model involving a monomer–dimer–tetramer–octomer system in rapid equilibrium.[903] The homologous proteins from the Rhesus monkey, in contrast, seemed to be best described by a rapidly equilibrating monomer–hexamer model.[904] In similar studies [905] human apo-A-II appeared to undergo a monomer–dimer–trimer association over the protein concentration range 0.8—1.5 mg ml^{-1}. The problem of the molecular weight of L-α-hydroxy-acid oxidase (published values vary from 89 000 to 430 000) was rationalized by the discovery from sedimentation equilibrium studies [906] that it self-associates. The monomer molecular weight (*i.e.* mol. wt. at infinite dilution) was 150 000, and the self-association could be described in terms of a monomer–dimer–tetramer relationship with $K_{\text{monomer-dimer}} = 5.4 \times 10^5$ l mol^{-1} and $K_{\text{dimer-tetramer}} = 1.7 \times 10^5$ l mol^{-1}.

Sickle-cell haemoglobin (HbS) was studied by sedimentation equilibrium methods with the aim of understanding the mode of action as anti-sickling agents of certain amino-acids and guanidine.[907] The non-ideality of concentrated haemoglobin solutions (up to 0.3 g ml^{-1}) was studied in detail. The dimer–tetramer association constants of the carbamoylated derivatives of haemoglobins A and S were measured by Williams and Kim [908] using an ultracentrifuge with modified scanner and on-line computer. They tentatively concluded that much of the change in oxygen affinity occurring upon carbamoylation could be accounted for without involving extensive structural changes in the unliganded molecule.

An interesting system studied by Blair and van Holde [909] is the association of the haemocyanin from an arthropod, *Callianassa*. They used the method developed by Dyson [910] in which the predicted and observed weight-average molecular weights are compared at each point in the cell, and the fraction of each species at each point is calculated. The monomer weight (431 000), the number of species to be utilized, and whether or not the system is to be considered ideal

[902] M. A. Calabro, J. T. Katz, and P. W. Holloway, *J. Biol. Chem.*, 1976, **251**, 2113.
[903] L. B. Vitello and A. M. Scanu, *J. Biol. Chem.*, 1976, **251**, 1131.
[904] D. L. Barbeau and A. M. Scanu, *J. Biol. Chem.*, 1976, **251**, 7437.
[905] L. B. Vitello and A. M. Scanu, *Biochemistry*, 1976, **15**, 1161.
[906] D. R. Phillips, J. A. Duley, D. J. Fennell, and R. S. Holmes, *Biochim. Biophys. Acta*, 1976, **427**, 679.
[907] J. A. Sophianopoulos, A. J. Sophianopoulos, J. S. Knowles, and R. J. Hill, *Arch. Biochem. Biophys.*, 1976, **173**, 517.
[908] R. C. Williams and H. Kim, *Biochemistry*, 1976, **15**, 2207.
[909] D. Blair and K. E. van Holde, *Biophys. Chem.*, 1976, **5**, 165.
[910] K. E. van Holde, G. P. Rossetti, and R. D. Dyson, *Ann. New York Acad. Sci.*, 1969, **164**, 279.

or non-ideal were input data. The association proceeded in two steps of which the first (monomer–dimer) was sensitive to Mg^{2+} concentration but temperature-insensitive, and the second (dimer–tetramer) was Mg^{2+}-insensitive but highly dependent on temperature. They suggest that this latter (high temperatures favouring association) may indicate that hydrophobic interactions play a major role in the dimer–tetramer reaction. Another haemocyanin system studied was the reversible reaction between whole (25S) and half (17S) molecules of lobster haemocyanin.[911] In this study it was demonstrated that it is feasible to detect and measure volumes of reaction in ultracentrifuge experiments even though direct measurements of changes in partial specific volume are practically impossible. Samples of the haemocyanin were subjected to accurately known nitrogen pressures in ultracentrifuge cells. The molecular weight was then determined at the liquid–gas meniscus by means of the Archibald method. The logarithmic dependence on pressure of the derived equilibrium constant then yielded the volume of reaction.

Gel Chromatography and Equilibrium Gel Permeation.—A single-photon counting spectrophotometer has been designed by Ackers *et al.*[912] for studying protein interactions by equilibrium gel permeation. Instead of scanning a chromatography column, the instrument records the optical densities of small flow cells packed with gel particles (*e.g.* Sephadex) which are at equilibrium with a solution whose properties are to be studied. For a mixture of interacting solutes the equilibrium gel permeation measurements yield the weight average of the species' partition cross-sections. Solute interaction parameters (stoicheiometry, equilibrium constants) can then be determined from the variation of this quantity with solute concentration. A single-photon counting spectrophotometer was developed because of the need to measure (i) very low absorbances, (ii) small differences between very large absorbances, and (iii) very high absorbances. For the instrument, using counting times of 10—1000 s and recording absorbancies of protein solutions at 220 nm, the standard deviation at an optical density of 4.0 was 0.005. The deviation from linearity in tests of Beer's law was less than these precision limits and, in addition, information could be obtained at protein concentrations of 1 μg ml^{-1} or less. This latter is important in the characterization of tightly associated systems: for example, for unliganded haemoglobin the equilibrium constant for dissociation of tetramers to dimers is near 10^{-10} M haem.

Cann and Hinman [913] have formulated a mass transport theory of the Hummel and Dreyer [914] gel chromatography method for measuring the binding of small molecules to macromolecules where there is ligand-mediated macromolecular association. Depending on the system, it is conceivable that association may enhance or even inhibit ligand binding, leading to an elution profile difficult to interpret. Cann and Hinman have calculated theoretical elution profiles for a large number of model systems to determine how the equilibrium constant and

[911] V. P. Saxena, G. Kegeles, and R. Kikas, *Biophys. Chem.*, 1976, **5**, 161.
[912] G. K. Ackers, E. E. Brumbaugh, S. H. C. Ip, and H. R. Halvorson, *Biophys. Chem.*, 1976, **4**, 171.
[913] J. R. Cann and D. Hinman, *Biochemistry*, 1976, **15**, 4614.
[914] J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 1962, **63**, 530.

the stoicheiometry of the binding reaction can best be determined. A typical profile showed a peak of ligand associated with the peak of macromolecule, followed by a peak of unbound ligand, in turn followed by a trough. They provide an explanation for this picture (which may indeed be diagnostic for ligand-mediated interactions) and also lay down guidelines for its unambiguous interpretation. The latter include the stipulations (i) that the area of the peak of bound ligand, rather than the area of the trough, should be used to calculate the ratio of ligand bound to macromolecule, (ii) that double-reciprocal plots should be avoided, (iii) that the intrinsic binding constant should be determined by extrapolating the apparent binding constant given by the Scatchard plot to infinite dilution of macromolecule, and (iv) that the extrapolation need not be linear. They go on to consider recently published data on the interactions of tubulin with GTP and with vinblastine in the light of their formulation.

The pH dependence of the apparent tetramer–dimer dissociation constants for haemoglobins A and Kansas in the oxy- and the deoxy-state were determined by Atha and Riggs[915] using gel chromatography and other methods. Hb Kansas is known to have an enhanced tetramer–dimer dissociation compared with that of HbA (normal human Hb). For HbA the dissociation constant decreased from 3.2×10^{-6} mol l^{-1} at pH 6.0 to *ca.* 3.2×10^{-8} mol l^{-1} at pH 8.5 and from the slope of the line it was calculated that dissociation was accompanied by the uptake of *ca.* 0.6 protons per tetramer. The corresponding dissociation constant for the deoxy-form increased linearly from 1.0×10^{-12} mol l^{-1} at pH 6.5 to *ca.* 1.0×10^{-5} mol l^{-1} at pH 11. This was associated with the release of *ca.* 1.6 protons. The dissociation constants for deoxy-Hb Kansas were identical with those for deoxy-HbA, but below pH 8.5 the constant for Hb Kansas was *ca.* 400 times greater than that of HbA in the absence of phosphate.

In a re-examination of nerve growth factor (NGF) over a wide range of concentrations by gel filtration, sedimention equilibrium, and sedimentation velocity, it was found that in aqueous solution NGF existed as a monomer–dimer system in rapidly reversible equilibrium.[916] At a concentration of 1 ng ml^{-1} (20—30 times the concentration shown to be biologically active) more than 99% of the material was however present as monomer, compared with 50% at 1.4 μg ml^{-1}. Gel filtration was used to study subunit interactions in the double-headed protease inhibitors from black-eyed peas.[917] A complex set of multiple equilibria was found. For example at pH 8.0 there was a rapid dimer–tetramer equilibrium, a distinct moderate rate dimer–tetramer equilibrium, and a very slow monomer–dimer equilibrium, the latter being unusual in that the dimer was stabilized by chaotropic ions. Gel filtration was used to show that upon binding two atoms of iron to a molecule of apo-transferrin to form the serum iron transport protein, transferrin, there was a change in the Stokes radius.[918] Differential sedimentation velocity measurements showed that there is a simultaneous change in the sedimentation coefficient of the same order of magnitude but of opposite sign.

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[916] M. Young, J. D. Saide, R. A. Murphy, and B. G. W. Arnason, *J. Biol. Chem.*, 1976, **251**, 459.
[917] L. S. Gennis and C. R. Cantor, *J. Biol. Chem.*, 1976, **251**, 747.
[918] P. H. Jarritt, *Biochim. Biophys. Acta*, 1976, **453**, 332.

Light-scattering.—Many reports have appeared in which intensity fluctuation spectroscopy of scattered laser light has been used to determine translational diffusion coefficients. The potential of this method for the determination of highly accurate values of D may be illustrated by reference to several recent papers on bovine liver glutamate dehydrogenase. Aggregation may play a vital role in the regulation of this allosteric enzyme, and its structure and properties have been reviewed.[919] Cohen and Benedek[920] developed their original models for analysing the detailed relationship between the distribution of enzyme polymers and the catalytic activity of the enzyme solution, and in a second paper[921] they compared predictions based on the models with the distribution of diffusion coefficients measured by light-scattering spectroscopy. The value of the polymerization constant obtained by this method was in good agreement with other published values, and they concluded that in the presence of ligands the mode of aggregation was one of reversible, linear end-to-end polymerization into rigid rods characterized by a single polymerization constant. Jullien and Thusius[922] performed similar experiments but obtained lower values for D_{monomer} and the polymerization constant. Cohen *et al.*[921] suggest that the lower value of D_{monomer} may result from the presence of small amounts of high molecular weight contaminant, and that this could have an effect on the polymerization constant. They also point out that light-scattering spectroscopy can be used to determine not only a mean diffusion coefficient, $\overline{D}$, from which relative amounts of monomer and polymer can be calculated, but also can be used to characterize the polydispersity of the proteins in solution.

Other workers have used laser-scattering spectroscopy to determine the diffusion coefficient of haemoglobin at concentrations approaching those in the human erythrocyte ($D = 4.25 \times 10^{-7}\ \text{cm}^2\,\text{s}^{-1}$, *cf.* $D^0 = 6.43 \times 10^{-7}\ \text{cm}^2\,\text{s}^{-1}$),[923] and for determining the electrophoretic velocity (and hence isoelectric point) of haemoglobin molecules in a specially constructed electrophoretic light-scattering chamber.[924]

Conventional (Rayleigh) light-scattering was used to study the self-association of deoxy-HbS.[925] Above concentrations of $2\ \text{g}\,\text{dl}^{-1}$ both M_n (obtained by osmometry) and M_w for deoxy-HbS were significantly different from the values for oxy-HbS, and the negative second virial coefficient of deoxy-HbS was consistent with self-association. In a study of the reaction of inositol hexaphosphate (IHP) with Hb by light scattering it was shown that the haemoglobin dimer can bind at least one molecule of IHP and that the nature of the binding was such as to prevent association to tetramers.[926] It was possible that the binding sites for IHP included half of the residues that bind IHP in the tetramer.

Transport Studies.—Claverie[927] has extended his theoretical treatment of the sedimentation of generalized systems of interacting particles to concentration-dependent sedimentation, and presented a simulation of a system exhibiting the

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[920] R. J. Cohen and G. B. Benedek, *J. Mol. Biol.*, 1976, **108**, 151.
[921] R. J. Cohen, J. A. Jedziniak, and G. B. Benedek, *J. Mol. Biol.*, 1976, **108**, 179.
[922] M. Jullien and D. Thusius, *J. Mol. Biol.*, 1976, **101**, 397.
[923] S. S. Alpert and G. Banks, *Biophys. Chem.*, 1976, **4**, 287.
[924] D. D. Haas and B. R. Ware, *Analyt. Biochem.*, 1976, **74**, 175.
[925] D. Elbaum, R. L. Nagel, and T. T. Herskovits, *J. Biol. Chem.*, 1976, **251**, 7657.
[926] S. L. White, *J. Biol. Chem.*, 1976, **251**, 4763.
[927] J.-M. Claverie, *Biopolymers*, 1976, **15**, 843.

Johnston–Ogston effect. Electrophoresis and molecular sieving are treated through the similarity of their continuity equations with the Lamm equation. The simulation procedure is capable of taking into account kinetically controlled reactions and non-ideality.

Edelstein *et al.*[928] have used their ultracentrifuge scanner–computer system to investigate a range of fish haemoglobins. The CO-Hbs of the species examined tended to dissociate much less than mammalian haemoglobins, and they concluded that the possession of unusually stable tetramers was a general property of fish haemoglobins (tetramer–dimer dissociation constant no more than 10^{-8} mol l^{-1}). As little was known of the properties of fish haemoglobins the sedimentation velocity method was used with a wide variety of experimental conditions. The computer system was set up to collect absorbance *versus* radius data for up to five cells at time intervals of 4 min or more. Successive data sets were treated by a subtraction method which, as well as removing much of the 'noise', yielded a series of roughly Gaussian curves with peaks at the radius positions corresponding to those of the sedimenting boundaries at times midway between the times of the two curves used in the subtraction (see also ref. 892). The data were then in a form that could readily be analysed by the computer to find the position of the peak of the difference curves. It was also arranged for there to be a current calculation, and continuous updating, of the sedimentation coefficient. When this value reached a desired accuracy the experiment was stopped.

The binding of immunoglobulin (IgG) to the C1q subunit of the first component of complement was studied by a sedimentation method.[929] Usually two ultracentrifuge cells were run simultaneously, one having a wedge window. One cell contained a mixture of C1q and IgG while the other contained C1q solution at the same concentration as a control. In the first cell two sedimenting boundaries (peaks) were observed, a slower one of IgG and a faster due to the IgG–C1q complex. After correction for the Johnston–Ogston effect (details appended to the paper), the amount of IgG bound in the complex could be obtained from the difference in areas between the complex peak and the control C1q peak. The accurate determination of binding parameters also required that sedimentation rates (corrected for hydrodynamic interactions) be determined. The equation of Bloomfield *et al.*[930] was used to calculate the translational frictional coefficient for a model of C1q. It seemed likely that more than ten IgG binding sites were present on the C1q molecule.

A number of investigators have used the sedimentation velocity method to study enzymes. For example, the cytidine 5′-triphosphate synthetase of calf liver had a sedimentation coefficient of 6.8S (mol. wt. 133 000), but in the presence of ATP or UTP the sedimentation coefficient increased to 10.1S (mol. wt. 263 000).[931] It is known that the corresponding bacterial enzyme also polymerizes in the presence of kinetically saturating levels of ATP and UTP. Another enzyme, glutaminase-asparaginase from *Pseudomonas* 7A, appeared to polymerize

[928] S. J. Edelstein, B. McEwen, and Q. H. Gibson, *J. Biol. Chem.*, 1976, **251**, 7632.
[929] V. N. Schumaker, M. A. Calcott, H. L. Spiegelberg, and H. J. Müller-Eberhard, *Biochemistry*, 1976, **15**, 5175.
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[931] R. P. McPartland and H. Weinfield, *J. Biol. Chem.*, 1976, **251**, 4372.

during zone sedimentation when the initial protein concentration was > 1 mg ml^{-1} and the buffer contained asparagine, glutamine, or 5-diazo-4-oxonorvaline.[932] In this work an extension of the active enzyme sedimentation method was used in which changes in absorption during hydrolysis of asparagine by low concentrations of enzyme were measured. Active enzyme sedimentation was also used to study the pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa* (34S).[933] It was shown that the previously identified filamentous aggregate of the enzyme ($\geqslant$121S) was inactive. Bartholmes *et al.*[934] showed by sedimentation methods that the apo-β_2 subunit of tryptophan synthetase of *E. coli* was monodisperse and dimeric down to a concentration of 0.02 mg ml^{-1} protein. *E. coli* nitrate reductase showed concentration-dependent sedimentation coefficients in sucrose gradients, ranging from 10S at low concentrations to 24S at high concentrations, suggesting that it is an associating–dissociating system.[935] Sedimentation velocity and other measurements were used by Powell and Brew[936] to investigate the interactions of bovine colostrum galactosyltransferase with ovalbumin and with α-lactalbumin which is the lactose synthase regulatory protein.

Several sedimentation studies on haemoglobins have appeared, including the measurement of the sedimentation coefficient of the liganded β-chains of human haemoglobin in the presence and absence of effectors,[937] and the observation that subunit dissociation in bullfrog tadpole haemoglobin was pH dependent over the range pH 8.0—10.5.[938]

Electron Microscopy.—Certain of the very large invertebrate haemoglobins have been studied by electron microscopy, for example that from the snail, *Heliosoma* (mol. wt. 1.7×10^6),[939] and those from the horseleech, *Haemopis*,[940] and a marine worm, *Eunice*[941] (both mol. wt. *ca.* 3.5×10^6). All appear to be ring-shaped molecules, although the snail protein is a ten-membered ring whereas the leech and worm proteins exist as stacks of a pair of six-membered rings. However, despite a clear electron microscopic characterization of these multisubunit structures, little information is available on the fundamental subunits. The size of the subunit(s) is not known with any certainty (although there is a suggestion that it might be 12 000 in the case of the worm haemoglobin),[942] and there is no sequence information for comparison with vertebrate haemoglobins and myoglobins. A review on the subunit structure of polychaete respiratory pigments has appeared.[943]

Other ring-shaped structures that have been investigated are that of the enzyme formiminotransferase-cyclodeaminase from pig liver (mol. wt. *ca.* 5×10^5)

[932] J. S. Holcenberg, D. C. Teller, and J. Roberts, *J. Biol. Chem.*, 1976, **251**, 5375.
[933] F. Widmer and N. O. Kaplan, *Biochemistry*, 1976, **15**, 4699.
[934] P. Bartholmes, K. Kirschner, and H.-P. Geschwind, *Biochemistry*, 1976, **15**, 4712.
[935] K. Lund and J. A. DeMoss, *J. Biol. Chem.*, 1976, **251**, 2207.
[936] J. T. Powell and K. Brew, *J. Biol. Chem.*, 1976, **251**, 3653.
[937] A. Salahuddin and E. Bucci, *Biochemistry*, 1976, **15**, 3399.
[938] T. Araki, K. W. K. Watt, and A. Riggs, *J. Biol. Chem.*, 1976, **251**, 4254.
[939] N. B. Terwilliger, R. C. Terwilliger, and E. Schabtach, *Biochim. Biophys. Acta*, 1976, **453**, 101.
[940] E. J. Wood, L. J. Mosby, and M. S. Robinson, *Biochem. J.*, 1976, **153**, 589.
[941] J. V. Bannister, W. H. Bannister, A. Anastasi, and E. J. Wood, *Biochem. J.*, 1976, **159**, 35.
[942] S. N. Vinogradov, B. C. Hall, and J. M. Shlom, *Comp. Biochem. Physiol.*, 1976, **53B**, 89.
[943] R. M. G. Wells and R. P. Dales, *Comp. Biochem. Physiol.*, 1976, **54A**, 387.

which exists as a planar ring of eight identical subunits,[944] and that of rat liver pyruvate carboxylase (mol. wt. 2.8×10^5),[945] which is a tetramer, the four subunits being arranged at the corners of a square. This latter enzyme was also investigated in the analytical ultracentrifuge and it was found that dissociation to trimers or dimers occurred at low temperatures and low protein concentrations.

Larger structures studied by electron microscopy include *Salmonella* flagellae,[946] the various polymorphic forms resulting from the assembly of tubulin (microtubule, twisted ribbon, sheet) at different pH values,[947] and those from the assembly of sickle-cell haemoglobin (HbS), [948] where the forms include linear strands of HbS molecules ('monofilaments'), helical fibres composed of multiple monofilaments, and larger associations of monofilaments into multi-stranded cables and two-dimensional sheets. The hand of the helical arrangement of subunits in the stacked-disc form of tobacco mosaic virus (TMV) protein has been determined,[949] and a TMV mutant was studied (PM6) [950] in which the coat protein was defective in that it could not encapsulate viral RNA. It did, however, form unusual wheel-like disks with a helical conformation. Another mutant virus studied was an aberrant bacteriophage lambda.[951] Such studies of large regularly arranged subunit arrays are important because many key processes such as enzyme regulation, assembly of complex subcellular structures, muscle contraction, *etc.*, are believed to be controlled by conformational changes in proteins. Shifts may thus lead to a 'domino effect' where a change in one subunit is transferred to all the other identical subunits hence becoming observable in the electron microscope. This becomes a very attractive proposition where multiple repetition of basic structural elements can be studied by optical filtration methods.[952]

Kinetic Studies.—*Protein–Protein Interactions.* Koren and Hammes [953] investigated the self-association of insulin, β-lactoglobulin, and α-chymotrypsin by means of stopped-flow and temperature-jump techniques, using changes in pH accompanying the self-association to monitor the course of the reaction with pH indicators. In each case conditions were chosen such that the reaction observed was a dimerization. The following values were obtained: for insulin at pH 6.8 and 23 °C, $k_1 = 1.14 \times 10^8\ \mathrm{l\,mol^{-1}\,s^{-1}}$, $k_{-1} = 1.48 \times 10^4\ \mathrm{s^{-1}}$; for β-lactoglobulin AB at pH 3.7 and 35 °C, $k_1 = 4.7 \times 10^4\ \mathrm{l\,mol^{-1}\,s^{-1}}$, $k_{-1} = 2.1\ \mathrm{s^{-1}}$; for α-chymotrypsin at pH 4.3 and 25 °C, $k_1 = 3.7 \times 10^3\ \mathrm{l\,mol^{-1}\,s^{-1}}$, $k_{-1} = 0.68\ \mathrm{s^{-1}}$. These kinetic data provide an independent determination of the monomer–dimer equilibrium constant and the values thus obtained were compared with published values. The association rate constant for insulin dimerization approached the value expected for a diffusion-controlled process,

[944] R. Beaudet and R. E. Mackenzie, *Biochim. Biophys. Acta*, 1976, **453**, 151.
[945] E.-M. Gottschalk, F. Mayer, A. Klostermann, and W. Seubert, *European J. Biochem.*, 1976, **64**, 411.
[946] H. Hotani, *J. Mol. Biol.*, 1976, **106**, 167.
[947] F. Matsumura and M. Hayashi, *Biochim. Biophys. Acta*, 1976, **453**, 162.
[948] R. Josephs, H. S. Jarosch, and S. J. Edelstein, *J. Mol. Biol.*, 1976, **102**, 409.
[949] A. C. Bloomer, J. N. Champness, and P. N. T. Unwin, *J. Mol. Biol.*, 1976, **105**, 453.
[950] J. J. Hubert, D. P. Boerque, and M. Zaitlin, *J. Mol. Biol.*, 1976, **108**, 789.
[951] M. Wurtz, J. Kistler, and T. Holm, *J. Mol. Biol.*, 1976, **101**, 39.
[952] 'Research News', *Science*, 1976, **192**, 360.
[953] R. Koren and G. G. Hammes, *Biochemistry*, 1976, **15**, 1166.

but the corresponding values for β-lactoglobulin and α-chymotrypsin were below those expected for a diffusion-controlled reaction. The dimerization of α-chymotrypsin was also studied using a stopped-flow method in which the enzyme initially at pH 7 is mixed with proflavin to give a final pH of 4.[954] There is a loss of enzyme active sites and a change in proflavin binding, monitored by a change in absorbance at 465 nm upon dimerization. The rate constants for formation and dissociation of dimer were 9.45×10^3 l mol^{-1} s^{-1} and 1.9 s^{-1}, respectively, under these conditions, and the kinetically-derived equilibrium constant (2.01×10^{-4} mol l^{-1}) compared well with that determined directly (1.44×10^{-4} mol l^{-1}).

The interaction between domains of the immunoglobulin molecule was studied by equilibrium (sedimentation equilibrium) and kinetic (temperature jump) methods [955] using the variable part of a Bence-Jones protein. This is of significance as the dimerization of light chains is an unwanted side-reaction in the study of the assembly of immunoglobulins from light and heavy chains, and may also be of physiological importance because it is believed that only monomeric light chains can bind to heavy chain dimers. The association and dissociation rate constants were 9×10^6 l mol^{-1} s^{-1} and 1.5×10^2 s^{-1} for the dimerization of the Bence-Jones fragments. However, the temperature-jump experiments showed the presence of two distinct kinetic phases. It was suggested that the reaction involved an association step followed by isomerization of the dimer, or the existence of two isomers of the monomer, of which only one type could dimerize, and the former explanation appeared to fit the data better. The two isomers of the dimer were present in almost equal amounts at equilibrium and the isomerization process had a half-time of *ca.* 0.15 s.

Fluorescence, stopped-flow, and enzyme activity measurements were used to study the kinetics of subunit dissociation and reassembly of rabbit muscle phosphofructokinase.[956] The dissociation of active tetramer in 0.8M guanidinium chloride appeared to occur in three phases: tetramer to dimer with a relaxation time of a few milliseconds, dimer to monomer with a relaxation time of a few seconds, and conformational change of the monomer with a relaxation time of several minutes. Under suitable conditions the monomers could be reassembled to give almost fully active enzyme. The renaturation process obeyed second-order kinetics and the regaining of activity was enhanced by the presence of ATP.

Several groups have made studies of the kinetics of the dissociation of human haemoglobin tetramers by the use of haptoglobulin binding. The dissociation of deoxyhaemoglobin tetramers into dimers is very slow with half-times of several hours, and it was shown that the absorbance changes in the Soret region that accompany the dissociation persist when the dimers bind (1 : 1) to haptoglobulin.[957] In the presence of 2,3-diphosphoglycerate the rate constant decreased in keeping with the proposed role of this compound in binding to both β chains thus stabilizing the deoxy-tetramers. In contrast, des-Arg-141-α-haemoglobin, in which half the constraining salt links in the dimer–dimer contact region are

[954] M. J. Gilleland and M. L. Bender, *J. Biol. Chem.*, 1976, **251**, 498.
[955] H. Maeda, J. Engel, and H. J. Schramm, *European J. Biochem.*, 1976, **69**, 133.
[956] G. R. Parr and G. G. Hammes, *Biochemistry*, 1976, **15**, 857.
[957] S. H. C. Ip, M. L. Johnson, and G. K. Ackers, *Biochemistry*, 1976, **15**, 654.

eliminated, the dissociation rate was increased by about three orders of magnitude. Combination of the dissociation rate constant for unmodified haemoglobin with the association rate constant determined by the stopped-flow method yielded a value for the equilibrium constant of 2.54×10^{10} l mol^{-1} (haem) in 0.1M-Tris–HCl, pH 7.4, containing 0.1M-NaCl and 1mM-edta at 21.5 °C. The intersubunit contact energy was −59 kJ per mol haem. This work was extended to determine the intersubunit contact energy changes between $\alpha\beta$ dimer pairs accompanying the binding of the first, the middle two, and the last oxygen molecules.[958] The results established that during the oxygenation–deoxygenation cycle, successive changes in ligand binding energy (the co-operative energy changes) are compensated by equal and opposite changes in the average intersubunit contact energy. In another study the reaction of haptoglobulin with haemoglobin covalently cross-linked between $\alpha\beta$ dimers (*i.e.* non-dissociable tetramers) was investigated by fluorescence quenching and peroxidase activity.[959] In an initial fast phase a complex was formed reversibly in which one of the $\alpha\beta$ dimers of the cross-linked haemoglobin was bound to one of the sites of the bivalent haptoglobulin molecule. In a second, slower step the product added another haemoglobin molecule or polymerized.

Protein–Small Molecule Interactions. Many papers have appeared on the kinetics of binding of ligands to haemoglobin. Sharma *et al.*[960] used the reaction between CO-Hb and reduced microperoxidase (which binds CO faster and more tightly than Hb) to determine the stepwise CO dissociation rate constants l_4, l_3, l_2, and l_1. The overall CO dissociation rate constant which they obtained was the same as the reported, statistically corrected value of l_4, and was not affected by the presence of 2,3-diphosphoglycerate. Compared to O_2-Hb the contribution to the co-operativity of the dissociation rate constants of CO-Hb was greatly reduced. DeYoung *et al.*[961] measured l'_4, the rate of combination of CO with the triliganded molecule by flash photolysis, and l_4, by ligand replacement. They found that L_4 ($= l'_4/l_4$) was pH-dependent and affected by 2,3-diphosphoglycerate. CO binding by a mutant haemoglobin, Hb(M)Iwate (α_2His → Tyrβ_2), was investigated.[962] The α_2(Met)β_2(CO) tetramer had a low affinity for CO which was bound non-co-operatively but the phosphate-free tetramer tended to dimerize extensively and appeared to have a high affinity for CO. Inositol hexaphosphate stabilized the tetramer and reduced the population of high-affinity dimers. CO binding has been used to investigate the rates of conformational change between rapidly reacting Hb* (the immediate product of complete photolysis) and normal (slowly reacting) deoxyhaemoglobin.[963] The rate of the ligand-free conformational change was 920, 6400, and 15 700 s^{-1} at 3, 20, and 30 °C, respectively, in borate buffers between pH 8.4 and 9.6. These results could be well fitted in terms of a two-state Monod–Wyman–Changeaux model which includes a decreasing of

[958] G. K. Ackers, M. L. Johnson, F. C. Mills, and S. H. C. Ip, *Biochem. Biophys. Res. Comm.*, 1976, **69**, 135.
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[961] A. DeYoung, R. R. Pennelly, A. L. Tan-Wilson, and R. W. Noble, *J. Biol. Chem.*, 1976, **251**, 6692.
[962] J. M. Salhany, C. L. Castillo, and S. Ogawa, *Biochemistry*, 1976, **15**, 5344.
[963] C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.*, 1976, **251**, 1533.

the rate of the conformational change by a constant factor for each bound ligand. Below pH 8 the behaviour was inconsistent with a two-state model. A new technique was used by Ferrone and Hopfield [964] to study the same conformational change. They used a mechanically chopped light beam to drive the CO–Hb out of equilibrium while a second beam passed through the sample to a photodetector. In this system, the sudden transient introduction of a non-equilibrium driving force (*e.g.* a photolysis flash) is replaced with a weak, periodic driving force. The populations of chemical species present then exhibit a periodic modulation whose amplitude and phase determine the kinetic constants. The kinetics of the binding of O_2 and CO to monomeric haem proteins have been investigated, for example, with myoglobin with a modified haem group,[965] and with the monomeric invertebrate haemoglobin from *Glycera dibranchiata*.[966] Haemoglobins with modified haem groups (deutero-, meso-) were also studied.[967] Moore and Gibson investigated the kinetics of dissociation of NO from haemoglobin using dithionite to remove free NO,[968] and concluded that if the equilibrium binding curve for NO could be determined experimentally it would show co-operativity with a Hill coefficient, n, at half-saturation of *ca.* 1.6. Other haemoglobin systems studied were the binding of inositol hexaphosphate to human methaemoglobin,[969] and the binding of NO and n-butyl isocyanide to human haemoglobin, where it was shown that the β-haems of deoxyhaemoglobin react much more rapidly with n-butyl isocyanide than the α-haems.[970]

A great deal is now known about the invertebrate respiratory protein, haemerytherin, including the amino-acid sequence [971] and the tertiary and quaternary structures.[972] Haemerythrin has a mol. wt. of 108 000 and consists of eight oxygen binding units each containing two non-haem iron(II) centres. The kinetics of the reaction of *Golfingia* haemerythrin with O_2 have been studied by stopped-flow.[973] At pH 8.2 and $I = 0.015$ mol l^{-1} over a range of protein concentrations the second-order association velocity constant, k_{-1}, was 51 s^{-1}. All the evidence indicated that the processes observed were simple and that octameric haemerythrin behaved kinetically as eight independent units. The kinetics of the reaction of met-haemerythrin with anions (N_3^-, SCN^-, F^-, Cl^-) have also been reported.[974]

Many reports have appeared dealing with the kinetics of binding of small molecules, including substrates and cofactors, to enzymes. The binding of glucose to the monomeric forms of yeast hexokinases P-I and P-II was investigated by taking advantage of the fact that binding is accompanied by quenching of intrinsic protein fluorescence.[975] In fluorescence temperature-jump experiments

[964] F. A. Ferrone and J. J. Hopfield, *Proc. Nat. Acad. Sci.* (*U.S.A.*)., 1976, **73**, 4497.
[965] M. Sono, P. D. Smith, J. A. McCray, and T. Asakura, *J. Biol. Chem.*, 1976, **251**, 1418.
[966] B. Seamonds, J. A. McCray, L. J. Parkhurst, and P. D. Smith, *J. Biol. Chem.*, 1976, **251**, 2579.
[967] D. W. Seybert, K. Moffat, and Q. H. Gibson, *J. Biol. Chem.*, 1976, **251**, 45.
[968] E. G. Moore and Q. H. Gibson, *J. Biol. Chem.*, 1976, **251**, 2788.
[969] J. S. Olson, *J. Biol. Chem.*, 1976, **251**, 447.
[970] P. Reisburg, J. S. Olson, and G. Palmer, *J. Biol. Chem.*, 1976, **251**, 4379.
[971] I. M. Klotz, G. L. Klippenstein, and W. A. Hendrickson, *Science*, 1976, **192**, 335.
[972] K. B. Ward, W. A. Hendrickson, and G. L. Klippenstein, *Nature*, 1975, **257**, 818; W. A. Hendrickson, G. L. Klippenstein, and K. B. Ward, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 2160; R. E. Stenkamp, L. C. Sieker, L. H. Jensen, and J. S. Loehr, *J. Mol. Biol.*, 1976, **100**, 23.
[973] D. J. A. De Waal and R. G. Wilkins, *J. Biol. Chem.*, 1976, **251**, 2339.
[974] D. R. Meloon and R. G. Wilkins, *Biochemistry*, 1976, **15**, 1284.
[975] J. G. Hoggett and G. L. Kellett, *European J. Biochem.*, 1976, **68**, 347.

only one relaxation time was observed with each of the isozymes, and the differences in stabilities of the complexes were attributable to glucose dissociating more slowly from its complex with P-I than of that with P-II. The data were incompatible with a slow 'prior-isomerization' pathway for substrate binding, but were consistent with a 'substrate-guided' pathway involving isomerization of the enzyme–substrate complex. Phosphofructokinase from rabbit muscle has also been studied in order to define the mechanism of the pH-dependent inactivation and reactivation and to correlate its regulatory kinetic and molecular properties.[976] For mitochondrial malate dehydrogenase it was found that the binding of NADH resulted in a small shift of the subunit dissociation curve towards monomer.[977] Binding of NAD^+ prevented dissociation of the dimer, indicating that the binding of reduced or oxidized cofactors results in different changes in conformation which are transferred to the subunit interface. Fluorescence energy transfer measurements were used to investigate the pyruvate dehydrogenase complex of *E. coli*.[978] Human erythrocyte pyruvate kinase showed a time-dependent activation in the progress curves, and evidence was presented indicating that this lag-phase resulted from a slow, phosphoenolpyruvate-mediated conversion of the enzyme to a more active form.[979] Single-photon counting pulse fluorometry was used to study certain complexes of calf liver glutamate dehydrogenase, the ternary complexes, enzyme–GTP–NADPH and enzyme–L-glutamate–NADPH, and the quaternary complex enzyme–GTP–L-glutamate–NADPH.[980] A theory for kinetic co-operativity in the concerted model for allosteric enzymes has been presented.[981]

An interesting development is the isolation of the lactose repressor protein from *E. coli* in sufficient amounts to allow the study of specific protein–sugar interactions responsible for the process of induction. The kinetics of the binding of the synthetic inducer, isopropyl β,D-thiogalactoside, were investigated with stopped-flow techniques, using the reporter group 2-mercuri-4-nitrophenol, as well as u.v. absorbance and fluorescence changes.[982]

Protein–Small Molecule Equilibria.—*Theory and Techniques.* Nichol and Winzor[983] have considered three models of ligand-induced polymerization, (i) dimerization of acceptor–ligand complex, (ii) cross-linking of monomer units *via* a ligand bridge to form dimer, and (iii) cross-linking to form linear chains. A binding equation was developed for each model, and the characteristics of the binding curves that emerged were correlated with those found by examining the dependence on ligand concentration of either $\bar{M}_w$ of the acceptor constituent, or a function of it. Practical implications of their findings are discussed in relation to experimental systems reported in the literature. Colosimo, Brunori, and Wyman[984] have discussed the ligand-linked conformational changes that

[976] P. E. Bock and C. Frieden, *J. Biol. Chem.*, 1976, **251**, 5630, 5644.
[977] J. D. Shore and S. K. Chakrabarti, *Biochemistry*, 1976, **15**, 875.
[978] G. B. Shepherd and G. G. Hammes, *Biochemistry*, 1976, **15**, 311.
[979] J. A. Badwey and E. W. Westhead, *J. Biol. Chem.*, 1976, **251**, 5600.
[980] J. C. Brochon, P. Wahl, J.-M. Jallon, and M. Iwatsubo, *Biochemistry*, 1976, **15**, 3259.
[981] A. Goldbeter, *Biophys. Chem.*, 1976, **4**, 159.
[982] B. E. Friedman, J. S. Olson, and K. S. Matthews, *J. Biol. Chem.*, 1976, **251**, 1171.
[983] L. W. Nichol and D. J. Winzor, *Biochemistry*, 1976, **15**, 3015.
[984] A. Colosimo, M. Brunori, and J. Wyman, *J. Mol. Biol.*, 1976, **100**, 47.

form the basis of many biological control systems, and have proposed the word 'polysteric' to distinguish the effects resulting from ligand-linked dissociation and association of a macromolecule from the more familiar allosteric ones (*i.e.* conformational change *without* change in molecular weight). There is in fact a close overall similarity between the two types of effect. When the number of binding sites is large, the induced co-operativity of the homotropic interactions can be very great even though the apparent free energy of interaction is arbitrarily small. Similarly the heterotropic control of the binding of one ligand by another can be very sharp. Looked at from another point of view the analysis can be thought of as a study of the control of macromolecular assembly. A new plot has been proposed by Watari and Isogai[985] for allosteric phenomena in place of the widely used Hill plot. They propose plotting $\ln[Y/(1-Y)] - \ln F$ as the ordinate (where F is the ligand concentration and Y is the fractional saturation) instead of $\ln[Y/(1-Y)]$. The Hill coefficient can then be determined with high accuracy. Dixon[986] has given a formula by which it is possible to calculate the extent to which a fairly tightly bound ligand can be removed from a macromolecule by gel filtration (where dialysis would be inefficient).

A new method for quantitative affinity chromatography has been devised for quantifying the interactions between two or three components of an interacting system, one of which is insoluble.[987] The method does not require elution volumes to be measured but depends upon measurements of the quantity of affinity-bound material. Using a Sepharose-myosin column a dissociation constant of 1.8 μmol l^{-1} was found for ATP^{4-}. Drewe and Winzor[988] have described a moving boundary electrophoretic method for measuring relatively weak interactions of ions with proteins. Using it to study the interaction of a single dibasic phosphate ion with ovalbumin at pH 6.1 and I = 0.1, they found an association constant of *ca.* 250 l mol^{-1}.

Examples. The binding to a variety of enzymes of substrates, substrate analogues, and allosteric effectors has been studied by a variety of techniques and only a few examples can be mentioned. Ridge *et al.*[989] investigated the heterogeneity of binding sites for carbamyl phosphate and fluorinated analogues of carbamyl phosphate in *E. coli* aspartate transcarbamylase. The use of fluorinated substrate analogues has obvious attractions for n.m.r. studies. Suter and Rosenbusch[990] have also studied this interesting and complex enzyme. Two NADPH-binding sites with slightly different dissociation constants were found in beef liver isocitrate dehydrogenase by ultracentrifugation, molecular sieving, ultrafiltration, and fluorescence methods.[991] The binding of NADH to *E. coli* citrate synthase was studied by fluorescence enhancement and gel filtration,[992] and the binding of NAD^+ analogues to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was studied from the point of view of whether they bound co-operatively or

[985] H. Watari and Y. Isogai, *Biochem. Biophys. Res. Commun.*, 1976, **69**, 15.
[986] H. B. F. Dixon, *Biochem. J.*, 1976, **159**, 161.
[987] R. C. Bottomley, A. C. Storer, and I. P. Trayer, *Biochem. J.*, 1976, **159**, 667.
[988] R. H. Drewe and D. J. Winzor, *Biochem. J.*, 1976, **159**, 737.
[989] J. A. Ridge, M. F. Roberts, M. H. Schaffer, and G. R. Stark, *J. Biol. Chem.*, 1976, **251**, 5966.
[990] P. Suter and J. P. Rosenbusch, *J. Biol. Chem.*, 1976, **251**, 5986.
[991] M. F. Carlier and D. Pantaloni, *Biochemistry*, 1976, **15**, 4703.
[992] H. W. Duckworth and E. K. Tong, *Biochemistry*, 1976, **15**, 108.

non-co-operatively, and whether such binding stabilized the enzyme against thermal denaturation.[993] Fructose bis-phosphate, an allosteric activator, and phosphoenolpyruvate, the substrate, bound in a co-operative manner to *E. coli* pyruvate kinase,[994] and Novak [995] investigated the binding of a series of alkylamines to the corresponding muscle enzyme. Yeast hexokinase isozymes P-1 and P-II appeared to differ in their regulatory properties, glucose binding co-operatively ($n = 1.6$) to the P-1 dimer but non-co-operatively to P-II under the same conditions.[996] The pH affected the relative binding of Zn^{2+} and Mg^{2+} to, and the activation of, leucine aminopeptidase.[997] The substrate of the enzyme thymidylate synthetase, dUMP, and the product, dTMP, appeared to compete for the same site in the enzyme, but folate increased the affinity of the enzyme for the substrate to a greater extent than for the product.[998]

Quantitative affinity chromatography was used in an investigation of ribonuclease–nucleotide interaction,[999] and in haemoglobin–haptoglobulin interaction.[1000]

A detailed study has been made of the effects of temperature and pH on the binding of thyroxine and tri-iodothyronine to human thyroxine-binding globulin.[1001] A single binding site was found for thyroxine, but there appeared to be two classes of binding site for tri-iodothyronine.

As usual much effort has been put into understanding the physiological function of haemoglobin by studies of the binding of small molecules. The dissociation constant of the inositol hexaphosphate (IHP)–deoxy Hb complex at pH 7.3 in 0.1M-NaCl at 20 °C was found [1002] to be 6×10^{-8} mol l^{-1}, and this is of relevance to the effect of IHP (and 2,3-diphosphoglycerate) in enhancing the Bohr effect.[1003] Myoinositol hexasulphate is a powerful allosteric effector of oxygen binding by haemoglobin.[1004] It binds to the same site as IHP and 2,3-diphosphoglycerate with an affinity intermediate between those of the two phosphate esters. The change in the Bohr effect in the presence of phosphates was also analysed by Szabo and Karplus,[1005] and the effect of IHP on the three low-spin ferrous compounds of haemoglobin was studied with O_2, CO, and NO.[1006] IHP was capable of switching nitrosyl-, but not carbonmonoxy- or oxy-haemoglobin A from the R to the T state. Evidence from i.r. studies was presented for IHP-induced cleavage of proximal histidine-to-iron bonds in two of the four subunits.[1007] IHP appeared to stabilize the tetrameric state of a mutant haemoglobin, Hb(M) Iwate.[962]

[993] D. Eby and M. E. Kirtley, *Biochemistry*, 1976, **15**, 2168.
[994] E. B. Waygood, J. S. Mort, and B. D. Sanwal, *Biochemistry*, 1976, **15**, 277.
[995] T. Nowak, *J. Biol. Chem.*, 1976, **251**, 73.
[996] E. L. Tickner, J. G. Hoggett, and G. L. Kellett, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 808.
[997] G. A. Thompson and F. H. Carpenter, *J. Biol. Chem.*, 1976, **251**, 53.
[998] J. H. Galivan, G. F. Maley, and F. Maley, *Biochemistry*, 1976, **15**, 350.
[999] I. M. Chaiken and H. C. Taylor, *J. Biol. Chem.*, 1976, **251**, 2044.
[1000] A. Tsapis, M. Rogard, A. Alfsen, and C. Mihaesco, *European J. Biochem.*, 1976, **64**, 369.
[1001] L. Korcek and M. Tabachnick, *J. Biol. Chem.*, 1976, **251**, 3558.
[1002] R. Edalji, R. E. Benesch, and R. Benesch, *J. Biol. Chem.*, 1976, **251**, 7720.
[1003] H. S. Rollema, S. H. DeBruin, and G. A. J. van Os, *Biophys. Chem.*, 1976, **4**, 223.
[1004] R. Benesch, R. Edalji, and R. E. Benesch, *Biochemistry*, 1976, **15**, 3396.
[1005] A. Szabo and M. Karplus, *Biochemistry*, 1976, **15**, 2869.
[1006] M. F. Perutz, J. V. Kilmartin, K. Nagai, A. Szabo, and S. R. Simon, *Biochemistry*, 1976, **15**, 378.
[1007] J. C. Maxwell and W. S. Caughey, *Biochemistry*, 1976, **15**, 388.

Two important papers have appeared which explore the oxygenation-linked subunit interactions in human haemoglobin.[1008] The concentration dependence of the oxygen binding curves was determined between 4×10^{-8} and 5×10^{-4} mol l^{-1} haem using the highly precise oxygenation cell designed by Imai *et al.*[1009] Pronounced changes in both the shape and the position of the curves occurred as the haemoglobin was progressively diluted, and there was a striking qualitative resemblance to the behaviour predicted for a linked dimer–tetramer system by Ackers and Halvorson.[1010] Combination of the data obtained with independently determined values for dissociation constants for unliganded and fully liganded haemoglobin permitted resolution of the seven parameters required for the definition of the linked binding and subunit association process. Such studies provide a powerful method of probing the intersubunit contact energy changes that accompany co-operative ligand binding.

Subunit Structure of Proteins.—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) continues to be exploited as a cheap, simple, and rapid method for estimating protein, and especially subunit, molecular weights. Many recent studies have shifted the method from an empirical to a more theoretically based analytical technique. Thus Dunker and Kenyon[1011] aimed to determine the relative contributions of SDS binding and conformation to the electrophoretic mobility of protein–SDS complexes. Using reduced and unreduced lysozyme aggregates prepared by HCHO cross-linking, they found that whereas the reduced aggregates were indistinguishable from 'normal' proteins, the unreduced aggregates migrated anomalously fast by *ca.* 14%, giving correspondingly lower apparent molecular weights. They failed to detect any anomalous surface-charge density or any unusual conformation for the unreduced aggregates by the diagnostic methods they used. Such studies have an obvious importance where bifunctional reagents are used to probe the neighbourhoods of the various polypeptide components of complex structures.[1012] Following studies of the c.d. spectra of a number of protein–SDS complexes, Mattice *et al.*[1013] proposed a model which assumes that arginyl, histidyl, and lysyl residues have an enhanced probability of propagating a helical segment in the presence of the detergent. They considered that their model could account for the abnormal transport properties of the SDS complexes of RNase A, collagen fragments, and certain histones and, even though some of the protein–SDS complexes had an apparent helical content as high as 50%, the overall conformation more closely approximated that of a random coil than a rod. An investigation of two independent electro-optical properties, the specific Kerr constants and the electric birefringence relaxation times of protein–SDS complexes, led Rowe and Steinhardt[1014] to discard the compact prolate ellipsoid

[1008] F. C. Mills, M. L. Johnson, and G. K. Ackers, *Biochemistry*, 1976, **15**, 5350; M. L. Johnson, H. R. Halvorson, and G. K. Ackers, *ibid.*, p. 5363.
[1009] K. Imai, H. Morimoto, M. Kotani, H. Watari, H. Waka, and M. Kuroda, *Biochim. Biophys. Acta*, 1970, **200**, 189.
[1010] G. K. Ackers and H. R. Halvorson, *Proc. Nat. Acad. Sci.* (*U.S.A.*), 1974, **71**, 4312.
[1011] A. K. Dunker and A. J. Kenyon, *Biochem. J.*, 1976, **153**, 191.
[1012] F. Iborra and J.-M. Buhler, *Analyt. Biochem.*, 1976, **74**, 503.
[1013] W. L. Mattice, J. M. Riser, and D. S. Clark, *Biochemistry*, 1976, **15**, 4264.
[1014] E. S. Rowe and J. Steinhardt, *Biochemistry*, 1976, **15**, 2579.

model proposed by Reynolds and Tanford.[1015] Tanford's group have studied protein–detergent complexes both by sedimentation equilibrium[1016] and by gel chromatography.[1017] They found that sedimentation equilibrium methods could be used to determine the molecular weight of the protein moiety of a protein–detergent complex without prior knowledge of detergent binding, by adjusting the solvent density by the addition of D_2O so as to counteract the contribution of bound detergent to the sedimentation potential. In gel filtration experiments with protein–SDS complexes they observed a large discrepancy between the apparent Stokes radius thus measured and the true value measured by hydrodynamic methods. In the course of this work they observed that all large asymmetric particles (even in the absence of detergent) may give anomalous Stokes radii on gel chromatography. For example, native fibrinogen, true Stokes radius 108 Å, appeared to have a radius of 71 Å when the column was calibrated with globular proteins, and they suggested that end-on insertion of the asymmetric particles into the gel pores contributed to the observed retardation.

Various practical devices and systems for molecular weight determinations by means of detergent-gels have been reported. These include gradient-gel systems[1018, 1019] which give improved resolving capacity and increase the molecular weight range, and an instantaneous monitoring system using a dark-field system allowing zones to be detected during the course of the electrophoresis.[1020] Other systems used to determine protein molecular weights were gel filtration on controlled pore glass beads in the presence of 6M guanidine hydrochloride[1021] and polyacrylamide gel electrophoresis in the presence of the non-ionic detergent, Triton X-100.[1022] The latter appears to have a number of advantages, including the ability to solubilize insoluble proteins nonetheless still allowing an estimate to be made of their charge as well as their molecular weights. A hepatic membrane protein isolated by Kawasaki and Ashwell,[1023] composed of two subunits of mol. wt. 48 000 and 40 000, respectively, as determined in the presence of SDS, showed a tendency to self-associate in aqueous solution. This tendency could be promptly and completely reversed by the addition of Triton X-100, allowing homogeneity to be established.

Many multisubunit proteins have been studied by detergent-gel methods and space prevents more than a few examples receiving mention (Table 5). In contrast to this fairly routine establishment of subunit structures, it was found that the erythrocyte membrane protein, glycophorin A, *aggregated* to form a stable dimer in the presence of SDS but could be dissociated partially by heat treatment.[1024] It was concluded that the subunits may interact *via* hydrophobic portions of the polypeptide chains.

[1015] J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 1970, **245**, 5161.
[1016] J. A. Reynolds and C. Tanford, *Proc. Nat. Acad. Sci. (U.S.A.)*, 1976, **73**, 4467.
[1017] Y. Nozaki, N. M. Schechter, J. A. Reynolds, and C. Tanford, *Biochemistry*, 1976, **15**, 3884.
[1018] K. Lorentz, *Analyt. Biochem.*, 1976, **76**, 214.
[1019] P. Lambin, D. Rochu, and J. M. Fine, *Analyt. Biochem.*, 1976, **74**, 567.
[1020] A. Elliott, *Biochem. J.*, 1976, **159**, 743.
[1021] A. A. Ansari and R. G. Mage, *Analyt. Biochem.*, 1976, **74**, 118.
[1022] V. J. Hearing, W. G. Klingler, T. M. Ekel, and P. M. Montague, *Analyt. Biochem.*, 1976, **72**, 113.
[1023] T. Kawasaki and G. Ashwell, *J. Biol. Chem.*, 1976, **251**, 1296.
[1024] H. Furthmayr and V. T. Marchesi, *Biochemistry*, 1976, **15**, 1137; M. Silverberg, H. Furthmayr, and V. T. Marchesi, *ibid.*, p. 1448.

Table 5 *Subunit structures of some enzymes and proteins*

Protein	*Structure*	*Mol. wt(s). of subunits*	*Comments*	*Ref.*
Hexosaminidase A	$\alpha_2\beta_2$	all 25—27 000	detergent/gels	*a*
Hexosaminidase B	β_4			*a*
Hexosaminidase S	α_4			*a*
UDP-Glucose dehydrogenase	α_2	47 000	detergent/gels; s.e.* in 6M-GuHCl	*b*
Protocatechuate 3,4-dioxygenase	$8(\alpha_2\beta_2)$	α 22 500 β 25 000	detergent/gels	*c*
Purine nucleoside phosphorylase	$\alpha\beta_2$	α 30—32 000 β 27—28 000	detergent/gels: g.f.* in 6M-GuHCl	*d*
Creatine kinase M-line protein	α_2	44 000	detergent/gels	*e*
Fatty acid synthetase (rabbit mammary gland)	α_3	350 000	detergent/gels	*f*

* S.e.=sedimentation equilibrium; g.f.=gel filtration.

(*a*) E. Beutler, A. Yoshida, W. Kuhl, and J. E. S. Lee, *Biochem. J.*, 1976, **159**, 541. (*b*) J. G. Schiller, F. Lamy, R. Frazier, and D. S. Feingold, *Biochim. Biophys. Acta*, 1976, **453**, 418. (*c*) R. Yoshida, K. Hori, M. Fujiwara, Y. Saeki, H. Kagamiyama, and M. Nozaki, *Biochemistry*, 1976, **15**, 4048. (*d*) K. Murakami and K. Tsushima, *Biochim. Biophys. Acta*, 1976, **453**, 205. (*e*) R. S. Mani and C. M. Kay, *ibid.*, p. 391. (*f*) N. Paskin and R. J. Mayer, *Biochem. J.*, 1976, **159**, 181.

Use of Cross-linking Reagents. Several groups have studied the arrangement of subunits in multisubunit protein complexes by cross-linking subunits chemically by means of bifunctional reagents followed by analysis.[1012] Such studies can obviously also give information about symmetry, and the principles upon which the method is based are discussed in some detail by Hajdu *et al.*[1025] with special reference to isologous tetramers. Their theoretical predictions appeared to be borne out by experiments in which rabbit muscle aldolase and pig muscle lactate dehydrogenase were cross-linked with di-imidoesters of increasing chain length. Similar methods were used by Satre *et al.*[1026] to investigate mitochondrial F_1-ATPase, and by Baird and Hammes[1027] to investigate chloroplast coupling factor 1, a complex of molecular weight 325 000 and subunit stoicheiometry $\alpha_2\beta_2\gamma\delta\varepsilon_2$. Cholera toxin, which consists of five similar B subunits (mol. wt. 10 600) and one A subunit (mol. wt. 29 000) was studied by Gill.[1028] Other workers have studied the arrangement of protein subunits in *E. coli* 30S and 50S ribosomes.[1029]

Examples of Associating–Dissociating Systems.—Many new macro-associations have been investigated, including the self-assembly of bovine epidermal keratin filaments,[1030] flagella formation,[946, 1031] polymerization of *Acanthamoeba* actin,[1032] the interaction of actin monomer with myosin,[1033] and the aggregation of TMV

[1025] J. Hajdu, F. Bartha, and P. Friedrich, *European J. Biochem.*, 1976, **68**, 373.
[1026] M. Satre, G. Klein, and P. V. Vignais, *Biochim. Biophys. Acta*, 1976, **453**, 111.
[1027] B. A. Baird and G. G. Hammes, *J. Biol. Chem.*, 1976, **251**, 6953.
[1028] D. M. Gill, *Biochemistry*, 1976, **15**, 1242.
[1029] A. Sommer and R. B. Trout, *J. Mol. Biol.*, 1976, **106**, 995; *ibid.*, **108**, 781; L. Gorelic, *Biochemistry*, 1976, **15**, 3579.
[1030] P. M. Steinert, *J. Mol. Biol.*, 1976, **108**, 547.
[1031] W. Marino, S. Ammer, and L. Shapiro, *J. Mol. Biol.*, 1976, **107**, 115.
[1032] D. J. Gordon, Y.-Z. Yang, and E. D. Korn, *J. Biol. Chem.*, 1976, **251**, 7474.
[1033] P. D. Chantler and W. B. Gratzer, *Biochemistry*, 1976, **15**, 2219.

protein.[1034] A monograph on the thermodynamics of the polymerization of proteins has appeared.[1035]

Sickle-cell Haemoglobin. A great deal of effort is being put into the study of the polymerization of deoxy-HbS and into investigating agents capable of preventing this as potentially therapeutic antisickling agents[1036] (see also refs. 907, 908, 925, and 948). Minton[1037] has extended his thermodynamic model for equilibrium aggregation of HbS. The tendency of HbS to aggregate decreased only slightly with increasing saturation with O_2 until the fractional saturation of non-aggregated haemoglobin exceeded one-half. The effect of inositol hexaphosphate upon the *in vitro* gelation of oxy-HbS has been investigated[1038] and kinetic and thermodynamic studies suggested that both the thermodynamics and the kinetics of gelation were controlled by the supersaturation ratio.[1039] The delay time (before gelation becomes detectable) was proportional to a high power (30—40) of the solubility. The interaction of cyanate, a known antisickling agent, with HbS was studied by c.d. and difference spectroscopy.[1040] However, the reaction of cyanate with HbS is slow and requires a large excess of the reagent, probably because the reactive species is actually isocyanic acid ($pK = 3.8$). In confirmation of this methyl isocyanate reacted rapidly with HbS and only a two-fold excess over HbS α-amino-groups was required to prevent sickling of erythrocytes.[1041] Methylcarbamylated HbS had a higher minimum gelling concentration than untreated HbS, and methylisocyanate-treated sickle erythrocytes had a higher O_2-affinity than untreated ones.

Microtubule Formation. McCammon and Deutch[1042] have considered the frictional properties of non-spherical multisubunit structures and their calculations may have application to microtubule structure (see also ref. 947). Sutherland and Sturtevant[1043] used stopped-flow microcalorimetry to determine the enthalpy change of chain propagation in the polymerization of brain tubulin and found it to be 0 ± 1 kcal mol^{-1} (6S dimer) at both 17 and 25 °C. Engelborghs *et al.*[1044] have investigated the effect of temperature and pressure on the polymerization equilibrium of neuronal microtubules. The main influence was on the nucleation equilibrium. The small pressure dependence observed at temperatures between 25 and 35 °C was considered likely to be a consequence of the dominance of hydrophobic interactions. The so-called 'microtubule-associated proteins' were investigated by Sloboda *et al.*[1045] and by Hines *et al.*,[1046] and a 20S minor compo-

[1034] D. Vogel and R. Jaenicke, *European J. Biochem.*, 1976, **61**, 423.
[1035] F. Oosawa and S. Asakwa, 'Thermodynamics of the Polymerization of Protein', Academic Press, London and New York, 1976.
[1036] E. J. Wood, in this series Vol. 7, p. 245.
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nent of brain tubulin was characterized by Doenges *et al.*[1047] Rat brain tubulin purified by affinity chromatography on colchicine-agarose contained protein kinase activity which could be separated from tubulin by chromatography on casein columns.[1048] Rappaport *et al.*[1049] also clearly separated cyclic AMP protein kinase activity from tubulin. Vinblastine-isolated chick muscle microtubule protein had an associated diglyceride kinase activity.[1050] Griseofulvin was found to interact with microtubules both *in vivo* and *in vitro*,[1051] and the rate of binding of colchicine to tubulin was enhanced by certain anions (sulphate, tartarate).[1052] Bromocolchicine was found to be an affinity label for tubulin,[1053] and tubulin–nucleotide interactions during polymerization and depolymerization were studied.[1054]

11 Spin Labels

Contributed by P. J. Seeley

Covalent Spin Labels.—Šentjurc *et al.* have presented a report of detailed experiments on labelling of membrane acetylcholinesterase with 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethylphosphonofluoridate.[1055] Their labelling conditions (2.5 μM spin label for 0.5 h) resulted in binding of the fluoridate only to active-site serines, as judged by competition and reactivation experiments. The bound radicals gave an immobile e.s.r. signal, in contrast to results obtained by Morrisett and colleagues.[1056] Morrisett's reaction conditions were rather severe however (33 mM spin label for 10 h), and appear to result in extensive and non-selective modification of the membranes used.

Bagshaw and Reed [1057] have investigated interactions of myosin subfragment 1 with adenosine diphosphate and manganous ion. The reactive thiol of the myosin was labelled with either five- or six-membered-ring iodoacetamide nitroxides. Binding of MgADP was detected by enhanced relaxation rate of aqueous solvent. The authors failed to detect dipolar interaction ('quenching') between the spin label and MnADP bound to the subfragment.

Specific attachment of a maleimide spin label to the lipoic acid cofactors of transacetylase was achieved by reduction of the pyruvate dehydrogenase complex with thiamine pyrophosphate and pyruvate or NADH in the presence of magnesium ion.[1058] E.s.r. spectra implied at least two types of lipoic acid environment for transacetylases of the *Azotobacter vinelandii* complex and interaction between pairs of lipoyl spins (*i.e.* proximity) for the complex dissolved in Tricine buffer. (Additional asymmetry in the multienzyme complex was detected by fluorescence spectroscopy.)

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Additional studies using spin-labelled proteins reflect diversity in application of the nitroxide technique: e.s.r. spectra were employed to detect protein–protein interaction,[1059] temperature-dependent conformational equilibria,[1060] and segmental motion in macromolecules.[1061] Kirkpatrick[1059] has used a well-characterized preparation of spin-labelled actin to detect changes in label mobility consequent on polymerization of spectrin on F-actin filaments. Steiner and Smith studied the conformational states of paramagnetically labelled phosphorylase *b* and their dependence on substrate and effector concentrations (glucose 1-phosphate, glycogen, and adenosine monophosphate). Käiväräinen and Nezlin were also concerned with protein–ligand interactions in their study of immunoglobulins labelled with 2,2,6,6-tetramethyl-4-amino(*N*-dichlorotriazine).[1061] Binding of antigen to the globulin resulted in changes in the mobility of some 'mobile' radicals and the 'immobilization' of others. The spectra were interpreted in terms of flexibility of the immunoglobulin molecule at domain boundaries.

Non-covalent Spin Labels.—*Substrate and Ligand Analogues.* A useful spin label has been added to the range of nitroxide analogues of biological molecules: a nicotinamide adenine dinucleotide (NAD) analogue with a piperidine nitroxide attached to the amino-group of the adenine ring.[1062] The spin-labelled NAD closely mimics the action of the parent molecule and has in this case been used as a substrate for glutamate dehydrogenase. Both homotropic and heterotropic co-operativity were studied with the coenzyme. For example, inorganic phosphate or its pyro- and tripoly-phosphate derivatives enhanced NAD binding (e.s.r. detected) in the presence of 2-oxoglutarate. There was strong positive co-operativity in NAD binding to the dehydrogenase-2-oxoglutarate–GTP complexes.

Fung, Gupta, and Mildvan have examined the specific binding of a nitroxide coenzyme-A analogue (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-1-oxy-CoA thioester) to transcarboxylase.[1063] Binding of the analogue to the enzyme caused a six-fold enhancement of the longitudinal relaxation rate of solvent protons and also resulted in paramagnetic relaxation of proton and carbon nuclei of pyruvate in the ternary complex transcarboxylase–CoA–pyruvate. The latter relaxation effects were used to calculate nitroxyl–pyruvate distances. Incorporation of data for Co^{II} transcarboxylase into the model allowed detailed speculation on the function of the biotin cofactor.

Nitroxide derivatives of morphine, spin-labelled at the 3- or 6-position, were described by Copeland and DeBaare in studies of synaptosomal opiate receptors from rat brain.[1064] The authors stressed the advantages of continuous e.s.r. monitoring of the binding process over conventional radioisotope techniques, and observed an unusual kinetic pattern in the form of relatively rapid complex formation after an initial lag period of several minutes. It was felt that nitroxide

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[1062] A. Zantema, W. E. Trommer, H. Wenzel, and G. T. Robillard, *European J. Biochem.*, 1977, **72**, 175.
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[1064] E. S. Copeland and L. DeBaare, *Biophys. J.*, 1976, **16**, 1245.

modification of morphine might have generated alterations in the time-course of opiate binding. Specificity and temperature of ligation were also examined.

Ruf and Gratzl have examined binding of doxyl stearates (5-, 12-, and 16-derivatives) to bovine serum albumin.[1065] They observed four equivalent sites with dissociation constants of *ca.* 1 μmol l^{-1}. The accessibility of sections of the stearate chain to the aqueous medium was assessed from dipolar interaction between the various spin labels and ferricyanide ion in the buffer. Both the head and tail of the fatty acid were found to be available to solvent.

Membranes and Fatty Acid Spin Labels.—*Model Systems.* E.s.r. data for mixed monomolecular films made up of 12-doxyl stearate and myristic acid have been interpreted in terms of two conformations for the nitroxide fatty acid, an 'erect' conformation and a 'bent' form for which both the carboxy-group and the oxazolidine ring lie at the air–water interface.[1066] The latter conformation is predominant in situations for which the ratio of label to host lipid is high. Presumably this behaviour is caused by the polar nature of the oxazolidine ring. (Synthesis of a new and less hydrophilic lipid nitroxide is described below.[1092])

Suckling and Boyd have studied packing effects of the cholesterol side-chain in egg lecithin liposomes.[1067] They determined order parameters for two steroid spin labels embedded in the membranes: a conventional steroid nitroxide, 3-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)]cholestane, and a novel steroid, 3β-hydroxy-26-nor-25[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)]cholestane which has the nitroxide in the side-chain. The molar percentage of cholesterol and cholesterol analogues incorporated into the liposomes was varied. Cholestene analogues with shortened side-chains caused less structuring of the bilayer than the parent molecule and, in the particular case of an analogue without a side-chain, the bilayer was hardly more ordered than for steroid-free liposomes under the same conditions.

Two reports of the interaction of small molecules with lipid bilayers are worth mentioning. The first concerns the localization of chlorophyll *a* in the membrane.[1068] Effects of additions of both the chlorophyll molecule itself and part structures (*e.g.* phytol, phytol ester) to vesicles were monitored as perturbations of e.s.r. spectra of several doxyl stearate probes. Changes indicated that the polycyclic ring of the chlorophyll was near the polar head groups of the lipids. This conclusion was ratified by examination of chlorophyll-mediated photodestruction of the nitroxides. The second study was concerned with the effect of tocopheryl acetate on bilayer structure of phosphatidylcholine vesicles.[1069] The membrane phase transition was appreciably broadened by the presence of tocopheryl acetate. But, provided the relative concentration of the acetate was low, tocopheryl acetate and phosphatidylcholine seemed to be completely miscible in both solid and liquid crystalline states of the membrane, at least as was revealed by partitioning of the TEMPO spin label.

A commonly studied model for lipid–protein interaction, the cytochrome *c*–phospholipid system, has been examined using the spin label method.[1070] Birrell

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and Griffith bound cytochrome *c* to diphosphatidylglycerol and diphosphatidylglycerol–phosphatidylcholine bilayers which were doped with a relatively high proportion of the 3-doxyl-5α-cholestane steroid spin label (1 : 20 molar ratio of spin label to lipid chains). E.s.r. signals were considerably broader than those for protein-free bilayers, an effect resulting from spin exchange between adjacent spin label molecules and previously described in detail by Sackmann and Träuble.[1071] Protein–lipid interaction thus resulted in phase separation within the membrane plane even though the cytochrome as an extrinsic protein contacted only the polar region of the bilayer. (The 'extrinsic' character of the protein–phospholipid complex was indicated by the very small effects on the order parameter of the 5-doxyl stearate sampling the hydrophobic region.)

Hemminga and Post employed the labels of the previous study in an examination of interaction between lipids and proteolipids extracted from the white matter of bovine brain.[1072] Steroid and stearate spin labels were incorporated into multi-bilayers formed by macroscopic orientation of the lipid–protein sample between glass plates. E.s.r. spectra were collected as a function of both temperature and orientation of the membrane plane with respect to the spectrometer magnetic field. Two classes of spin label signal were superposed and these were assigned to membrane lipids oriented perpendicular to the membrane plane and those with no preferential inclination. The unoriented lipids (about two thirds of the total) were thought to occupy 'disrupted areas in the lipid bilayer structure' in which the proteolipid was located. Evidence for this suggestion was adduced from electron microscopy and the temperature dependence of spin label order parameters.

Three papers from McConnell's laboratory continue a series of biophysical investigations in immunochemistry. Brûlet, Humphries, and McConnell have prepared antibodies directed against nitroxide spin labels by injecting rabbits with a limpet haemocyanin alkylated with an iodoacetamide spin label.[1073, 1074] These antibodies were then applied to liposomes containing a lipid hapten nitroxide, the product of reaction of a piperidinyl iodoacetamide nitroxide with dipalmitoylphosphatidylethanolamine.[1075] The efficiency of complement fixation for this system was found to depend on the lateral mobility of the lipid hapten at low hapten concentrations (less than 0.1 mol %). Lipid mobility was not so important at high hapten concentrations. The fixation could be modulated by the presence of cholesterol in the liposome membrane; cholesterol both increased the 'exposure' of hapten to antibody and altered membrane fluidity properties.

Biological Membranes. It is appropriate to precede a review of spin label applications to biological membranes with a study of potential artefactual effects of the spin label method by Butterfield *et al.*[1076] These workers have extended the

[1071] E. Sackmann and H. Träuble, *J. Amer. Chem. Soc.*, 1972, **94**, 4492.
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[1075] P. Brûlet and H. M. McConnell, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2977.
[1076] D. A. Butterfield, C. C. Whisnant, and D. B. Chesnut, *Biochim. Biophys. Acta*, 1976, **426**, 697.

examination by Bieri *et al.*[1077] of structural disruption of erythrocyte membranes by 5-doxyl stearate. Red cell lysis, scanning electron microscopy, and e.s.r. were used to study the state of the spin-labelled erythrocyte. They conclude: "Morphological changes are not reflected in e.s.r. spectra at low label concentrations and, more importantly, that whatever model of molecular motion one wishes to employ to analyse the e.s.r. results, valid data must be obtained at sufficiently low molar ratios of spin label-to-lipid."[1076]

Mitochondrial adenosine triphosphatase has been examined by e.s.r., *X*-ray diffraction, and enzyme activity in its membrane-bound and Triton-solubilized forms.[1078] Lipid extracted along with ATPase protein appeared by functional criteria to be the same lipid that determined the properties of the enzyme in the mitochondrial membrane.

Marsh *et al.* have carried out a classical study of the chromaffin granule membrane using stearate, iodoacetamide, maleimide, and 2,2,6,6-tetramethylpiperidine-1-oxyl spin labels.[1079] Plots of the order parameter for membrane-intercalated 1-, 5-, and 12-doxyl stearates as a function of temperature showed slope discontinuities at *ca.* 32 °C. This temperature was also observed as that for discontinuities in Arrhenius plots of membrane-bound ATPase and NADH oxidase activities. Comparison of results from one doxyl stearate to the next allowed the probabilities of *trans* and *gauche* conformers of individual C—C bonds to be calculated. Changes in these conformation probabilities at the transition temperature were perhaps associated with segregation of cholesterol in the membrane. Spectra from covalently bound iodoacetamide spin labels also showed a transition at *ca.* 32 °C. A third form of e.s.r. measurement, the partitioning of a simple piperidine nitroxide between membrane and aqueous solution, indicated structural changes at 34 and 49 °C. Calcium ions abolished these latter transitions and decreased the extent of motion of stearate spin labels. These alterations were, however, only induced by concentration increments of the order of 100 mmol l^{-1}.

E.s.r. detection of the effects of metal ions on membrane structure has been much used during the past year. Adams *et al.* have studied irreversible calcium damage to erythrocyte membranes during haemolysis using fatty acid and covalent protein nitroxides.[1080] Data for fatty acid spin labels were presented as order parameters and motional amplitudes which gave different concentration dependences for the different probes. The largest response (39—45° change in motional amplitude over 0—2 mmol l^{-1} calcium ions) occurred with 5-doxyl stearate. The immobilization of protein-bound nitroxide with increasing calcium ion concentrations was thought to be caused by aggregation of membrane proteins, an aggregation which allowed simultaneous increases in fluidity of lipid regions of the membrane.

The effect of calcium and magnesium ions on synaptosomal plasmalemma and synaptic vesicles was studied by Viret and Leterrier.[1081] (This paper includes

[1077] V. G. Bieri, D. F. H. Wallach, and P. S. Lin, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4797.
[1078] E. Bertoli, J. B. Finean, and D. E. Griffiths, *F.E.B.S. Letters*, 1976, **61**, 163.
[1079] D. Marsh, G. K. Radda, and G. A. Ritchie, *European J. Biochem.*, 1976, **71**, 53.
[1080] D. Adams, M. E. Markes, W. J. Leivo, and K. L. Carraway, *Biochim. Biophys. Acta*, 1976, **426**, 38.
[1081] J. Viret and F. Leterrier, *Biochim. Biophys. Acta*, 1976, **436**, 811.

a useful table of spin label parameters for a number of biological membranes.) Both 1- and 12-doxyl stearates were incorporated into membranes and a transition in order parameter observed between 15 and 19 °C for the 12- (head group) nitroxide. Since the order parameter for the hydrophobic nitroxide (1-doxyl stearate) was indeterminate for part of the temperature range studied, apparent rotational tumbling times were calculated using the Henry–Keith formula.[1082] There was no transition in the latter parameter. Calcium ions (1 mmol l^{-1}) caused significant increases in membrane rigidity at physiological temperature. Uyesaka *et al.* have also examined metal ion effects on synaptosomal membranes (from rat brain cortex).[1083] Fatty acid spin label signals were 'broadened' by lanthanum, cerium, cadmium, and mercuric ions, but not substantially affected by the alkaline earth series calcium, strontium, and barium. Calcium binding and its competition by lanthanum have previously been observed by the same workers. The reduction in motion of polar lipid head groups effected by lanthanum ions was therefore mediated *via* non-calcium binding sites.

Calcium ion binding to sarcoplasmic reticulum membranes has been studied by e.s.r. of covalently bound iodoacetamide nitroxide.[1084] The e.s.r. spectrum has both mobile and immobile spin label components. For the immobile component, $2T_{\parallel}$ was perturbed specifically by addition of calcium ions to the membranes. The half-point for the transition was at *ca.* 1—3 μmol l^{-1} and corresponded to the half-point for phosphorylation of the reticulum. There was evidence therefore for e.s.r. detection of Ca^{2+} binding to its specific site on the Ca^{2+}-ATPase.

Landsberger and Compans have carried out nitroxide studies of the lipid structure of vesicular stomatitis virus.[1085] They concluded that the viral lipid formed a bilayer structure and that the fluidity of this bilayer was dependent on the integrity of glycoproteins making up the 'spikes' on the outer surface of the virus. Stomatitis virus was grown on two types of cell line which had different lipid compositions for their cell surface membranes. Fluidity of the viral bilayer was dependent on the character of the cell line from which the bilayer was derived. Since viral lipid was considerably less fluid than the plasmalemma of the parent cell, the authors made deductions about the structuring effect of membrane proteins on the lipid portions of the bilayer.

Lyles and Landsberger have studied agglutination of erythrocytes using lipid spin labels (doxyl stearates and a hydrophobic derivative of oxazolidine, 2-butyl-2-pentyl-4,4-dimethyl-*N*-oxyl oxazolidine).[1086] Measurements of $2T_{\parallel}$ for spin labels incorporated into the erythrocyte membrane indicated increased fluidity of lipid on agglutination of erythrocytes by Sendai or influenza viruses or by the lectins conA or wheatgerm agglutinin. Extensive protease or neuraminidase treatment of the red cell membrane did not alter its fluidity but did abolish Sendai virus-mediated effects. Differential action between human and chicken

[1082] S. Henry and A. Keith, *Chem. Phys. Lipids*, 1971, **7**, 245.
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[1084] P. Champeil, F. Bastide, C. Taupin, and C. M. Gary-Bobo, *F.E.B.S. Letters*, 1976, **63**, 270.
[1085] F. R. Landsberger and R. W. Compans, *Biochemistry*, 1976, **15**, 2356.
[1086] D. S. Lyles and F. R. Landsberger, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3497.

erythrocytes and drug sensitivity for the chicken erythrocyte system implicated microtubules in the alterations in membrane structure caused by agglutination.

Incorporation of fatty acid spin labels and a stearamide derivative of 2,2,6,6-tetramethylpiperidine-1-oxyl into the membranes of various strains of *Streptococcus pyogenes* has been used to provide a structural readout, principally in the form of the order parameter.[1087] The membranes of wild-type *Streptococcus* were substantially more fluid than those of osmotically fragile forms of the bacterium and also responded differently to temperature variation. Plots of signal intensity *versus* temperature showed two transitions in the dependent variable, and there were differences of *ca.* 10 °C in transition temperatures of strains of the bacterium with different osmotic stabilities. Comparison of results from different doxyl stearates and the head group stearamide label indicated the greatest variation in membrane order at the lipid–water interface. This correlated with known deficiencies in membrane glycerol teichoic acid structure for the osmotically labile form of *pyogenes*.

Spin Labels as General Biological Probes and New Spin Labels.—The small spin label 2,2,6,6-tetramethylpiperidinyl-1-oxycholine (TEMPO-choline) has been used as a marker for the permeability of sonicated vesicles of dimyristoylphosphatidylcholine.[1088] Spin label permeability, measured by reduction of 'outside' nitroxide with ascorbate at the impermeable temperature of 0 °C, was found to be at a maximum at the phase transition temperature of the vesicles. The ability of TEMPO-choline to move through the vesicle membrane correlated with the fraction of lipid molecules lying at interfacial boundaries between gel and liquid-crystalline regions of lipid. Permeability was thus interpreted as being due to phase-boundary 'mismatch' between ordered and fluid phospholipids.

There have been two reports of elegant measurements of membrane surface potential using the partitioning of amphiphilic spin labels.[1089, 1090] Castle and Hubbell used *NN*-dimethyl-*N*-nonyl-*N*-tempoylammonium cation as their probe.[1089] Gaffney and Mich's spin labels are oxazolidine derivatives of a C_{15} hydrocarbon chain terminated by either a methylene trimethylammonium or a carboxylate group.[1090] Since probe molecules bound to lipid bilayers are partially immobilized by their incorporation, the partition ratio $(\text{spin label})_{\text{bilayer}}/(\text{spin label})_{\text{solution}}$ can be derived directly from the e.s.r. spectrum or its integrations.[1089] Variations in surface charge of the vesicle and composition of the aqueous electrolyte showed that spin label partitioning reflected surface potential of the bilayer.[1089, 1090] Both pairs of authors verified that surface potential was accurately predicted by the Gouy equation.

Henry *et al.* have examined fatty acid starvation in mutant forms of yeast.[1091] Small, water-soluble spin labels were used to probe the yeast cytosol and doxyl stearates to examine the cell membrane. Withdrawal of fatty acids from mutants lacking fatty acid synthetase resulted in a well-documented loss of cell viability which correlated with a two-fold increase in restriction on the rate of small-molecule rotation imposed by the cytoplasm. No change in membrane structure

[1087] M. Chevion, C. Panos, and J. Paxton, *Biochim. Biophys. Acta*, 1976, **426**, 288.
[1088] D. Marsh, A. Watts, and P. F. Knowles, *Biochemistry*, 1976, **15**, 3570.
[1089] J. D. Castle and W. L. Hubbell, *Biochemistry*, 1976, **15**, 4818.
[1090] B. J. Gaffney and R. J. Mich, *J. Amer. Chem. Soc.*, 1976, **98**, 3044.
[1091] S. A. Henry, A. D. Keith, and W. Snipes, *Biophys. J.*, 1976, **16**, 641.

could be detected from e.s.r. spectra of the lipophilic spin labels. Alterations in the state of the aqueous interior of the cells were tentatively related to aberrant protein synthetic activity during the starvation process.

Keana, Lee, and Bernard have reported the synthesis of a new series of lipid spin labels, namely the 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl, or 'proxyl', nitroxides, for which the oxygen of the usual oxazolidine ring has been replaced by a methylene grouping.[1092] This substitution not only rendered the nitroxide less susceptible to decomposition (*e.g.* in cytochrome *c* doped liposomes), but also decreased the polarity of the nitroxyl probe portion of the hydrocarbon (as judged from dodecane–water partitioning experiments). The hyperfine splitting constant was also more responsive to solvent polarity than that for the corresponding doxyl derivative.

A radical anion, tetracyanoquinodimethane (TCNQ), (not a nitroxide) has been used as a novel spin probe for the polar head region of the phospholipid bilayer.[1093] Absence of hydrogen coupling in the spectrum of $TCNQ^{\bullet-}$ bound to dimyristoylphosphatidylcholine vesicles was taken as evidence for interaction of the quinone portion of the molecule with NMe_3^+ head groups of the bilayer. Plots of hyperfine splitting constant and linewidth against temperature showed discontinuities at *ca.* 18 °C. Since the temperature for ordered-fluid transition of the lipid chains is 23 °C, TCNQ appeared to be detecting *pre*transitional effects at the surface of the bilayer.

Some original observations on thionitroxide analogues of the conventional spin labels[1094] have been carefully followed up by Danen and Newkirk.[1095] The similarities between nitroxide and thionitroxide spectra have been discussed in terms of hyperfine interaction with the nitrogen-14 nucleus.[1096] Ready dimerization of thionitroxides to form disulphide-bonded structures, and the reversal of this process by photolysis in solution, may result in the development of these spin labels as biochemical probes.

There have also been reports of examination of the point-dipole approximation for nitroxide biradicals,[1097] nitroxide hyperfine splitting as a polarity parameter,[1098] and spin delocalization from nitroxides to halogenated molecules in non-covalent interactions.[1099]

[1092] J. F. W. Keana, T. D. Lee, and E. M. Bernard, *J. Amer. Chem. Soc.*, 1976, **98**, 3052.
[1093] P. Delhaes, C. Lussan, M.-O. Valiron, and J. Amiell, *F.E.B.S. Letters*, 1976, **69**, 252.
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3
Peptide Synthesis

BY E. ATHERTON AND R. C. SHEPPARD
Appendices compiled by
A. V. STACHULSKI

1 Introduction

Progress towards the rational synthesis of large polypeptides continues, and this year's Report includes descriptions of syntheses of fully protected peptide chains corresponding in sequence to α-bungarotoxin (74 amino-acid residues) and to the first 75 residues of a lysozyme analogue. The comments in last year's Report regarding difficulty of protecting-group removal may still apply, and in this respect further progress in the syntheses mentioned above will be followed with great interest since they involve very different protecting-group combinations. On the solid-phase front, a further synthesis of pancreatic trypsin inhibitor (58 residues) is noteworthy for its careful chemical and biological assessment of the product. Use of solid-phase methods as an adjunct to solution synthesis is exemplified in an assembly of the 1—47 sequence of urogastrone by sequential addition to a polystyrene support of four classically synthesized fragments. Here the solid-phase principle is being used largely as an isolation tool since relatively few of the peptide bonds are formed directly in the solid phase. Further development of methods for the solid-phase assembly of purified solid-phase-synthesized peptides is still awaited and anticipated.

The general arrangement of this chapter is the same as in previous volumes. The Proceedings of the Fourth American [1] and Fourteenth European [2] Peptide Symposia were published during the period under review, and selected papers are discussed as appropriate. The present chapter includes in the form of Appendices the customary lists of synthetic peptides and useful amino-acid derivatives.

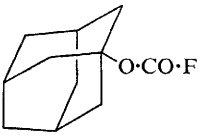
2 Methods

Protective Groups.—*Established Methods of Amino-group Protection.* A further report [3] has appeared of explosion during the preparation of t-butoxycarbonyl azide, and relevant thermodynamic data have been published.[4] Detonation of the azide yields 45% of the energy of an equivalent amount of TNT. In view of this, it is fortunate that other reagents are available for the preparation of t-butoxycarbonyl amino-acids. Attention is drawn particularly to the pyrocarbonate (1), which is now commercially available (Fluka) and which has been

[1] 'Peptides: Chemistry, Structure and Biology', Proc. 4th American Peptide Symposium, ed. R. Walter and J. Meienhofer, Ann Arbor, Mich., 1975.
[2] 'Peptides 1976', Proc. 14th European Peptide Symposium, ed. A. Loffet, Université de Bruxelles, 1976.
[3] P. Feyen, *Angew. Chem. Internat. Edn.*, 1977, **16**, 115.
[4] *Chem. and Eng. News*, 1976, **54**, 3.

described as an 'ideal' reagent.[5] It reacts with amino-acids in dioxan–aqueous sodium carbonate or sodium hydroxide mixtures within 30 min at room temperature to give good yields of Boc-derivatives. 2-(t-Butoxycarbonylimino)-2-phenylacetonitrile (2)[6] is also now commercially available (Aldrich). It reacts more slowly than (1) but the yields of Boc-amino-acids are equally high.

(1) $Bu^tO{\cdot}CO{\cdot}O{\cdot}CO{\cdot}OBu^t$
(2) $Bu^tO{\cdot}CO{\cdot}ON{=}C(CN)Ph$

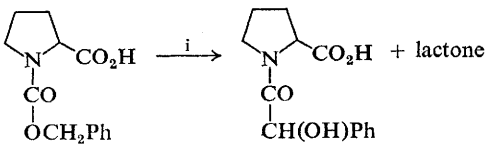


(3)

1-Adamantylfluoroformate (3) has been prepared from 1-adamantol and fluorophosgene and reacts with amino-acids to give consistently high yields of adamantyloxycarbonyl derivatives.[7] It is claimed to be ideally reactive for acylation of the imidazole function of histidine and histidyl peptides.[7]

Methyl trifluoroacetate and ethyl formate react smoothly with amino-acids in the presence of tetramethylguanidine to give trifluoroacetyl and formyl derivatives, respectively.[8] Benzyloxycarbonyl derivatives are rapidly and selectively cleaved from peptides by catalytic transfer hydrogenation.[9] Cyclohexene is used as the hydrogen donor in the presence of relatively large amounts of Pd–C catalyst. Reaction is complete within 15 min in boiling ethanol or methanol (7—10 min for *p*-methoxybenzyloxycarbonyl derivatives). Methionine derivatives are not reduced.[9] ^1H N.m.r. spectroscopy has been used to follow the cleavage of benzyloxycarbonyl derivatives (and benzyl esters) during hydrogenolysis.[10]

Benzyloxycarbonyl amino-acids may undergo an oxo-Wittig rearrangement when treated with strong bases. Benzyloxycarbonylproline, for example, is converted into the mandelamide derivative (4), together with its lactone, by lithium di-isopropylamide in THF (Scheme 1).[11]



(4)

Reagent: i, $LiNPr^i_2$–THF

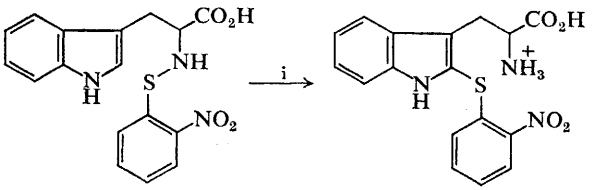
Scheme 1

The formation of *N*-benzylated side-products has been detected during the cleavage of α and side-chain benzyloxycarbonyl derivatives by strong acids, even

[5] L. Moroder, A. Hallett, E. Wünsch, O. Keller, and G. Wersin, *Z. physiol. Chem.*, 1976, **357**, 1651.
[6] See Vol. 8 of these Reports, p. 249.
[7] L. Moroder, L. Wackerle, and E. Wünsch, *Z. physiol. Chem.*, 1976, **357**, 1647.
[8] W. Steglich and S. Hinze, *Synthesis*, 1976, 399.
[9] A. E. Jackson and R. A. W. Johnstone, *Synthesis*, 1976, 685.
[10] V. W. Monnier, *Helv. Chim. Acta*, 1976, **59**, 348.
[11] P. A. Crooks, R. H. B. Galt, and Z. S. Matusiak, *Chem. and Ind.*, 1976, 693.

in the presence of anisole.[12] Most probably these reactions occur by intramolecular pathways, cautioning assumptions that amino-groups liberated in strongly acidic media will be protected from electrophilic attack. t-Butoxycarbonyl derivatives are apparently less prone to this side-reaction, and the nature of the acidic medium is also important. Liquid hydrogen fluoride did not promote benzylation in solution or under solid-phase conditions (p. 343).

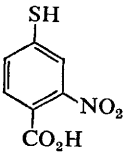
The problems encountered in cleavage of *o*-nitrophenylsulphenyl (Nps) derivatives have been discussed at length.[13] Rearrangement of the N_α–Nps group of tryptophan derivatives into the 2-position of the indole ring (Scheme 2)



Reagent: i, 0·04N-HCl, 30 min, 20 °C

Scheme 2

on acidic deprotection was confirmed, and the structure of the product established from spectroscopic data. This rearrangement can be avoided by use of non-acidic cleavage conditions (*e.g.* thiolysis), and several new reagents of this type were studied. 3-Nitro-4-mercaptobenzoic acid (5) and its methyl



(5)

ester were considered most favourable, causing complete cleavage of simple Nps-amino-acids and peptides within 1 min at room temperature without affecting a wide range of other protecting groups. Disulphide co-products were freely soluble and easily eliminated. Selenourea and sodium hydroselenide were also suitable reagents for cleavage of Nps-derivatives, with the special feature that excess reagent can be readily separated by air oxidation and precipitation of free selenium.

New Methods of Amino-group Protection. The rates of acidolysis of an extensive range of α- and ring-substituted benzyloxycarbonyl derivatives have been studied (Table 1).[14] In contrast to the α,α (geminally) substituted compounds previously studied by Sieber and Iselin,[15] which led to the introduction of the biphenylisopropopoxycarbonyl protecting group, most of the present series bear a single

[12] A. R. Mitchell and R. B. Merrifield, *J. Org. Chem.*, 1976, **41**, 2015.
[13] M. Juillerat and J. P. Bargetzi, *Helv. Chim. Acta*, 1976, **59**, 855.
[14] G. R. Matsueda and J. M. Stewart, in ref. 1, p. 333.
[15] P. Sieber and B. Iselin, *Helv. Chim. Acta*, 1968, **51**, 614.

Table 1 *Rate of deprotection of* 1-*arylethoxycarbonyl- and related urethane protected glycine ethyl esters*

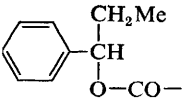
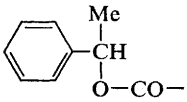
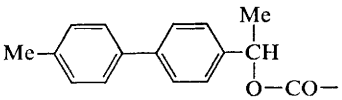
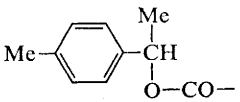
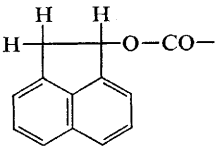
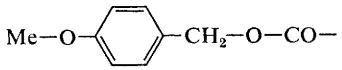
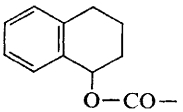
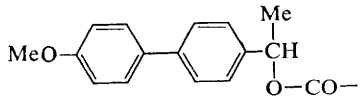
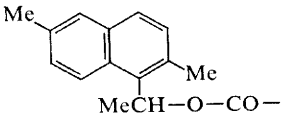
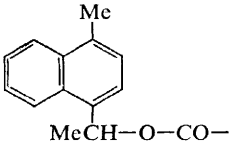
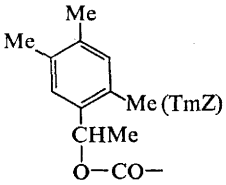
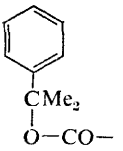
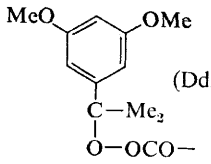
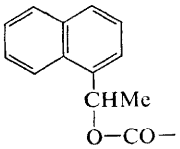
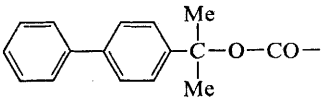
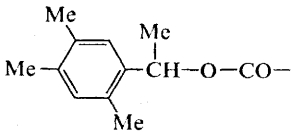
Structure of urethane protecting group	$t_{\frac{1}{2}}$	*Rate relative to* 'TmZ'
	100 h [a]	1/8400
	50 h [a]	1/4200
Bu^tO-CO-	50 h [a]	1/4200
	2.7 h [a]	1/220
	18 min [a]	1/25
	17 min [a]	1/23
	12.5 min [a]	1/17
	8 min [a]	1/11.4
	4.4 min [a]	1/5.9
	3.0 min [a]	1/4.2
	2.7 min [a]	1/3.8

Table 1 (*cont.*)

Structure of urethane protecting group	$t_{\frac{1}{2}}$	*Rate relative to* 'TmZ'
	42 s [a] 420 s [b]	(1)
	120 s [b]	3.4
 (DdZ)	75 s [b]	5.4
	~30 s [b, c]	~10
	~10 s [b, c]	~30

[a] Deprotection with 3% (v/v) TFA in chloroform. [b] Deprotection with 0.5% (v/v) TFA in chloroform. [c] Calculated by assuming plateau time approximates $t_{99.9\%}$

(alkyl) substituent on the benzylic carbon atom. They are therefore asymmetric. The requirement for an optically resolved reagent may severely limit the availability of protected amino-acids based on these groups, although it is envisaged [14] that the new chiral centre may in certain cases confer some advantage. Thus *physical purity* of the diastereoisomeric protected amino-acids should ensure also *optical purity* at the amino-acid α-carbon. Rates of acidolysis by trifluoroacetic acid were markedly sensitive to solvent, the fastest reactions being obtained in dichloromethane. The α,2,4,5-tetramethylbenzyloxycarbonyl (TmZ) group (6)

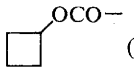
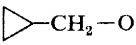
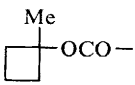
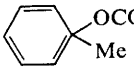
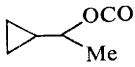


(6)

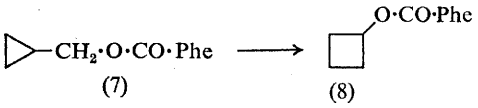
was selected for further study in connection with solid-phase synthesis (p. 339) on the basis of its rate of deprotection (4000 × Boc), ease of preparation, and the crystallizability of amino-acid derivatives.

A similar survey of cleavage rates has been carried out for a number of primary, secondary, and tertiary alkoxycarbonyl derivatives (Table 2).[16] The 1-methyl-

Table 2 *Acid-mediated cleavage of various* N*-protected derivatives of phenylalanine and phenylalanylalanine methyl ester*

X	$t_{\frac{1}{2}}$(X-Phe)/min (25 °C) TFA	HCOOH	$t_{\frac{1}{2}}$(X-Phe-Ala-OMe)/min (25 °C) TFA	HCOOH
$C_6H_5CH_2$—OCO—(Cbz)	300			
 (cBoc)	>300			
 (cPoc)	40		~50	
 (McBoc)	2		3	180
Bu^tOCO—(t-Boc)	1	4	<2	10
	1	2		
		1.5		

cyclobutoxy group is considered advantageous *vis à vis* t-butoxycarbonyl in situations (*e.g.* purification by gel filtration) where prolonged stability to aqueous acetic acid is essential. The authors also point out dangers inherent in the use of certain alkoxycarbonyl derivatives, which may undergo competitive acid-catalysed rearrangement to more stable urethanes. The cyclopropylmethylene derivative (7), for example, is cleaved by trifluoracetic acid with an apparent half life of 40 min, but the reaction does not approach completion in the expected time owing to simultaneous rearrangement to the more stable cyclobutyl derivative (8) (Scheme 3). The authors also comment on the effect of scavenging



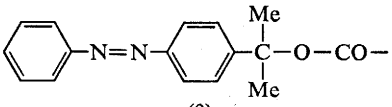
(7) (8)

Scheme 3

additives on cleavage rate, particularly on the relative cleavage rate of protecting groups of different types. Thus the presence of dimethyl sulphide as scavenger reduces the selectivity of protecting-group removal in peptides containing both t-butoxycarbonyl and benzyloxycarbonyl functions.[16]

[16] J. F. Brady, R. Hirschmann, and D. F. Veber, *J. Org. Chem.*, 1977, **42**, 143.

A colourful variant of the biphenylisopropoxycarbonyl protecting group has been described.[17] The *p*-phenylazophenylisopropoxycarbonyl (9) or Azoc group



(9)

is easily introduced *via* the stable, crystalline phenyl ester and azide reagents. It has essentially the same lability to acids as the Bpoc group. Physical characteristics of 32 amino-acid derivatives have been reported (Appendix II). The usual advantages associated with coloured (or strongly u.v. absorbing) protecting groups are noted, but the effect of the phenylazo residue on solubility in this series has yet to be established.

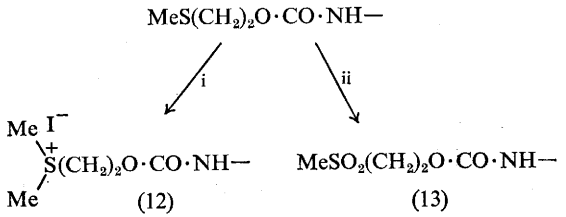
As noted in last year's Report, there is increasing interest in protecting groups cleavable by base-catalysed β-elimination (but see p. 325 for a note of caution). Kunz has described [18] the preparation of a series of 2-(methylthio)ethoxycarbonyl (Mtc) amino-acids by way of the phenyl (10) or *p*-nitrophenyl (11) carbonates.

$$MeS(CH_2)_2O\cdot CO\cdot OC_6H_4X$$

(10) X = H

(11) X = NO_2

The derivatives are stable in moderately alkaline or acidic media, but may be labilized to base-catalysed elimination by either methylation to the sulphonium salt (12) or oxidation to the sulphone (13) (Scheme 4). Both methods of



Reagents: i, MeI; ii, H_2O_2 and ammonium molybdate

Scheme 4

activation have previously been applied to methylthioethoxy-esters.[19, 20] The latter appears to be favoured and the Mtc group therefore provides a 'safety catch' variant of the methylsulphonylethoxycarbonyl (Msc) group discussed in detail in last year's Report (p. 254).

Kunz has also examined [21, 22] some phosphorus derivatives (14) analogous to the foregoing sulphonium compounds. The crystalline chloroformate (15) may

[17] A. Tun-Kyi and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 1642.
[18] H. Kunz, *Chem. Ber.*, 1976, **109**, 3693.
[19] J. S. A. Amaral, G. C. Barrett, H. N. Rydon, and J. E. Willett, *J. Chem. Soc.* (*C*), 1966, 807.
[20] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *Tetrahedron Letters*, 1968, 2525.
[21] H. Kunz, *Chem. Ber.*, 1976, **109**, 2670.
[22] H. Kunz, *Annalen*, 1976, 1674.

$$Ph_3\overset{+}{P}(CH_2)_2O\cdot CO\cdot NH-\quad X^-$$
(14)

$$Ph_3\overset{+}{P}(CH_2)_2O\cdot CO\cdot Cl\quad Cl^-$$
(15)

be prepared from the corresponding hydroxyethylphosphonium salt but is apparently too reactive to allow direct preparation of amino-acid derivatives under aqueous conditions.[21] These may be obtained indirectly *via* amino-acid esters or *NO*-disilylated amino-acids. The protected amino-acids and derived peptides show the expected stability to acids and lability to bases. When cleaved under mildly basic conditions and in the absence of added nucleophiles, back-

$$Ph_3\overset{+}{P}(CH_2)_2O\cdot CO-Leu-OH \xrightarrow{pH\ 8.4} Ph_3\overset{+}{P}CH{=}CH_2 + H-Leu-OH$$
$$\longrightarrow Ph_3\overset{+}{P}(CH_2)_2-Leu-OH$$
Scheme 5

addition of the eliminated alkenyl residue was a significant side-reaction (Scheme 5). This may be avoided by using ammonia or dimethylamine as the basic reagent.

An alternative route to phosphonioethoxycarbonyl derivatives (14) is by way of the 2-bromoethoxycarbonyl precursors (16).[22] Like the phosphonium derivatives themselves, these are apparently best prepared *via* silylated amino-acids. They react with tertiary phosphines, particularly methyldiphenylphosphine to give phosphonium derivatives analogous to (14).

$$Br(CH_2)_2O\cdot CO\cdot NH-$$
(16)

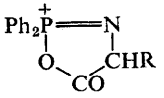
$$Ph_2P(=O)Cl$$
(17)

$$Ph_2PO\cdot NH\cdot CHR\cdot CO_2H$$
(18)

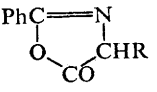
Some new non-urethane acid-sensitive amino-protecting groups based on derivatives of phosphinic acid have been devised.[23] Diphenylphosphinyl-chloride (17) reacts smoothly with amino-acid methyl or benzyl esters (but not with free amino-acids) to yield phosphinamides, from which the protected amino-acids (18) may be obtained by saponification or hydrogenolysis. The diphenyl-phosphinyl (Dpp) group is slightly more acid labile than t-butoxycarbonyl, and may be cleaved selectively in the presence of ε-benzyloxycarbonyllysine functions and even t-butyl esters under controlled conditions. Since no carbonium ions are produced during the cleavage, scavengers are not required. In contrast to simple benzoyl derivatives, diphenylphosphinylamino-acids are not sensitive to racemization when coupled by mixed anhydride or dicyclohexylcarbodi-imide procedures. This is to be expected from stereoelectronic considerations, which preclude stabilization of the enolic or anionic forms of phosphorus analogues (19) of oxazolones (20) through π-delocalization.[23]

In contrast to diphenylphosphinyl chloride (17), the corresponding thio-compound (21) is sufficiently stable in aqueous alkaline media to allow direct

[23] G. W. Kenner, G. A. Moore, and R. Ramage, *Tetrahedron Letters*, 1976, 3623.

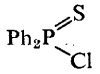


(19)



(20)

preparation of diphenylthiophosphinyl (Ppt) amino-acid derivatives (22).[24] These are cleaved in 80% acetic acid more rapidly ($t_{\frac{1}{2}}$ ~ 20 min) than t-butoxy-

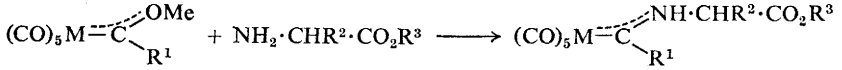


(21)

(22)

carbonyl derivatives ($t_{\frac{1}{2}}$ ~ 90 min), but more slowly than the sulphur-free analogues (18). Like the latter, the thiophosphinylamino-acids are resistant to racemization.

A further report has appeared on the preparation of tosylaminocarbonyl (Tac) amino-acids.[25] Maleylglycine has been used synthetically in the oxytocin field.[26] Both of these topics were discussed in last year's Report (pp. 255 and 256). A full paper has appeared on the application of aryl 2-hydroxyketimine derivatives of amino-acids to peptide synthesis.[27] Pentacarbonyl(methoxyorganylcarbene) complexes of chromium and tungsten react with amino-acid esters (Scheme 6) to give complexes of type (23).[28] The utility of such complexes in peptide synthesis has been suggested.[28]

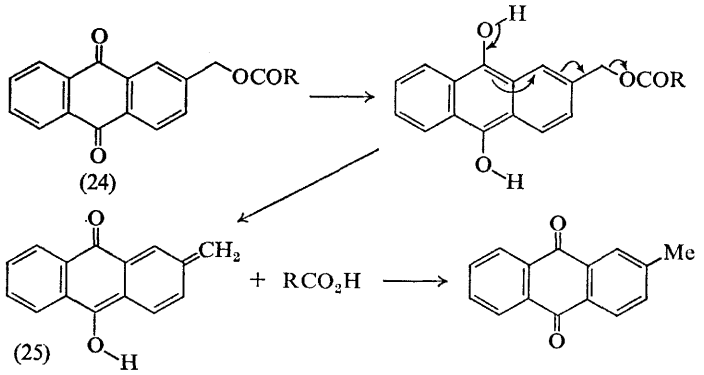


R^1 = Me or Ph, R^3 = Me or Et (23)
R^2 = range of amino-acid side-chains

Scheme 6

Protection of Terminal Carboxy-groups. An ingenious new carboxy-protecting group based on anthraquinone has been devised by Kemp and Reczek.[29] Esters (24) of 2-hydroxymethyleneanthraquinone are resistant to acid-catalysed alkyl–oxygen cleavage because of the electron-withdrawing effect of the quinone carbonyl groups. However, the quinone group is exceptionally easily reduced to the quinol which is then able to undergo spontaneous fragmentation (arrows, Scheme 7) with cleavage of the ester bond. Reagents investigated for effecting this reductive cleavage are aqueous sodium dithionite (pH 7—8), photolysis in the presence of *N*-methylmorpholine, 9-hydroxyanthrone, and cross-linked polystyrene resin functionalized with 9,10-dihydroxyanthracene residues. In all cases, 99—100% cleavage was observed. A particularly attractive feature of this system is that the quinone methide (25) formed initially in the fragmentation

[24] M. Ueki and S. Ikeda, *Chemistry Letters*, 1976, 827.
[25] B. Weinstein, T. N.-S. Ho, R. T. Fukura, and E. C. Angell, *Synth. Comm.*, 1976, **6**, 17.
[26] M. Krojidlo, T. Barth, K. Blaha, and K. Jost, *Coll. Czech. Chem. Comm.*, 1976, **41**, 1954.
[27] A. P. Hope and B. Halpern, *Austral. J. Chem.*, 1976, **29**, 1591.
[28] K. Weiss and E. O. Fischer, *Chem. Ber.*, 1976, **109**, 1868.
[29] D. S. Kemp and J. Reczek, *Tetrahedron Letters*, 1977, 1031.



Scheme 7

step is able to tautomerize to the less reactive 2-methylanthraquinone (Scheme 7). Thus no trapping reagent is necessary to prevent reaction of (25). The tautomerism is evidently extremely rapid, for even in the presence of an efficient trapping agent, the methylanthraquinone is formed quantitatively. Hydroxymethyleneanthraquinone (Maq) esters are unaffected by trifluoracetic acid (1 h, 20 °C), hydrogen chloride in dichloromethane (1 h, 20 °C), or triethylamine in aqueous dioxan (2 eq., 24 h). They are cleaved slowly by saturated hydrogen bromide in acetic acid ($t_{\frac{1}{2}} \sim 65$ h) and rapidly by catalytic hydrogenolysis and the reducing conditions mentioned above. Their practical value has been tested in syntheses of Boc-enkephalin and an angiotensin analogue. Maq esters and the corresponding N-protecting urethane would seem to have considerable future potential.

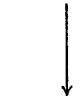
A report has appeared [30] on the cleavage of β-halogenated alkyl esters by supernucleophilic cobalt(I)-phthalocyanine (*cf.* Vol. 8 of these Reports, p. 258). Trichloroethyl esters (and carbamates) are cleaved by zinc in THF–aqueous buffer under very mild conditions.[31]

Protection and Reactions of Side-chain Carboxy and Carboxamide Groups. Side-reactions of β-aspartyl esters and free carboxy-groups continue to cause concern. Activation of unprotected aspartyl carboxy-groups [32] during active ester synthesis has been confirmed by isolation of a further byproduct.[33] β-Phenacyl esters selectively cleavable by thiophenoxide ion have been used in solid-phase synthesis (*q.v.*) to avoid HF-induced cyclization problems associated particularly with the -Asp(OBzl)-Gly- sequence.[34] The extreme base-sensitivity of *C*-terminal asparaginyl α-esters has been noted in connection with the use of base-labile N-protecting groups.[35] Even t-butyl esters may be attacked by the amide side-chain during brief (90 s) treatment with aqueous alkali; with the hexapeptide derivative (26), removal of the Msc group under very mild conditions gave quantitative conversion into the *C*-terminal imide (27) (Scheme 8).

[30] H. Eckert and I. Ugi, *Angew. Chem. Internat. Edn.*, 1976, **15**, 681.
[31] G. Just and K. Grozinger, *Synthesis*, 1976, 457.
[32] See Vol. 8 of these Reports, p. 260.
[33] S. Natarajan and M. Bodanszky, *J. Org. Chem*, 1976, **41**, 1269.
[34] C. C. Yang and R. B. Merrifield, *J. Org. Chem.*, 1976, **41**, 1032.
[35] W. König and A. Volk, *Chem. Ber.*, 1977, **110**, 1.

Msc-Leu-Glu(OBu^t)-Asn-Tyr(Bu^t)-Cys(Trt)-Asn-OBu^t

(26)



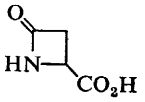
H-Leu-Glu(OBu^t)-Asn-Tyr(Bu^t)-Cys(Trt)-Asp-NH

(27)

Conditions: Aq. 4N-NaOH (12), dioxan (170), methanol (58), 90 s, 0 °C

Scheme 8

4-Carboxyazetidinone (28) (the aspartyl analogue of pyroglutamic acid) has been used as an amino and side-chain protected derivative of aspartic acid in a



(28)

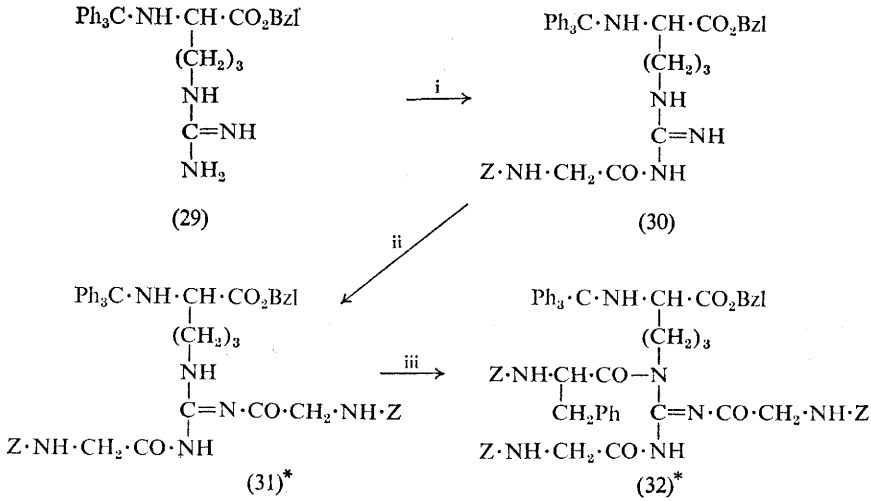
synthesis of the sweetening agent aspartam (aspartylphenylalanine methyl ester).[36] The β-lactam ring is opened by treatment with the stoicheiometric amount of hydrogen chloride in methylene chloride.

γ-*p*-Chloro- and *p*-nitro-benzyl esters of glutamic acid have been evaluated for use in situations (*e.g.* in solid-phase synthesis, p. 339) where stability to acids greater than that afforded by benzyl ester protection is required.[37]

Protection of the Arginine–Guanidine Group. The N_ω-toluene-*p*-sulphonyl group has achieved some popularity for the side-chain protection of arginine residues, but its removal requires vigorous conditions (sodium–ammonia or liquid hydrogen fluoride). The methoxybenzene-*p*-sulphonyl group is more satisfactory in this respect,[38] being removed within 1 h by treatment with methanesulphonic acid at room temperature or with boron tristrifluoroacetate at 0 °C, conditions which leave N_ω-toluene-*p*-sulphonyl groups largely unaffected. It is sufficiently stable to other acidic reagents (*e.g.* trifluoroacetic acid) and to hydrogenolysis to allow its use in combination with both N_α-butoxycarbonyl and benzyloxycarbonyl groups. Adequate protection of the guanidine function was demonstrated by syntheses of bradykinin and tuftsin.[38]

Photaki and Yiotakis have shown [39] that the free (unprotonated) guanidine group of arginine derivatives is very easily acylated under typical peptide synthesis conditions, *e.g.* the N_α- and carboxy-protected arginine (29) gave a side-chain monoacylated product (30) with benzyloxycarbonylglycine hydroxysuccinimide ester, and the diacyl derivative (31) with the corresponding *p*-nitrophenyl ester (Scheme 9). Furthermore, the diacyl derivative (31) could be further acylated by benzyloxycarbonylphenylalanine and dicyclohexylcarbodi-imide to the peracyl derivative (32). The importance of these results may lie not only in

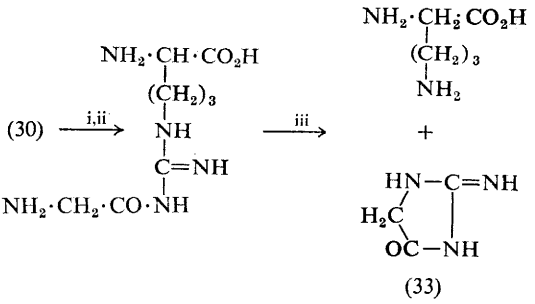
[36] H. Pietsch, *Tetrahedron Letters*, 1976, 4053.
[37] R. L. Prestidge, D. R. K. Harding, and W. S. Hancock, *J. Org. Chem.*, 1976, **41**, 2579.
[38] O. Nishimura and M. Fujino, *Chem. and Pharm. Bull. Japan*, 1976, **24**, 1568.
[39] I. Photaki and A. Yiotakis, *J.C.S. Perkin I*, 1976, 259.



Conditions: i, Z-Gly-ONSu, 1 h, 0 °C, then RT overnight; ii, Z-Gly-ONp, 1 h, 0 °C, then RT overnight; iii, Z-Phe-OH, DCCI, 1 h, 0 °C, then RT overnight
*The *N*-acyl substituents were not located in these compounds.

Scheme 9

the caution they indicate in the use of protonation for side-chain protection, but also in the demonstration that one or even two ω-acyl groups may not convey adequate protection to the guanidine function. Several protected arginine derivatives of this type have been proposed and used in the past. The diacyl derivative (31) is easily cleaved to (30) by cold alkali; deprotection of (30) affords the ω-linked dipeptide which decomposes spontaneously (Scheme 10) to free



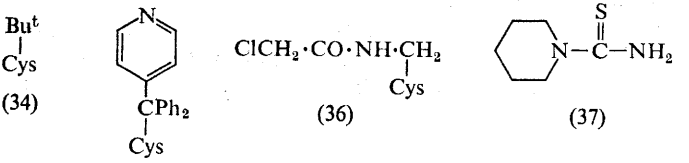
Reagents: i, HCl–$MeNO_2$; ii, H_2–Pd; iii, spontaneous

Scheme 10

ornithine and the imino-imidazolone (33). Ornithine derivatives are common contaminants in the synthesis of arginine peptides.

Protection of Thiol Groups and the Synthesis of Cysteine Peptides. A new method for the cleavage of *S*-*p*-methoxybenzyl- and *S*-t-butyl-cysteine derivatives promises to be of value. Reaction with mercuric trifluoroacetate in aqueous

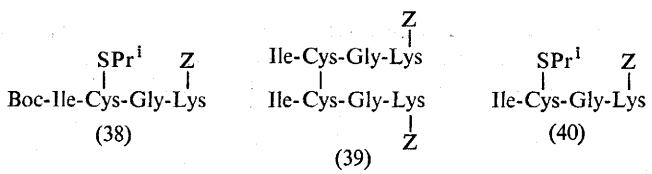
acetic acid or mercuric acetate in trifluoroacetic acid affords the corresponding mercaptides quantitatively.[40] These can be decomposed with thiols in the usual way. The *S*-t-butyl group has long been considered excessively stable, requiring very vigorous conditions for its cleavage by acids.[41] However, the protected cysteine derivative (34) is readily available [41, 42] and the potential for cleavage by electrophilic reagents other than protons provides a new incentive to explore its use in synthesis.



(34) (35) (36) (37)

The replacement of phenyl by 4-pyridyl residues greatly enhances acid stability, and the pyridyl analogue (35) of *S*-tritylcysteine provides another example.[43, 44] It is stable to trifluoroacetic acid and 45% hydrogen bromide in acetic acid during 48 h at room temperature, but is readily cleaved by iodine, mercuric acetate, zinc and acetic acid, or electrolytic reduction. Another acid-stable S-protecting group with specific cleavage conditions is illustrated in the chloroacetamidomethylcysteine (36).[45] Like other chloroacetamide derivatives,[46] this is cleaved by thioureas, *e.g.* 1-piperidinethiocarboxamide (37).

Isopropylsulphenyl derivatives may provide inadequate protection for the thiol group of cysteine residues under acidic conditions.[47] Deprotection of (38) with hydrogen chloride in acetic acid gave substantial amounts of the dimeric symmetrical disulphide (39) as well as the desired side-chain-protected tetrapeptide (40). Boron trifluoride ethereate was a superior reagent in this respect, but some disulphide interchange still took place.[47]



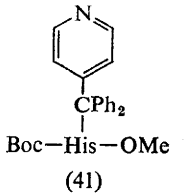
(38) (39) (40)

Protection of the Histidine Imidazole Group. A further paper has appeared on the use of N_{im}-tosylhistidine derivatives in solution.[48] This protecting group has already won some popularity for histidine protection in solid-phase synthesis.

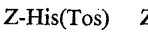
[40] M. Fujino and O. Nishimura, *J.C.S. Chem. Comm.*, 1976, 998.
[41] H. Kofod, in 'Peptides 1974', Proc, 13th European Peptide Symposium, 1974, Wiley, New York, 1975, p. 57.
[42] A. Chimiak, *Ann. Soc. Chim. Polonorum*, 1964, **38**, 883.
[43] S. Coyle and G. T. Young, in ref. 2, p. 205.
[44] S. Coyle and G. T. Young, *J.C.S. Chem. Comm.*, 1976, 980.
[45] P. Hermann and G. Hoffmann, in ref. 2, p. 121.
[46] W. Steglich and H. G. Batz, *Angew. Chem.*, 1971, **83**, 83.
[47] P. Harker, *Z. Physiol. Chem.*, 1976, **357**, 1683.
[48] T. Fujii, T. Kimura, and S. Sakakibara, *Bull. Chem. Soc. Japan*, 1976, **49**, 1595.

As expected, the *im*-tosyl group has reactivity towards nucleophilic reagents and is removed by, *e.g.*, hydroxybenzotriazole, ammonia, *etc.* The preparation of hydroxysuccinimide esters requires caution, and the azide coupling method is is inapplicable. There is also risk of partial removal by amino-components with formation of contaminating N_{α}-tosyl derivatives. Liquid hydrogen fluoride is apparently still the method of choice for final removal, although it is suggested that selective removal (by hydroxybenzotriazole) immediately after coupling may be advantageous in certain cases.[48]

The 4-pyridyldiphenylmethyl derivative (41) analogous to *im*-tritylhistidine has been prepared and its use in synthesis investigated.[43, 44] The pyridyldiphenylmethyl group is removed by catalytic or electrolytic reduction, or by zinc and acetic acid.



(41)



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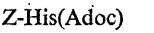
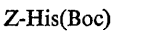


(43)



(44)

New preparations of N_{α}-t-butoxycarbonyl-[49] and N_{α}-benzyloxycarbonylhistidine[50] have been described. The latter has been converted into the side-chain-protected derivatives (42), (43), and (44).

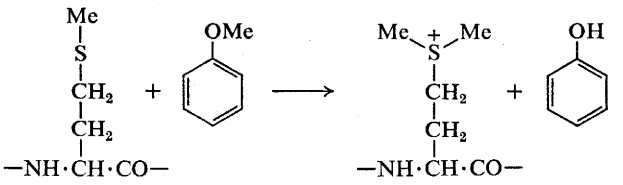
Protection of other Functional Side-chains and the General Deprotection of Peptides. Conditions have been devised for the preparation of N_{in}-benzyloxycarbonyltryptophan derivatives.[51] Acylation of the indole nitrogen is achieved by reaction of Boc-Trp-OEt with *p*-nitrophenylbenzyl carbonate in acetonitrile containing di-isopropylethylamine and potassium fluoride, the latter solubilized by a crown ether. Presumably the high basicity of the unsolvated fluoride anion in organic solvents is the essential feature. The benzyloxycarbonyl group is removed by catalytic hydrogenation, hydrazine, or liquid hydrogen fluoride, but is stable to the acidic conditions used for removal of butoxycarbonyl groups.

Further studies have been carried out on alternatives to liquid hydrogen fluoride for the general deprotection of peptides (see also solid-phase synthesis section, p. 341). Up to 70% hydrogen fluoride can be dissolved in pyridine giving stable solutions of pyridine polyhydrogen fluoride, which are safer and easier to use than the liquefied gas.[52] All the usual t-butyl- and benzyl-based protecting groups except N_{im}-benzylhistidine are cleaved within 1 h at room temperature by this reagent. The side-chain tosyl derivatives of histidine and arginine are also cleaved but nitroarginine is stable.

The use of boron tristrifluoroacetate for general deprotection has been discussed further with particular reference to the need for carbonium-ion-scavenging reagents.[53] Thioanisole was the preferred scavenger for this cleavage agent as well as for liquid hydrogen fluoride.

[49] D. D. Van Batenburg and K. E. T. Kerling, *Internat. J. Peptide Protein Res.*, 1976, **8**, 1.
[50] H. Eckstein, *Annalen*, 1976, 1289.
[51] M. Chorer and Y. S. Klausner, *J.C.S. Chem. Comm.*, 1976, 596.
[52] S. Matsura, C.-H. Niu, and J. A. Cohen, *J.C.S. Chem. Comm.*, 1976, 451.
[53] W. Bauer and J. Pless, in ref. 1, p. 341.

When methane- or trifluoromethane-sulphonic acid is used for general deprotection in the presence of anisole, the latter may be decomposed with transfer of its methyl group to methionine residues (Scheme 11).[54] This break-



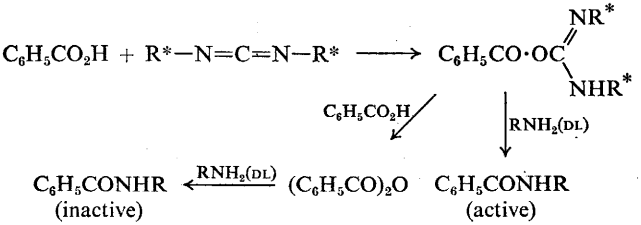
Reagent: $MeSO_3H$ or CF_3SO_3H–CF_3CO_2H

Scheme 11

down of the aromatic ether apparently does not occur in the absence of methionine or a similar acceptor molecule. It is evident that liberal addition of anisole to strongly acidic deprotection reagents will not always be advantageous. The reaction with methionine may be circumvented by use of the sulphoxide.

Papers relating to the protection of aliphatic[56] and aromatic[57] hydroxy-groups as methylthiomethyl ethers, and the general cleavage of esters and ethers using iodotrimethylsilane[58] may be of interest.

Formation of the Peptide Bond.—Some interesting new probes for investigating the mechanisms of acylation reactions have been devised.[59–61] Use of optically active carbodi-imides[59] has confirmed previous conclusions concerning the duality of mechanism involving these reagents. Thus reaction between an achiral carboxylic acid, racemic amine, and a limiting amount of chiral carbodi-imide (Scheme 12) gives optically inactive amide under solid-phase synthesis conditions

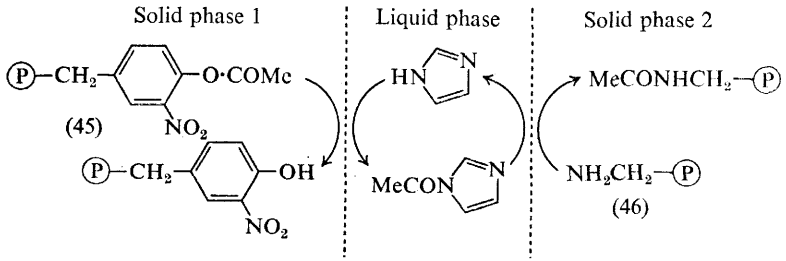


Scheme 12 R* = asymmetric group

(excess carboxylic acid) but optically active amide when the amine is initially in excess. These results are consistent with acylation by an achiral symmetrical anhydride under the first set of reaction conditions but by a chiral isourea with asymmetric induction under the second (Scheme 12).

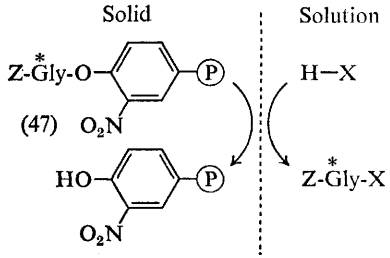
[54] H. Irie, N. Fujii, H. Ogawa, and H. Yajima, *J.C.S. Chem. Comm.*, 1976, 922.
[55] V. G. Mairanorsky, *Angew. Chem. Internat. Edn.*, 1976, 15.
[56] P. M. Pojer and S. J. Angyal, *Tetrahedron Letters*, 1976, 3067.
[57] R. A. Holton and R. G. Davis, *Tetrahedron Letters*, 1977, 533.
[58] T.-L. Ho and G. A. Olah, *Angew. Chem. Internat. Edn.*, 1976, **15**, 774.
[59] J. Rebek, D. Brown, and S. Zimmerman, in ref. 1, p. 371.
[60] J. Rebek and D. Brown, in ref. 2, p. 61.
[61] J. Rebek, D. Brown, and S. Zimmerman, *J. Amer. Chem. Soc.*, 1975, **97**, 454.

The occurrence of acyl transfer during nucleophilic catalysis of acylation reactions has been demonstrated using polymeric reagents in admixture.[60, 61] Polymer-bound acetyl-*o*-nitrophenate (45) does not react with polymeric amine (46) when the two resins are suspended in the same solvent, or when they are



Scheme 13

ground together in a mortar. However, addition of a catalytic amount of nucleophile, *e.g.* imidazole, results in smooth acyl transfer (Scheme 13). Hydroxybenzotriazole, hydroxyphthalimide, and hydroxysuccinimide were all effective but pyridine, acetoxime, and 1,2,4-triazole were not.[61] The radiolabelled



H–X = nucleophilic catalyst; Gly = radiolaballed glycine

Scheme 14

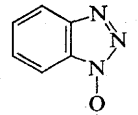
polymeric ester (47) has been used to measure the relative rates of active ester deacylation (Scheme 14) by amines and nucleophilic catalysts (Table 3).

Phosphorus derivatives continue to attract much attention as coupling reagents for peptide synthesis. An alternative preparation of the hydroxybenzotriazolyl

Table 3 *Relative rates for deacylation of polymer-bound benzyloxycarbonylglycyl o-nitrophenate* (47) *in the presence of* 0.05M-NEt_3/NEt_3–HBF_4 [60]

Nucleophile	*Solvent* THF	DMF
Imidazole	1.00	0.88
1,2,4-Triazole	0.42	0.38
1-Hydroxybenzotriazole	0.55	0.2
N-Hydroxyphthalimide	1.2	1.4
N-Hydroxysuccinimide	1.2	1.4
Mercaptoethanol	0.02	0.35
o-Nitrophenol	0.003	—
Gly-OMe	—	1.45

phosphonium salt (48) from hexamethylphosphoric triamide (rather than from the less accessible phosphorus triamide) has been developed [62] and the reagent used in a fragment condensation synthesis of somatostatin. Very high yields were obtained but model couplings indicated a significant degree of racemization. In an independent study,[63] the tetrafluoroborate of the same cation has been obtained from the bisphosphonium salt (49). It gave 29% racemization in the Izumiya test whereas direct use of (49) in the presence of hydroxybenzotriazide gave <1% racemization (see also Vol. 8, p. 265). These results pose an interesting mechanistic problem.



$(Me_2N)_3\overset{+}{P}-O$

PF_6^-

(48)

$(Me_2N)_3\overset{+}{P}-O-\overset{+}{P}(NMe_2)_3$ $2BF_4^-$

(49)

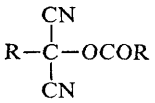
The structure and properties of diethyl phosphorocyanidate (DEPC) have been further studied.[64] Material freshly prepared by Arbuzov reaction between cyanogen bromide and triethyl phosphate shows two nitrile peaks in the i.r. spectrum, and these have been assigned to cyanide (50) and isocyanide (51)

$(EtO)_2P(O)C\equiv N$

(50)

$(EtO)_2P(O)\overset{+}{N}\equiv\overset{-}{C}$

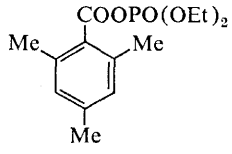
(51)



(52) R = Ph or CH_2CH_2Ph

RCOCN

(53) R = Ph or CH_2CH_2Ph



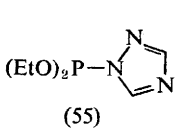
(54)

derivatives. The latter may be removed by treatment with a complexing agent such as cobalt(II) chloride, followed by re-distillation. The purified reagent (50) converted benzoic and phenylpropionic acids into dimers (52) of the intermediate acyl cyanides (53), but the sterically hindered mesitoic acid yielded the stable acyl phosphate (54). High yields of simple amides were obtained in coupling reactions using (50), but only moderate yields of esters.

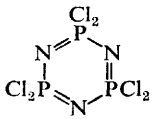
Simple peptides have been obtained in high yields using phosphorus trichloride or alkyl derivatives (*e.g.* diethylchlorophosphine) in the presence of imidazole.[65] These reagents also provide an excellent route for the preparation of isolated acyl imidazolides. Phosphorus diester triazolides (55) have been obtained from 1-trimethylsilyl-1,2,4-triazole and used in the synthesis of simple peptides and polymers.[66] Phosphonitrilic chloride (56) has been used in a stepwise synthesis

[62] B. Castro, J. R. Dormoy, G. Evin, and C. Selve, in ref. 2, p. 79.
[63] I. J. Galpin, P. F. Gordon, R. Ramage, and W. D. Thorpe, *Tetrahedron*, 1976, **32**, 2417.
[64] T. Shiori, Y. Yokoyama, Y. Kasai, and S. Yamada, *Tetrahedron*, 1976, **32**, 2211.
[65] M. Brenner, U. Giger, R. Nyfeler, P. Schenk, and A. Tschopp, in ref. 2, p. 65.
[66] H. R. Kricheldorf, M. Fehrle, and J. Kaschig, *Angew. Chem. Internat. Edn.*, 1976, **15**, 305.

of the tetrapeptide tuftsin.[67] The oxidation–reduction–condensation method involving conversion of P^{III} into P^{V} derivatives has been authoritatively reviewed.[68]



(55)



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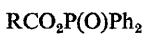
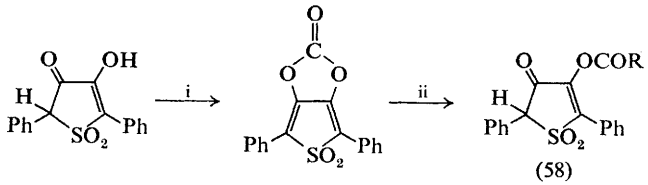


(57)

Diphenylphosphinic acid has been used as a new partner in mixed anhydrides (57) with protected amino-acids.[69] The high specificity for attack by amines at the carbonyl group (as opposed to the phosphorus atom) is demonstrated by reaction of the sterically hindered pivalic-diphenylphosphinic anhydride (57; R = Bu^t) with phenylethylamine. The pivalamide derivative was formed exclusively. The method has been used successfully for the synthesis of a number of short peptides. Racemization in the Izumiya test was 5.7%.

Interest in the mechanisms of non-ribosomal peptide biosynthesis has prompted re-investigation of simple alkylthiol esters as coupling agents.[70] The reactivity of these esters is apparently much increased by weakly acidic (pivalic acid) or bifunctional (2-hydroxypyridine) catalysts, and they combine with amines in high yield and with little if any racemization. The rather low intrinsic reactivity of alkylthiol esters in the absence of catalysts permits their use as temporary carboxy-protecting groups during coupling by other methods.



(58)

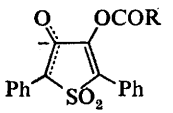
Reagents: i, $COCl_2$–pyridine; ii, RCO_2H–pyridine

Scheme 15

Every year brings its crop of novel activated amino-acid derivatives and this year is no exception. Among the most interesting are the 4-acyloxy-3-oxo-2,5-diphenyl-1,1-dioxides (58), readily available by the route shown in Scheme 15.[71] Esters of type (58) may be regarded as vinylogous mixed anhydrides between carboxylic and sulphonic acids, but are sufficiently stable to be isolated and crystallized in a number of cases. They react smoothly with amino components in the presence of tertiary base to give very high yields of acyl derivatives. The system is self indicating in that the starting activated derivative (58) forms a

[67] J. Martinez and F. Winternitz, in ref. 2, p. 551.
[68] T. Mukaiyama, *Angew. Chem. Internat. Edn.*, 1976, **15**, 94.
[69] A. G. Jackson, G. W. Kenner, G. A. Moore, R. Ramage, and W. D. Thorpe, *Tetrahedron Letters*, 1976, 3627.
[70] S. Yamada, Y. Yokoyama, and T. Shiori, *Experientia*, 1976, **32**, 967.
[71] O. Hollitzer, A. Seewald, and W. Steglich, *Angew. Chem. Internat. Edn.*, 1976, **15**, 444.

coloured anion (59) under basic conditions, the disappearance of which indicates completion of the reaction. Couplings are evidently very rapid, for competitive acylation of benzylamine by a mixture of the very hindered pivaloyl derivative (58; R = Bu^t) and *p*-nitrophenyl acetate gave 97% *N*-benzylpivalamide and only 3% *N*-benzylacetamide. This high reactivity may be attributable to intramolecular base catalysis of acylation by the neighbouring enolate group in the anion (59).



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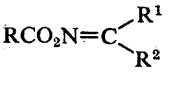
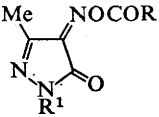
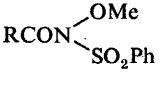


(60)



(61)



(62)

Other activating groups examined include *O*-acyl oximino (nitroso) derivatives (60) of activated methylene compounds, *e.g.* acetylacetone, R^1, R^2 = MeCO; benzoylacetophenone, R^1, R^2 = PhCO; dimethylmalonate, R^1, R^2 = CO_2Me; malononitrile, R^1, R^2 = CN; and nitroethane, R^1 = Me, R^2 = NO_2.[72] A further variant is the oximinopyrazolone (61; R^1 = Me or Ph).[73] *N*-Acyl derivatives of *N*-benzenesulphonyl-*O*-methylhydroxylamine (62) are also sufficiently reactive to function in peptide synthesis.[74]

Several further papers have appeared [75–77] on the four-component condensation method of synthesis (which in part uses optically inactive non-amino-acid starting materials), including a highly stereoselective synthesis of tetravaline.[77]

Racemization.—Data relating to racemization by individual coupling methods are discussed in the previous section.

The rates of racemization of a range of S-protected benzyloxycarbonylcysteine pentachlorophenyl esters by triethylamine in chloroform have been measured (Table 4).[78] Although previous investigations of *S*-benzylcysteine have shown [79] that the α-proton never becomes fully detached during racemization, there seems to be no alternative but to suggest that the sulphur atom assists racemization by accepting some negative charge from the α-carbon (β with respect to the sulphur atom) into its *d*-orbitals. The resistance of thiazolidine-4-carboxylic acid and the six-membered ring analogue to racemization may then be due to prevention of effective *p–d* orbital overlap by ring constraints.

A study of a range of aryl esters of phthaloylphenylalanine and phthaloylglycylphenylalanine generally supports the supposition that the rate of base-

[72] A. G. Veronese, G. Cavicchioni, P. Giori, M. P. Quaglio, and M. Guarneri, *Gazzetta*, 1976, **106**, 179.
[73] M. Guarneri, C. B. Vicentini, M. P. Quaglio, and P. Giori, in ref. 2, p. 209.
[74] L. Nakonieczna, Z. Makowski, I. Berezowska, and E. Taschner, in ref. 2, p. 117.
[75] I. Ugi, H. Aigner, B. Beijer, D. Ben-Efraim, H. Burghard, P. Bukall, G. Eberle, H. Eckert, D. Marquarding, D. Rehn, R. Urban, L. Wackerle, and H. von Zychlinsky, in ref. 2, p. 159.
[76] G. Eberle and I. Ugi, *Angew. Chem. Internat. Edn.*, 1976, **15**, 492.
[77] R. Urban, G. Eberle, D. Marquarding, D. Rehn, H. Rehn, and I. Ugi, *Angew. Chem.*, 1976, **88**, 644.
[78] M. Barber and J. H. Jones, in ref. 2, p. 109.
[79] J. J. Cram, 'Fundamentals of Carbanion Chemistry', Academic Press, London, 1968, p. 38.

catalysed racemization parallels the electron-withdrawing character of the parent substituted phenol.[80] 2-Hydroxyphenyl esters are exceptional in racemizing appreciably faster than phenyl esters, whereas 2-methoxyphenyl derivatives racemize more slowly. Experimental racemization rate constants for an extensive range of Boc- and Z-amino-acid and dipeptide active esters have been tabulated and compared with predicted rates using a simple additivity principle.[81] Generally good agreement was obtained.

Table 4 *Second-order rate constants* ($l\ mol^{-1}\ s^{-1} \times 10^4$) *for racemization by triethylamine in chloroform at* 20 °C. (*Thz = thiazolidine-2-carboxylic acid; Thm = thiamorpholine-2-carboxylic acid; Btm = benzylthiomethyl*)

Active ester	*Rate constant*
Z-Thz-OPcp	< 0.05
Z-Thm-OPcp	< 0.05
Z-Ala-OPcp	< 0.05
Z-Phe-OPcp	0.19
Z-Met-OPcp	0.18
Z-Ser(Bzl)-OPcp	0.61
Z-Cys[Bzl(4-OMe)]-OPcp	1.86
Z-Cys(Trt)-OPcp	2.30
Z-Cys(Me)-OPcp	2.33
Z-Cys(Bzl)-OPcp	2.45
Z-Cys(Bzh)-OPcp	3.10
Z-Cys[Bzl(4-Cl)]-OPcp	3.84
Z-Cys(Btm)-OPcp	4.06
Z-Cys[Bzl(4-CN)]-OPcp	6.63
Z-Cys[Bzl(4-NO_2)]-OPcp	8.50

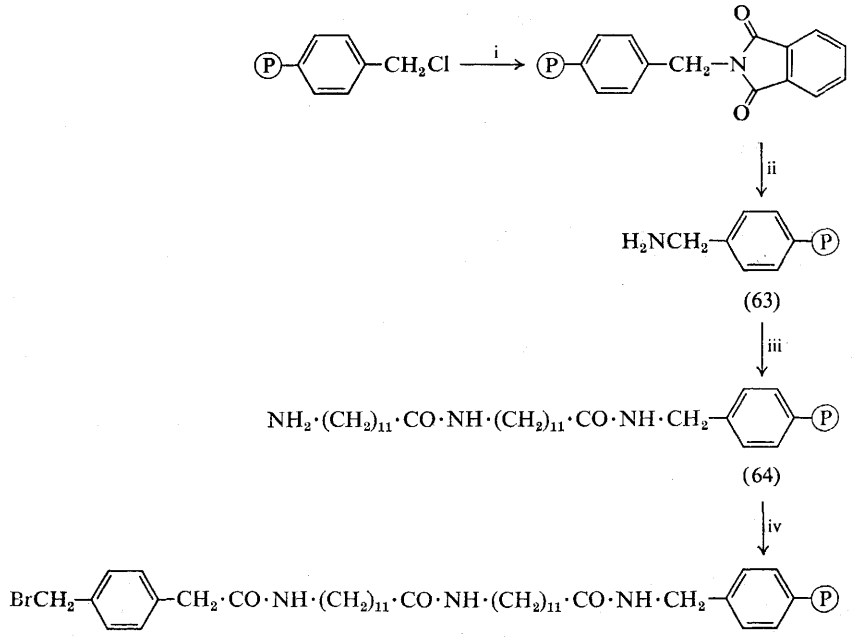
The coupling of tritiated formyl-D-phenylalanine with L-phenylalanine methyl ester and separation of diastereoisomeric products by t.l.c. has been used as a simple racemization test.[82] *N*-Hydroxysucciminide, hydroxyphthalimide, and hydroxybenzotriazole reduced racemization in dicyclohexylcarbodi-imide couplings, whereas addition of 2-hydroxypyridine, 8-hydroxyquinoline, and *p*-nitrophenol tended to increase it. The hydroxylamine derivatives also reduced *N*-acylurea formation. Aminolysis of nitrophenyl and pentachlorophenyl esters of benzyloxycarbonyl phenylalanine has been shown to proceed without racemization at temperatures up to 90 °C.[83] Aminolysis (by valine methyl ester) is accelerated ten-fold.

The optical purity of histidine in peptide hydrolysates has been determined by ozonolysis to aspartic acid and g.l.c. separation of the *N*-trifluoroacetyl (+)-3-methylbutan-2-ol esters.[84] Up to 5% D-histidine (10% racemization) was found in peptide derivatives prepared by way of benzyloxycarbonylhistidine hydrazide. The resistance to racemization of *im*-tosyl-*N*-butoxycarbonylhistidine in solid-phase synthesis has been confirmed.[85]

[80] K. Bankowski, A. Misicka, and S. Drabarek, *Roczniki Chem.*, 1976, **50**, 2079.
[81] J. Kovacs, R. Cover, G. Jham, Y. Hsieh, and T. Kalas, in ref. 1, p. 317.
[82] A. Arendt, A. Kolodziejczyk, and T. Sokolowska, *Tetrahedron*, 1976, **32**, 447.
[83] A. Schuttler, W. Meltzow, J. Fohles, and H. Zahn, *Z. physiol. Chem.*, 1976, **357**, 741.
[84] W. Rahn, H. Eckstein, and W. A. König, *Z. physiol. Chem.*, 1976, **357**, 1223.
[85] R. E. Gabardy, H. E. Bleich, P. Zugler, and L. C. Craig, *Biochemistry*, 1976, **15**, 2303.

Repetitive Methods of Peptide Synthesis.—*Solid-phase Synthesis.* Two reviews have appeared during the year.[86, 87] One gives a basic description of the method outlining its main features and shortcomings;[86] the other is a fully comprehensive account,[87] which also outlines the scope of solid supports in other aspects of organic chemistry. It is a valuable addition to the library of any scientist practising the solid-phase method.

The derivatization of polystyrene with chloromethyl groups, besides necessitating the use of the potent carcinogen chloromethyl methyl ether, can be accompanied by side-reactions. A functionalized resin has now been made by co-polymerization of 4-chloromethylstyrene with styrene and divinylbenzene.[88] In practice the new resin gave similar results to those obtained from polystyrene which had been functionalized conventionally. Separation of the resin backbone by a long aliphatic spacer molecule is claimed to give superior results in the synthesis of a 19-residue peptide.[89, 90] Reaction of chloromethylated polystyrene with potassium phthalimide followed by treatment with hydrazine gave the aminomethyl resin (63) (Scheme 16) converted into (64) by addition of two



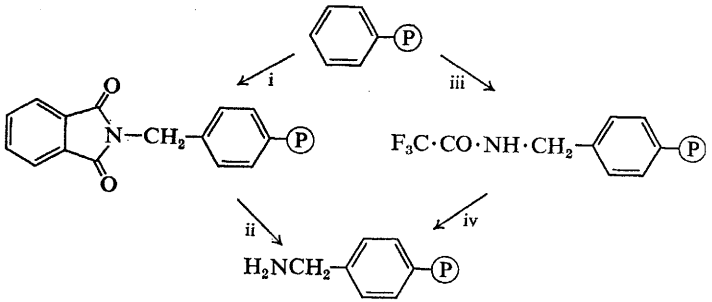
Reagents: i, Potassium phthalate; ii, Hydrazine; iii, Boc-11-aminohendecanoic acid–DCC, followed by 25% TFA–CH_2Cl_2 and repeated; iv, *p*-bromomethylphenylacetic acid–DCC

Scheme 16

[86] M. Manning, *Encyclopaedia of Polymer Science and Technology*, Suppl. 1, 1976, 492.
[87] B. W. Erickson and R. B. Merrifield, in 'The Proteins', ed. H. Neurath, R. C. Hill, and C.-L. Boeder, Academic Press, New York, 1976, 3rd ed., Vol. III, p. 257.
[88] R. Arshady, G. W. Kenner, and A. Ledwith, *Makromol. Chem.*, 1976, **177**, 2911.
[89] J. T. Sparrow, *J. Org. Chem.*, 1976, **41**, 1350.
[90] J. T. Sparrow, in ref. 1, p. 419.

residues of 11-aminohendecanoic acid. A DCC mediated coupling with (*p*-bromomethylphenyl)acetic acid allowed the resin to be used with a conventional benzyl ester linkage to the peptide. This resin has been employed in the synthesis of the 57-residue peptide apolipoprotein C-I (see p. 353).[91]

Other routes to aminomethyl-polystyrene (63) more direct than that outlined in Scheme 16 have been proposed.[92] These involve either direct phthalimidomethylation or trifluoroacetamidomethylation according to Scheme 17. The

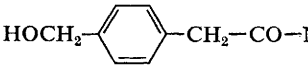


(63)

Reagents: i, *N*-(Hydroxymethyl) or *N*-(chloromethyl) phthalimide with an acid catalyst; ii, Hydrazine; iii, *N*-(hydroxymethyl) trifluoroacetamide with an acid catalyst; iv, 10% KOH in ethanol

Scheme 17

usefulness of these resins has been illustrated by the preparation of a model tetrapeptide. The loss of peptide during a solid-phase synthesis by acidolysis of the benzyl ester peptide–resin linkage is troublesome, and two groups have advocated the use of a *p*-carbamylmethylbenzyl ester linkage derived from (65).[93, 94] Because of the electron-withdrawing influence of the *p*-substituent the



(65)

Lys_5-Glu_3-Leu_2-Trp(Nps)-Phe

(66)

benzyl ester is stable to the acid conditions used during the assembly of the peptide but is labile enough to allow removal by anhydrous HBr or HF. During preparation of the model peptide (66) it was estimated spectrophotometrically that on average only 0.01% of peptide was removed each cycle.[93] Using a model tetrapeptide attached to the resin it was independently found that the *p*-substituent in (65) increased the ester stability to 50% TFA–CH_2Cl_2 100-fold over the normal benzyl ester linkage.[94]

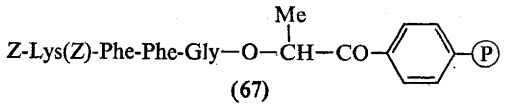
[91] G. F. Sigler, A. K. Soutar, L. C. Smith, A. M. Gotto, jun., and J. T. Sparrow, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1422.

[92] A. R. Mitchell, S. B. H. Kent, B. W. Erickson, and R. B. Merrifield, *Tetrahedron Letters*, 1976, 3795.

[93] J. Blake and C. H. Li, *J.C.S. Chem. Comm.*, 1976, 504.

[94] A. R. Mitchell, B. W. Erickson, M. N. Ryabtsev, R. S. Hodges, and R. B. Merrifield, *J. Amer. Chem. Soc.*, 1976, **98**, 7357.

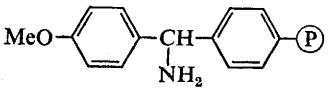
In order to facilitate the preparation of protected peptide fragments a photolabile α-methylphenacyl ester linkage has been advocated.[95] The derivatized resin was obtained by Friedal–Crafts acylation of copolystyrene-2% divinylbenzene with 2-bromopropionyl chloride. The *C*-terminal Boc-protected amino-acid was added as its caesium salt and elaborated to (67) using normal solid-



(67)

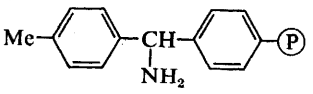
phase procedures. Irradiation at 350 nm released 70% of the tetrapeptide in 72 h, while hydrazinolysis appeared to be complete in less than 1 h. The *o*-nitrobenzyl ester linkage (see Vol. 8, p. 270) was less labile to photolysis than the phenacyl ester linkage but hydrazinolysed extremely rapidly.[95]

Benhydrylamine polymers have been particularly valuable for the preparation of peptide amides (TRH, LH-RH calcitonin, or oxytocin) with *C*-terminal glycine or proline residues. For molecules which terminate in different residues lower cleavage yields have sometimes been obtained.[96, 97] This may be due to the mode of preparation of the benzhydrylamine resin.[96] Resin prepared by metal

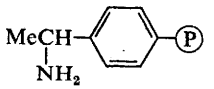


(68)

hydride reduction of the oxime of benzoylpolystyrene was found to contain 50—60% *secondary* amine, presumably produced by Curtius rearrangement. Better results were obtained using the Leuckart reaction, and this route was used in preparing a *p*-methoxybenzhydrylamine resin (68). It was used to prepare



(69)



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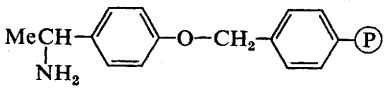
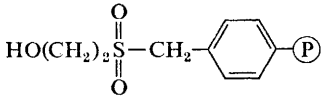


(71)

a number of simple peptide amides in high yield, including TRH. Another investigation, of the resins (69—71), came to the conclusion that only (69) is compatible with the Boc group for the synthesis of peptide amides.[97]

[95] S.-S. Wang, *J. Org. Chem.*, 1976, **41**, 3258.
[96] R. C. Orlowski, R. Walter, and De L. Winkler, *J. Org. Chem.*, 1976, **41**, 3701.
[97] J. M. Stewart, C. Pëna, G. R. Matsueda, and K. Harris, in ref. 2, p. 285.

A base-labile resin linkage analogous to the Msc amino-protecting group (see Vol. 8, p. 254) has now been fully developed.[98, 99] 2-Mercaptoethanol was treated with chloromethylpolystyrene in liquid ammonia in the presence of sodium hydroxide, and the product oxidized with *m*-chloroperbenzoic acid to the sulphone resin (72). Boc-protected amino-acids could then be esterified to the resin with DCC and normal solid-phase procedures followed. Investigations on a model peptide (73) revealed that cleavage by β-elimination was complete in



(72)

Z-Ala-Phe-Leu-Gly-OH
(73)

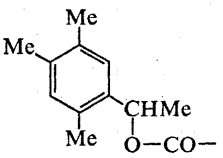
45 s with dioxan–methanol–4N-NaOH (30 : 9 : 1), whereas organic bases and, surprisingly, 0.1N-NaOH in dioxan were relatively ineffective.[98] The sulphone resin (72) was compared with a normal benzyl ester resin in the synthesis of protected α-ACTH (5—10) (74).[99] Using the dioxan–methanol–4N-NaOH

Z-Glu(OBu^t)-His-Phe-Arg(NO_2)-Trp-Gly-OH
(74)

mixture, 66% of the desired peptide acid was released from the sulphone resin in one 3 min treatment, whereas only 77% was released from the benzyl ester resin during four such treatments. Further experience will be necessary to establish the general inertness of side-chain ester and amide groups to these alkaline conditions.

Some oxytocin analogues with thiazolidine-4-carboxylic acid in place of proline have been prepared utilizing the novel dehydroalanine resin linkage (see also Vol. 8, p. 269).[100] In this synthesis the ethylcarbamoyl (Ec) group was used for cysteine protection, without any apparent difficulty; peptide bond formation was by repeated DCC–HOBt reactions. The peptide was released from the resin with 1N-HCl in acetic acid containing one equivalent of water at 50 °C for 30 min; removal of the Ec group followed by oxidation gave highly pure oxytocin analogues. A detailed study of the effect of acid concentration in the CF_3CO_2H–CH_2Cl_2 removal of Boc groups in solid-phase synthesis has been reported.[101] Using the *C*-terminal tri- and tetra-peptides of RNase A as models it was established that the optimum concentration of trifluoroacetic acid was 40%. Removal of the Boc group was troublesome in the synthesis of a sequence of human growth hormone[97, 102] even using a double deprotection cycle, and this has prompted the suggestion that incomplete deprotection is a major source of truncated sequences in solid-phase synthesis. Use of the highly acid-labile optically active α,2,4,5-tetramethylbenzyloxycarbonyl group (Tmz) (75) has

[98] G. I. Tesser, J. T. W. A. R. M. Buis, E. Th. M. Wolters, and E. G. A. M. Bothé-Helmes, *Tetrahedron*, 1976, **32**, 1069.
[99] J. T. W. A. R. M. Buis, G. I. Tesser, and R. J. F. Nivard, *Tetrahedron*, 1976, **32**, 2321.
[100] J. D. Rosamund and M. F. Ferger, *J. Medicin. Chem.*, 1976, **19**, 873.
[101] R. E. Reid, *J. Org. Chem.*, 1976, **41**, 1027.
[102] C. Pẽna, J. M. Stewart, A. C. Paladini, J. M. Dellacha, and J. A. Santomé, in ref. 1, p. 523.



(75)

been suggested to circumvent the problem.[103] Its rate of removal is 4000 times greater than that of the Boc group and $\frac{1}{30}$ that of Bpoc. A 3% solution of TFA in dichloromethane was sufficient to remove the Tmz group completely from resin-bound glycine within 3 min.

p-Chlorobenzyl esters for the side-chain protection of aspartic and glutamic acids have been proposed as alternatives to the commonly used benzyl esters.[104] They possess greater acid stability yet are removed easily with HF. However, they proved too labile for use in the preparation of protected peptide fragments when HBr in acetic acid was employed to remove the peptide from the resin. The more acid-resistant *p*-nitrobenzyl esters were used successfully.

Inclusion of trifluoroethanol has been shown to increase coupling efficiency during the synthesis of the model peptide (66).[105] The five lysine residues serve to magnify the effect of incomplete reaction since omission of any one of them gives rise to the same failure sequence. This shortened peptide was easily separable from the target sequence allowing easy estimation of coupling efficiency. Using symmetrical anhydrides for acylation the average coupling yield of each lysine residue was 98.3% in methylene chloride but 99.7% in 20% trifluoroethanol. The higher yield is associated with a two-fold increase in peptide–resin volume. This must be due to a solvation effect on the peptide since with chloromethyl resin itself no such increase in volume is observed. Rather surprisingly, accelerated active ester coupling rates have been observed in the preparation of *N*-benzyloxycarbonyl-*SS'*-dibenzyloxytocin when the reaction medium was toluene.[106] Generally, couplings were ten times faster than in DMF. More groups are now using catalysed active-ester condensations in solid-phase synthesis, *e.g.* in the preparation of analogues of oxytocin, hydroxysuccinimide and *p*-nitrophenyl esters were used successfully employing 1,2,4-triazole as catalyst.[107]

o-Nitrophenylsulphenyl-*N*-carboxyanhydrides have found application in solid-phase synthesis.[108] No racemization was found during the solid-phase coupling of Nps-NCA's in model experiments and they were used in the preparation of Gly^5,Gly^{10}-gramicidin S. However, analysis of the linear and cyclic products from this synthesis by hydrolysis followed by g.l.c. indicated substantial racemization. Whether this is due to the coupling method or to the hydrolysis step is still uncertain. Further details have appeared on the synthesis

[103] G. R. Matsueda and J. M. Stewart, in ref. 1, p. 333.
[104] R. L. Prestidge, D. R. K. Harding, and W. S. Hancock, *J. Org. Chem.*, 1976, **41**, 2579.
[105] D. Yamashiro, J. Blake, and C. H. Li, *Tetrahedron Letters*, 1976, 1469.
[106] S. A. Khan and K. M. Sivanandaiah, *Synthesis*, 1976, 614.
[107] R. Walter, R. T. Havran, and I. L. Schwartz, *J. Medicin. Chem.*, 1976, **19**, 328.
[108] T. Christensen, J. Halstrøm, K. Kovacs, P. Villemoes, and K. Brunfeldt, in ref. 2, p. 213.

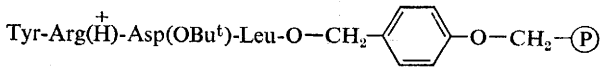
of ACTH (1—24) by the oxidation–reduction method.[109, 110] The use of high performance liquid chromatography to estimate completeness of amine uptake is a novel feature of the method which may find wider application.

The crystalline peptide hydrazide (76) has been prepared rapidly and on a large scale by a fully automated technique.[111] The procedure involves DCC

Boc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-NH-NH_2

(76)

coupling of the *C*-terminal amino-acid to hydroxymethyl-polystyrene in the presence of 4-dimethylaminopyridine, blocking of unchanged hydroxy-groups, four cycles of conventional solid-phase synthesis and hydrazinalysis from the resin. This last step regenerated the starting resin which was re-used with approximately equal efficiency (60%) four times. In the absence of side-chain-protected aspartic and glutamic acid residues, this procedure may become the prototype for rapid preparation of protected peptide hydrazides suitable for fragment condensation strategies. 8-Argininevasopressin[112] and residues 1—47 of the sequence assigned to urogasterone[113] have been synthesized by fragment



(77)

condensation techniques. The urogastrone sequence was assembled[113] on a *p*-alkoxybenzyl resin first by stepwise amino-acid addition to the tetrapeptide stage (77) and then by sequential condensation with the four protected fragments (78)—(81), previously prepared by solution methods. Protonation was used for side-chain protection of arginine throughout, avoiding the use of strong acids

Bpoc-Glu(OBu^t)-Arg(H^+)-Cys(Acm)-Gln·OH

(78)

Bpoc-Cys(Acm)-Asn-Cys(Acm)-Val-Val-Gly-Tyr-Ile-GlyOH

(79)

Bpoc-Val-Cys(Acm)-Met-Tyr(Bu^t)-Ile-Glu(OBu^t)-Ala-Leu-Asp(OBu^t)-Lys(Adoc)-Tyr(Bu^t)-Ala-OH

(80)

Boc-Asn-Ser(Bu^t)-Asp(OBu^t)-Ser(Bu^t)-Glu(OBu^t)-Cys(Acm)-Pro-Leu-Ser(Bu^t)-His-Asp(OBu^t)-Gly-Tyr(Bu^t)-Cys(Acm)-Leu-His-Asp(OBu^t)-Gly-OH

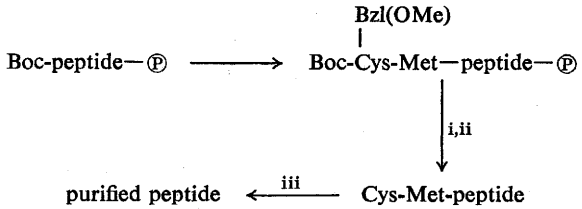
(81)

[109] R. Matsueda, H. Maruyama, E. Kitazawa, H. Takahagi, and T. Mukaiyama, in ref. 1, p. 403.
[110] H. Maruyama, R. Matsueda, E. Kitazawa, H. Takahagi, and T. Mukaiyama, *Bull. Chem. Soc. Japan*, 1976, **49**, 2259.
[111] J. K. Chang, M. Shimizu, and S.-S. Wang, *J. Org. Chem.*, 1976, **41**, 3255.
[112] L.-E. Larsson, P. Melin, and U. Ragnarsson, *Int. J. Peptide Protein Res.*, 1976, **8**, 39.
[113] R. Camble and N. N. Petter, in ref. 2, p. 299.

for deprotection. Chain branching by acylation of arginine side-chains was suppressed by use of only one equivalent of *N*-methylmorpholine at each neutralization step. Repeated DCC–HOBt couplings were used with only 1.2 equivalents of the first three protected peptide fragments (78)—(80), giving incorporations of 95, 92, and 76%, respectively. Similar yields were obtained in solution couplings. The large *N*-terminal fragment (81) proved more difficult to incorporate and a yield of only 16—19% was obtained by the DCC–HOBt procedure. Other methods gave inferior results. Cleavage from the alkoxybenzyl resin by CF_3CO_2H or 50% CF_3CO_2H–CH_2Cl_2 gave yields of 61 and 75% of crude product, which was purified more easily than that from a similar solution synthesis. This careful and detailed study of a major fragment condensation synthesis illustrates the advantages and difficulties of this approach.

Pyridinium polyhydrogen fluoride (70% HF–pyridine) is a relatively stable reagent which cleaves most common protecting groups including peptide–resin benzyl and benzhydrylamine linkages, thus allowing HF-type reactions to be carried out in polyethylene tubes.[114] Boron tristrifluoroacetate (BTFA) has been used to cleave peptide–resin benzyl esters in conjunction with the scavenger thioanisole.[115] Its value is illustrated in a synthesis of somatostatin.[115]

Two devices have been suggested to facilitate purification of solid-phase products.[116, 117] One advocates addition of a basic or acidic residue to the desired sequence to ease purification by ionic exchange techniques;[116] the other more sophisticated approach makes use of the affinity of free cysteine residues for organomercurial agarose.[117] This affinity chromatography technique is dependent on the peptide under investigation being free of internal methionine or cysteine residues, since these two residues are added at the end of the assembled target sequence (Scheme 18). After cleavage from the resin with HF and a



Reagents: i, HF; ii, gel filtration followed by affinity chromatography on organomercurial Sepharose; iii, CNBr in 70% formic acid

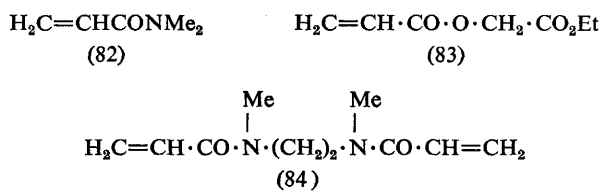
Scheme 18

preliminary gel filtration the product was passed down an organomercurial-agarose column which bound peptides containing cysteine, all truncated sequences passing directly through the column. The desired peptide could then be eluted with a thiol reagent and the superfluous residues removed using cyanogen bromide. The method is illustrated by the preparation of ribonuclease A(111—124) and histone H4 (1—37) when a six-fold increase in purity was achieved.[117]

[114] S. Matsuura, C.-H. Niu, and J. S. Cohen, *J.C.S. Chem. Comm.*, 1976, 451.
[115] W. Bauer and J. Pless, in ref. 1, p. 341.
[116] K. Suzuki, Y. Sasaki, and N. Endo, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 1.
[117] D. E. Krieger, B. W. Erickson, and R. B. Merrifield, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3160.

Supports used for Solid-phase Synthesis. Further examples of the use of the polydimethylacrylamide resin have been reported[118] (see also Vol. 8, p. 273). The essential structural features for a polar resin which can be used for solid-phase operations in polar media are discussed, and further details given on the chromatography of the previously described synthesis of acyl carrier protein residues 65—74. Some of the minor impurities have been ascribed to side-products arising during removal from the resin with liquid HF, *e.g.* an impurity analysing correctly for all the consistent amino-acids except tyrosine probably arises from intramolecular rearrangement of the O-protecting group. Undeca- and heptadeca-peptides related to the *C*-terminus of the ribonuclease barnase have also been prepared on the polyamide resin, and provide additional evidence for the value of this support.[118]

Another polyamide support has been described based on the monomers (82)—(84).[119] It swells to four times its dry bed volume in dimethylformamide. No applications to peptide synthesis have been reported.



(82) (83)

(84)

Sephadex LH-20 has been used for the synthesis of an N-protected penta-peptide composed of residues 5–9 of substance P.[120] A previous synthesis of bradykinin has been recorded using the same support.[121] In the current work, glycine was attached to the polysaccharide support with *NN'*-carbonyldi-imidazole, Boc-groups were removed with toluene-*p*-sulphonic acid (esterification of residual hydroxy-groups on the support by the sulphonic acid was also thought to occur), and couplings were judged to be 90—100% complete using three-fold excesses of *o*-nitrophenyl esters for coupling. The peptide was removed by alkaline hydrolysis using 0.1N methanolic NaOH in 45% yield.

Techniques for Monitoring Solid-phase Synthesis. A computer-controlled synthesizer has been described.[122] The computer controls all operations including a feed-back analytical evaluation of coupling and deprotection by titration with perchloric acid. A further illustration of the use of this technique, involving titration of large (1 g) dried and weighed resin samples instead of the total reaction vessel contents has been reported.[123]

The use of picric acid to monitor individual coupling and deprotection steps must be approached with some caution.[124] It has been determined that as the support becomes more polar due to the increased peptide content picric acid

[118] E. Atherton, D. L. J. Clive, D. A. East, and R. C. Sheppard, in ref. 2, p. 291.
[119] H. Köster and W. Heidmann, *Angew. Chem. Internat. Edn.*, 1976, **15**, 546.
[120] A. Orlowska, K. Bankouski, and S. Drabarek, *Roczniki Chem.*, 1976, **50**, 1701.
[121] G. P. Vlasov, A. Y. Bilibin, Y. N. Kuznetsova, I. Ditkovskaya, and V. N. Lashkov, *Chem. Zeitung*, 1973, **97**, 236 (*Chem. Abs.*, 1973, **79**, 42 833).
[122] P. Villemoes, T. Christensen, and K. Brunfeldt, *Z. physiol. Chem.*, 1976, **357**, 713.
[123] O. Schou, *Acta Chem. Scand.* (*B*), 1976, **30**, 991.
[124] W. S. Hancock, J. E. Battersby, and D. R. K. Harding, *Analyt. Biochem.*, 1975, **69**, 497.

may become increasingly difficult to wash out quantitatively. The reagent also cleaves 1.5% of Boc groups per hour from Boc-Ile-polymer. 2,4,6-Trinitrobenzenesulphonic acid (TNBS) has been advocated as an alternative to the well-known ninhydrin test for monitoring the completeness of coupling reactions.[125] The TNBS reaction is relatively mild and gives the same deep red colour when reacting with free amino-groups no matter which amino-acid is *N*-terminal. Fluorescamine is unsuitable for monitoring completeness of coupling reactions on nitrated polystyrene since this support quenches the fluorescence.[126]

The u.v.-absorbing properties of the α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz) group have been used to monitor continuously the assembly of the resin peptide (85) when using the oxidation–reduction coupling method.[127]

Ddz-Arg(NO_2)-Leu-Arg(NO_2)-Leu-Leu-Gln(Mbh)-Gly-Leu-Val-Ⓟ

(85)

Trianisylphosphine was used as the reducing agent since it has been reported to be superior to triphenylphosphine for coupling urethane-protected nitroarginine residues. Incorporation of individual residues was measured quantitatively during the deprotection cycle. Complete acylation was apparently achieved at each cycle but Edman degradation revealed the presence of failure sequences amounting to 4.3% of the total product.

Side-reactions in Solid-phase Synthesis. The observation that the background picric acid titration values rise as synthesis progresses led to investigation of possible *N*-alkylation peptides (Scheme 19) during the acidolytic removal of the

$$Bu^tO\cdot CO\cdot NH\cdot CHR\cdot CO\text{—peptide—Ⓟ} \xrightarrow{i} Bu^tNH\cdot CHR\cdot CO\text{—peptide—Ⓟ}$$

Reagent: i, TFA–CH_2Cl_2

Scheme 19

Boc group [128] (for an alternative explanation see ref. 124). This side-reaction was not observed during removal of the Boc-group from the model dipeptide (86)

Boc-Gly-Lys(Z)—Ⓟ

(86)

(with CF_3CO_2H–CH_2Cl_2) but cleavage of the benzyloxycarbonyl group gave rise to small amounts of *N*-benzylated product. The reaction is intramolecular since N_α-protected lysine gives only the N_α-benzyl side product and N_ε-protected lysine gives only the N_ε-side product. It does not occur when liquid HF is used as the cleavage agent.

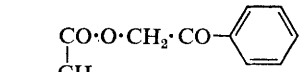
During a solid-phase synthesis of the decapeptide antamanide an increase in perchloric acid titration occurred after incorporation of valine onto resin-bound proline. This was shown to be due to *N*-alkylation of the terminal proline

[125] W. S. Hancock and J. E. Battersby, *Analyt. Biochem.*, 1976, **71**, 260.
[126] J. A. Smith, *Analyt. Biochem.*, 1976, **74**, 477.
[127] C. Birr, M. Ueki, and R. Frank, in ref. 1, p. 409.
[128] A. R. Mitchell and R. B. Merrifield, *J. Org. Chem.*, 1976, **41**, 2015.

residues by excess chloromethyl groups on the resin.[129] The amount of proline remaining attached to the resin after removal of the peptide was consistent with the increase in perchloric acid titration value.

Reaction of t-butylcarbonium ions with peptides containing methionine, even in the presence of the scavenger anisole, has been shown to occur during HF removal of peptides from polystyrene-based resins.[130] During a synthesis of ovine prolactin residues 37—55 an unstable by-product was obtained which slowly reverted to the main product. It was not found if the *N*-terminal Boc protecting group was removed with CF_3CO_2H before release of the peptide from the resin with HF. The by-product is thought to be the *S*-t-butylsulphonium derivative, and the authors recommend removal of Boc groups from peptide resins before exposure to HF if the peptide sequence includes methionine.[130]

Aspartic acid β-esters are troublesome in solid-phase synthesis because of the tendency to acid-catalysed cyclic imide formation, especially when the adjacent residue is glycine or serine. Since the side-chain-unprotected aspartic acid derivatives show little tendency to cyclize, use of β-phenacyl esters (87) which can be selectively cleaved under non-acidic conditions has been proposed.[131, 132] This side-chain ester can be removed from resin-bound peptides with sodium thiophenoxide prior to HF treatment. In the case of the model peptide (88) this procedure reduced the amount of cyclic product from 99 to 2.4%. Side-reactions of glutamyl peptides in HF have again been discussed[133] (see Vol. 8, p. 259).



(87)

Glu-Asp-Gly-Thr

(88)

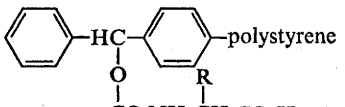
Other Repetitive Methods. A programmed synthesizer has been described for the liquid-phase method of peptide synthesis.[134] This method utilizes a linear soluble polymer, usually poly(ethylene glycol) (PEG), to which protected amino-acids can be added in a stepwise manner. Reactions are carried out in solution and the product is isolated by precipitation. Relative reaction rates for the coupling steps have been determined and the effects of side-chain protecting groups, amino and carboxyl component residues, coupling method, reaction solvent, and polymer molecular weight investigated. These results should be generally useful since rates obtained in the soluble polymer system are claimed to be the same as those for analogous non-polymer bound reactants in solution.

The PEG-soluble polymer system is optically transparent to 190 nm, and conformational changes during assembly of the peptide chain may be followed by circular dichroism measurements.[135]

[129] O. Schou, D. Bucher, and E. Nebelin, *Z. physiol. Chem.*, 1976, **357**, 103.
[130] R. L. Noble, D. Yamashiro, and C. H. Li, *J. Amer. Chem. Soc.*, 1976, **998**, 2324.
[131] C. C. Yang and R. B. Merrifield, *J. Org. Chem.*, 1976, **41**, 1032.
[132] C. C. Yang and R. B. Merrifield, in ref. 1, p. 447.
[133] R. S. Feinberg and R. B. Merrifield, in ref. 1, p. 455.
[134] E. Bayer, M. Mutter, and G. Holzer, in ref. 1, p. 425.
[135] M. Mutter, H. Mutter, R. Uhmann, and E. Bayer, *Biopolymers*, 1976, **15**, 917.

The liquid-phase soluble polymer method has also been used in conjunction with insoluble polymeric N-protected amino-acid active esters.[136] Best results were obtained when a five-fold excess of polymer-bound *o*-nitrophenyl ester was used; three couplings with the soluble polymeric amino-component were needed to achieve quantitative acylation. The method was illustrated by the preparation of several tripeptides containing ornithine and alanine.

An alternative to the purely liquid-phase soluble polymer protocol is the alternating liquid–solid system (see Vol. 8, p. 279). This has been developed further [137] using soluble PEG esters as amino-components and insoluble polymeric diphenylmethoxycarbonyl derivatives (89) as carboxyl-components. After the acylation step unchanged amino-component is removed by filtration from insoluble coupled product. Unchanged carboxyl-component is removed by gel filtration after the deprotection step. The technique was illustrated by preparation of the pentapeptide (90), a superior product being obtained than by the liquid-



(89)

Gly-Val-Gly-Ala-Pro

(90)

phase method. 5β-Dihydrocholesterol has found application as a high molecular weight *C*-terminal protecting group in a repetitive method. Product separation was by LH-20 chromatography. The *C*-terminal pentapeptide of angiotensin II was prepared as an illustration of its use.[138]

Full details of the impressive synthesis of secretin by the repetitive excess mixed anhydride (REMA) method have appeared,[139] as has an account of a similar synthesis of LH-RH.[140] In the latter, the water-solubility of *N*-benzyloxycarbonyl-L-prolylglycinamide and also the danger of lactam formation when coupling arginine derivatives to *N*-terminal proline residues prompted preparation of the tripeptide (91), as in Scheme 20. The remaining residues were then added

$$\text{Z-Arg(NO}_2\text{)-Pro-OH} + \text{H-GlyOEt} \xrightarrow{\text{i}} \underset{(91)}{\text{Z-Arg(NO}_2\text{)-Pro-GlyOEt}}$$

Conditions: i, isobutyl chloroformate, *N*-methyl morpholine, −15 °C

Scheme 20

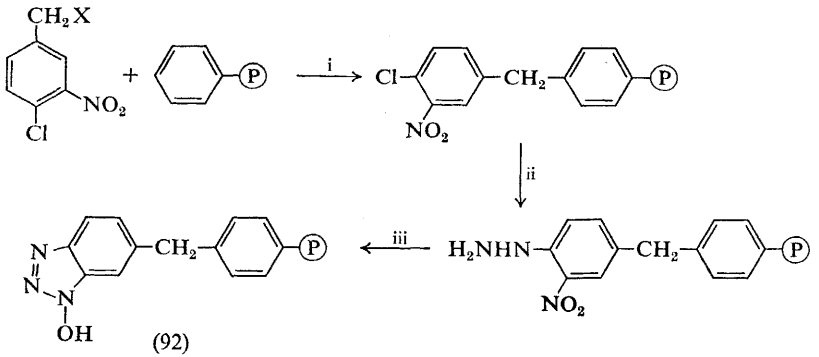
sequentially, the peptide converted by ammonolysis into the amide at the heptapeptide stage (without apparently greatly degrading the nitroarginine residue) and after deprotection with HF, LH-RH was obtained by CM-Sephadex chromatography.

[136] G. Jung, G. Bovermann, W. Göhring, and G. Heusel, in ref. 1, p. 433.
[137] H. Frank, H. Meyer, and H. Hagenmaier, in ref. 1, p. 439.
[138] J. Burton, in ref. 1, p. 365.
[139] A. van Zon and H. C. Beyerman, *Helv. Chim. Acta*, 1976, **59**, 1112.
[140] H. C. Beyerman, T. Rammelov, J. F. N. Renirie, J. L. M. Syrier, and A. van Zon, *Reuceil*, 1976, **95**, 143.

The REMA method has been compared with the solid-phase and pentafluorophenyl active ester methods, using as a model the *C*-terminal heptapeptide of gastrin.[141] At the end of each synthesis benzyl side-chain protecting groups (and in the case of the solid-phase synthesis the benzydrylamine resin) were removed by boron tristrifluoroacetate. The pentafluorophenyl ester rapid synthesis gave the highest yield (39%) of fully active material, whereas the solid-phase method gave 16% and the REMA method 10.2% yields. Comparisons of the three methods were also made during the synthesis of ACTH (1—24) from five fragments.

Polymeric *N*-hydroxysuccinimide esters of Nps-amino-acids have been prepared[142] and used in the synthesis of a protected tetrapeptide. Studies have also been carried out on the coupling rates of these derivatives.[143] A polymerization method of preparing polymeric *N*-hydroxysuccinimide esters has been proposed.[144] This involves preparation of Boc-amino-acid *N*-hydroxy-*maleimide* esters and their polymerization in the presence of styrene, divinylbenzene, and *N*-(*p*-chlorophenyl)maleimide (to aid the swelling characteristics of the polymers). The use of these activated polymeric esters was illustrated by the preparation of several dipeptides, but the polymerization step required for the preparation of each individual Boc-amino-acid derivative makes the method of low appeal.

Hydroxybenzotriazole-bound polystyrene (92) has been prepared according to Scheme 21.[145] Boc- or Z-protected amino-acids can be coupled to give the corresponding polymeric active esters with substitution levels between 0.6 and 1.3 mmol g^{-1}. The superiority of this method of activation over polymeric *o*-nitrophenyl esters was demonstrated by the comparative synthesis of a tetrapeptide. Even hindered couplings were complete within 20 min with HOBT resin esters, whereas the *o*-nitrophenyl esters needed 1½ days.



Conditions: i, $AlCl_3$–$PhNO_2$; ii, NH_2—NH_2; iii, HCl

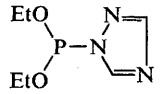
Scheme 21

[141] B. Penke, P. Pallai, K. Kovacs, and L. Balaspiri, in ref. 2, p. 101.
[142] Yu. A. Davidovich and V. Gut, *Coll. Czech. Chem. Comm.*, 1976, **41**, 1805.
[143] V. Gut and Yu. A. Davidovich, *Coll. Czech. Chem. Comm.*, 1976, **41**, 780.
[144] M. Akiyama, K. Shimizu, and M. Narita, *Tetrahedron*, 1976, 1015.
[145] M. Fridkin, R. Kalir, A. Warshawsky, and A. Patchornik, in ref. 1, p. 395.

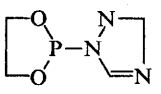
Acylation by the oxidation–reduction method has been achieved using polymer-bound triphenylphosphine.[146] The polymer can be prepared with a phosphorus content of 2.6—2.9 mmol g^{-1}, and optimum results were obtained when two equivalents of this reagent were used with 1.5 equivalents of 2,2′-dipyridyl disulphide over the amino- and carboxyl-components in boiling methylene chloride.

Synthesis of Polymeric Models for Studies in Protein Chemistry.—A simple procedure for the synthesis of amino-acid *N*-carboxyanhydrides has been described.[147] Amino-acids suspended in dry THF are treated with an excess of a standardized solution of phosgene in benzene at 65 °C. Yields are in the 80—95% range.

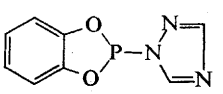
1-(Trimethylsilyl)-1,2,4-triazole can be treated with chlorophosphites to give compounds (93)—(95), which are useful polymerization reagents.[148] They were



(93)



(94)



(95)

applied to the preparation of poly-(N_ε-benzyloxycarbonyl-L-lysine), poly-(N_α-benzyloxycarbonyl-L-lysine), and polymers of several di- and tri-peptides. The resulting polymers were easily freed from phosphorus compounds. A new method for preparing polymers has been described which involves polymerization on a solid support.[149] The trifluoroacetate salts of tripeptide pentachlorophenyl esters were deposited on celite and heated at 165 °C *in vacuo*. The degree of racemization was assessed [149] by polymerization of (96) and (97) and estimation

$CF_3CO_2^-$ $\overset{+}{H}_2$·Gly-Val-Ala-OPcp (96)

$CF_3CO_2^-$ $\overset{+}{H}_2$·Val-Ala-Gly-OPcp (97)

of the amount of D-alanine in acid hydrolysates by the Manning and Moore method.[150] Both polymers yielded 9% D-alanine but since in (97) glycine is the activated amino-acid this amount of racemate was attributed to the acid hydrolysis conditions and not to polymerization.

The question of racemization during the preparation of monomer units for polymerization and during the polymer-forming step itself has been investigated with collagen models.[151] All steps during the synthesis of the tripeptide monomers were racemization-free, but activation to form *o*-nitrophenyl or pentachlorophenyl esters resulted in some racemization. D-Valine (36%) was found in hydrolysates of (98) after digestion with L-amino-acid oxidase. With the exception

Z-Pro-Gly-Val-OPcp (98)

Pro-Gly-Phe-ONp (99)

[146] K. Horiki, *Tetrahedron Letters*, 1976, 4103.
[147] W. D. Fuller, M. S. Verlander, and M. Goodman, *Biopolymers*, 1976, **15**, 1869.
[148] H. R. Kricheldorf, M. Fehrle, and J. Kaschig, *Angew. Chem. Internat. Edn.*, 1976, **15**, 305.
[149] M. Sakarellos-Daitsiotis, C. G. C. Sakarellos, and M. Goodman, *J. Amer. Chem. Soc.*, 1976, **98**, 7105.
[150] J. M. Manning and S. Moore, *J. Biol. Chem.*, 1968, **243**, 5591.
[151] R. S. Rapaka, R. S. Bhatnagar, and D. E. Nitecki, *Biopolymers*, 1976, **15**, 317.

of (99) which gave 8% D-phenylalanine, polymerization did not introduce additional racemization. Fractionation of the polymerized products revealed that the higher molecular weight fractions contained less racemized product, indicating that the polymerization is stereoselective.

This method of forming activated peptide units is clearly not the one of choice. A better method is the 'backing off' procedure[152] where the monomer unit is synthesized by adding N-protected amino-acids to the pre-activated *C*-terminal amino-acid; this method gave optically pure pentachlorophenyl ester (100) whereas activation of the preformed tripeptide resulted in 45% racemization.[149]

$CF_3CO_2^-$ $\overset{+}{H}_2$·Val-Val-Ala-OPcp (100)

$(Pro\text{-}Gly\text{-}Phe)_n$ (101)

Optically pure *N*-hydroxysuccinimide esters can, however, be formed directly from tripeptide acids,[153] but excess base in the polymerization mixture must be avoided. In the preparation of (101) from the optically pure *N*-hydroxysuccinimide ester one equivalent of polymerization-initiating triethylamine gave no racemic product, but two equivalents gave 8%.[151]

A series of protected oligopeptides having alternating γ-methyl-L-glutamyl and L-phenylalanyl residues have been prepared utilizing Nps-amino-acid *N*-carboxy-anhydrides.[154] Peptides up to the octapeptide were synthesized in a stepwise manner, but no evidence of optical purity was presented. The method has also found application in the synthesis of oligopeptides of type (102).[155]

$(Ala\text{-}Leu\text{-}Gly)_n$ (102) $n = 1—6$

$Gly\text{-}X\text{-}(Gly)_n\text{-}Trp\text{-}Gly$ (103)

In a study of protein–nucleic acid interactions a block oligopeptide has been prepared which has a tyrosine residue flanked on each side by three alanine and three or six lysine residues.[156] The compound was prepared by stepwise synthesis of the individual blocks and then fragment condensation. The conformation of the peptides was studied by c.d. and fluorescence techniques. In a u.v. study of oligopeptides of the type (103) compounds with X = Phe have been prepared.[157]

Preparation of a polymer containing *N*-chloroacetyl groups, as a potential ligand for protein thiol groups, has been described.[158] This was made by initiating the polymerization of alanine *N*-carboxyanhydride with N_α-chloro-acetyl-lysine n-butylamide. Its affinity for the thiol group of cysteine was demonstrated.

The solid-phase method has been used to synthesize some oligo-L-proline peptides[159] which are in principle monodisperse. Peptides up to 40 residues long have been made by condensing Boc-tri-L-proline fragments onto the solid

[152] M. Goodman and C. Steuben, *J. Amer. Chem. Soc.*, 1959, **81**, 3980.
[153] R. S. Rapaka, R. S. Bhatnagar, and D. E. Nitecki, *Biopolymers*, 1976, **15**, 1585.
[154] R. Katakai and Y. Nakayama, *Biopolymers*, 1976, **15**, 747.
[155] R. Katakai, *Biopolymers*, 1976, **15**, 1815.
[156] R. Guarnaccia, V. Rizzo, P. Gionola, and P. L. Luisi, *Biopolymers*, 1976, **15**, 1103.
[157] P. L. Luisi, R. Guarnaccia, V. Rizzo, G. P. Lorenzi, P. Wiget, and P. Skrabal, in ref. 1, p. 255.
[158] C. H. Moussebois, J. F. Heremans, P. Osinski, and W. Rennerts, *J. Org. Chem.*, 1976, **41**, 1340.
[159] M. Rothe, H. Rott, and J. Mazanek, in ref. 2, p. 309.

support in yields close to 100%, this procedure circumventing problems of diketopiperazine formation. As with oligo-(γ-methyl-L-glutamates) (see Vol. 8, p. 280) organized structure appeared at the heptapeptide level.[160]

The hydroxysuccinimide ester of Lys(Z).Glu(OBzl) and the β-benzyl aspartate analogue have been prepared and polymerized.[161] C.d. and i.r. studies showed that the right-handed sense of the α-helix was retained in both sequential polypeptides.[162]

A cross-linked tropoelastin-like polypentapeptide has been made from the monomers (104)—(106).[163] The basic monomer (104) was polymerized in the presence of lower amounts of (105) or (106), and the products cross-linked using water-soluble carbodi-imide. The physical properties[164] of the cross-linked polymer and its ability to induce calcification[165] have been studied extensively.

Val-Pro-Gly-Val-Gly-ONp (104)

Val-Pro-Gly-Lys(Z)-Gly-ONp (105)

Val-Pro-Gly-Glu-Gly-ONp (106)

poly(Lys-Gly-Pro-Pro-Gly-Pro)HBr (107)

Random and block polymers of lysine and leucine[166] and lysine and valine[167] have been prepared for studies of the interaction between DNA and polypeptides. Some basic sequential polypeptides, prepared as histone models, have been described.[168] The polymer (107) is the first example of a collagen model with an ionizable side-chain.[169] It formed collagen-like structures although its melting temperature was substantially lower than that of collagen. Other collagen models have been synthesized and their physical properties studied.[170–172]

Synthetic Operations with Peptides of Biological Origin.—Offord and his colleagues have described in detail their extensive semisynthetic studies using lysozyme as a model protein.[173–175] The approach adopted is that of large-scale fragmentation of partially side-chain-protected (ε-maleyl-lysine and cysteine *S*-sulphonate) protein by tryptic digestion at arginine residues. The resulting mixture of 11 N_ε-maleyl peptide sulphonates is fractionated according to molecular size (by gel filtration) and charge (by ion-exchange chromatography).

[160] W. Hecq, R. Brasseur, J. Caspers, and A. Loffet, *Biopolymers*, 1976, **15**, 1425.
[161] H. Doust and S. St.-Pierre, *J.C.S. Perkin I*, 1976, 1453.
[162] K. Itoh, B. M. Foxman, and G. Fasman, *Biopolymers*, 1976, **15**, 419.
[163] K. Okamoto and D. W. Urry, *Biopolymers*, 1976, **15**, 2337.
[164] D. W. Urry, K. Okamoto, R. D. Harris, C. F. Hendrix, and M. M. Long, *Biochemistry*, 1976, **15**, 4083.
[165] D. W. Urry, M. M. Long, C. F. Hendrix, and K. Okamoto, *Biochemistry*, 1976, **15**, 4089.
[166] E. C. Ong, C. Snell, and G. D. Fasman, *Biochemistry*, 1976, **15**, 468.
[167] R. Mandel and G. D. Fasman, *Biochemistry*, 1976, **15**, 3122.
[168] K. Blaha, S. Stokrova, B. Sedlacek, and J. Sponar, *Coll. Czech. Chem. Comm.*, 1976, **41**, 2273.
[169] G. F. Sigler, C. R. McMillin, and A. G. Walton, *Biopolymers*, 1976, **15**, 2005.
[170] V. S. Ananthanarayanan, S. K. Brahmachari, R. S. Rapaka, and R. S. Bhatnagar, *Biopolymers*, 1976, **15**, 707.
[171] R. S. Rapaka and R. S. Bhatnagar, *Int. J. Peptide Protein Res.*, 1976, **8**, 371.
[172] G. Heyer and E. R. Heidemann, *Makromol. Chem.*, 1976, **177**, 3299.
[173] A. R. Rees and R. E. Offord, *Biochem. J.*, 1976, **159**, 467.
[174] R. E. Offord, H. T. Storey, A. R. Rees, C. F. Hayward, W. H. Johnson, M. H. Pheasey, and D. A. Wightman, *Biochem. J.*, 1976, **159**, 480.
[175] A. R. Rees and R. E. Offord, *Biochem. J.*, 1976, **159**, 487.

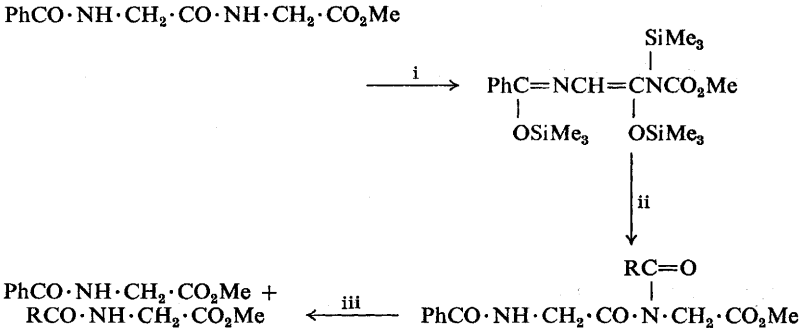
The individual peptides are then further protected at their *N*-termini by t-butoxycarbonylation and at side-chain and terminal carboxy-groups by esterification. The esterification step uses diazo-compounds, notably 1-diazo-2,2-dimethylpropane, the primary carbonium ion from which rearranges before reaction with carboxy-groups to yield t-amyl esters. Perhaps the key step in the whole scheme is the selective unmasking of the terminal carboxy-group by further digestion with trypsin under special conditions. This gives rise to a series of individual peptide acids which in principle are suitable for stepwise reassembly from the carboxy-terminus. A sequence of 45 residues has been prepared in this way using dicyclohexylcarbodi-imide-hydroxybenzotriazole as the coupling agent.

Some general points might be made about this type of approach to protein synthesis in relation to the totally synthetic approach. In the opinion of these Reporters, the undoubted strength of semisynthesis is likely to stem from the *minimum* fragmentation of natural protein starting materials, *i.e.* selective excision of individual amino-acid residues or peptide sequences so that the number of reassembly steps is minimized. It is just these assembly steps involving the coupling of large protected fragments and the subsequent removal of protecting groups which are currently presenting the greatest problems in the totally synthetic approach to proteins. The present scheme of semisynthesis meets these same problems under the less favourable circumstances dictated by the inflexibility of the fragment preparation procedures. The strength of total synthesis lies at present in the preparation of small and medium-sized protected peptide fragments, starting with pure side-chain-protected amino-acids. In the semisynthesis approach the side-chain protecting groups are introduced at a very late stage. It is unrealistic to expect that side-chain protection procedures (especially those involving carbonium ions) will proceed with absolute selectivity when applied to complex polyfunctional proteins and their fragments, and the intractability of the products may render their purification excessively difficult. It seems that the most fruitful application of the powerful semisynthetic approach will involve combination between one or more short synthetic peptides and a single large protein-derived fragment, the latter bearing the minimum of protecting groups. In this way the best features of both total and partial synthesis may be realized. The key step will now be the appropriate selective scission of the starting protein.

A novel way of interchanging peptide fragments by silylation, acylation, and methanolysis (Scheme 22) at non-hindered peptide bonds is being investigated.[176] This could be a useful way of introducing synthetic fragments into natural peptides. Model experiments indicated that acylation occurs preferentially at glycine amide bonds, particularly within -Gly-Gly- sequences.

A chain-cleaved derivative (108) of basic pancreatic trypsin inhibitor has been prepared which has proved useful for a number of semisynthetic transformations.[177] Selective reduction of the disulphide link between cysteine residues 14 and 38 permits cleavage of the active site Lys^{15}-Ala^{16} amide bond by trypsin; reoxidation then gives (108), which can be reconverted into the native inhibitor by the same enzyme acting in reverse. Treatment of the modified inhibitor (108)

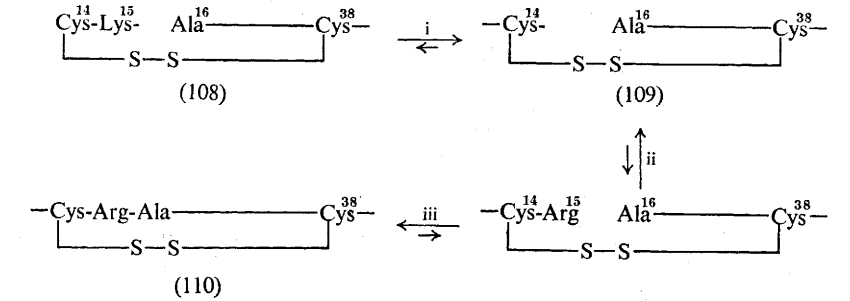
[176] V. B. Angadi, J. S. Davies, K. H. Hopkins, and R. K. Merrit, in ref. 1, p. 375.
[177] H. Jering and H. Tschesche, *European J. Biochem.*, 1976, **61**, 443.



Conditions: i, Trimethylsilylchloride; ii, RCOCl; iii, MeOH

Scheme 22

with aminopeptidase K (to which the natural *N*-terminus is inert) yields the inactive des(Ala[16], Arg[17]) derivative, while carboxypeptidase B treatment of (108) gives the inactive des-Lys[15] compound (109) (Scheme 23). This last derivative was used [178] to prepare three analogues of the inhibitor with arginine, phenylalanine, or tryptophan replacing Lys[15]. When (109) was incubated with arginine in the presence of carboxypeptidase B and trypsin, the first enzyme catalysed re-attachment of the arginine residue and the analogue was then trapped by



Conditions: i, Carboxypeptidase B followed by chromatographic isolation; steps ii and iii are achieved simultaneously in the presence of carboxypeptidase B, arginine, and trypsin

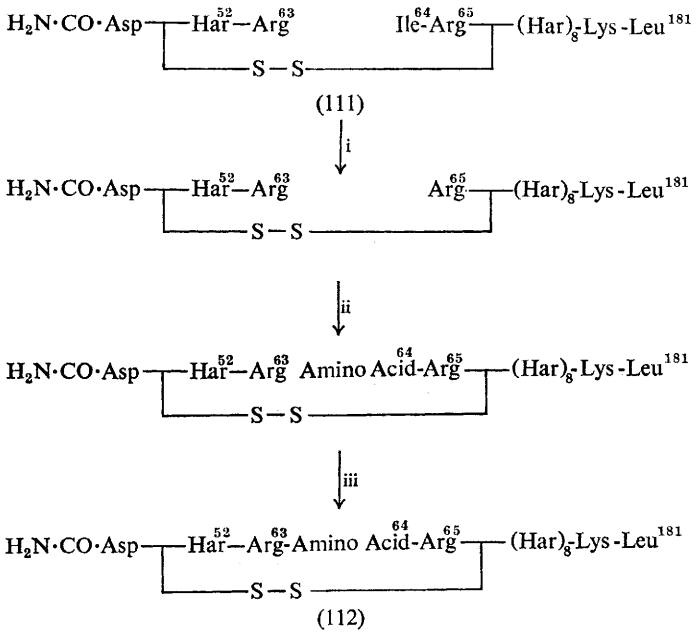
Scheme 23

complex formation with trypsin, which also reformed the amide bond between Arg[15] and Ala[16] to give (110). Carboxypeptidase A and α-chymotrypsin were used in a like manner to make the phenylalanine and tryptophan analogues. The inhibitory activities of the analogues made were investigated and are fully discussed in the paper.

Both enzymic and chemical methods have been used to prepare a series of derivatives of soyabean trypsin inhibitor (STI).[179, 180] A modified inhibitor, with the active centre bond hydrolysed (111), was obtained by successive

[178] H. Jering and H. Tschesche, *European J. Biochem.*, 1976, **61**, 453.
[179] D. Kowalski and M. Laskowski, jun., *Biochemistry*, 1976, **15**, 1300.
[180] D. Kowalski and M. Laskowski, jun., *Biochemistry*, 1976, **15**, 1309.

guanylation of eight of the nine side-chain lysine amino-groups (one is inert), carbamylation of the *N*-terminus, and digestion with trypsin.[179] This modified PTI molecule was then converted (Scheme 24) into derivatives in which Ile[64]



Conditions: i, PhNCS followed by trifluoroacetic acid; ii, Boc-amino-acid *N*-hydroxysuccinimide ester followed by trifluoroacetic acid; iii, trypsin attached to sepharose

Scheme 24

was replaced by Ala or Gly (112). The alanine analogue isolated by affinity chromatography on trypsin-Sepharose contained 90% of chain reconstituted product and was as active against trypsin as the guanylated and carbamylated natural material. The properties of these analogues are fully discussed[179] in terms of evolutionary aspects of trypsin inhibitors. In a subsequent study[180] the modified inhibitor (111) was treated with the Boc-amino-acid *N*-hydroxysuccinimide esters of isoleucine and alanine and also the *N*-carboxyanhydride of glutamic acid, thus providing analogues with additional residues added at the active site of the inhibitor. These were found to be inactive as trypsin inhibitors but were substrates for trypsin since the active-site peptide bond was partially reconstituted in all three cases.

Insulin has again received much attention from the semisynthesis viewpoint (see Vol. 8, p. 281). A review of the chemistry of insulin has been published.[181] Since human insulin is not available from natural sources considerable efforts have been made to convert readily available porcine insulin (which differs from human in having B-chain carboxyl terminal alanine in place of threonine in human) into human insulin. Porcine insulin has been converted into crystalline

[181] R. Geiger, *Chem. Zeitung*, 1976, **100**, 111.

human insulin by initial cleavage with trypsin to give the desoctapeptide (B22—30) analogue, which was subsequently treated with Boc-ONSu giving the di-t-butoxycarbonyl derivative.[182] A DCC-HOBt mediated reaction of this compound with an equimolar amount of synthetic octapeptide, corresponding to the human sequence, gave a product which, after deprotection, had 25—40% of insulin-like activity. This was achieved even though five carboxy-groups on the insulin-derived molecule were unprotected. Extensive purification involving Sephadex LH-20, ion exchange on Sephadex QEA A-25 (which separated more, basic higher molecular weight species), and Bio-Gel P-6 chromatography gave crystalline human insulin in 10—15% yield from the *N*αA1,*N*αB1-di-t-butoxycarbonyldesoctapeptide insulin. Des-(Ala B30) porcine insulin pentamethyl ester, a possible intermediate in the conversion of porcine into human insulin, has been isolated, together with other porcine insulin derivatives with shortened B-chain insulins.[183]

In another approach, a previously described method[184] of preparing des(octapeptide) porcine insulin pentamethyl ester has been modified to make analogues with the B-chain extended from the B23 arginine residue by addition of a range of synthetic peptides corresponding to the natural sequence and the natural sequence terminating in alanine.[185] Coupling yields, using DCC–HONSu activation, were in the 80% region and after deprotection (removal of Boc groups by trifluoroacetic acid followed by alkaline saponification) it was shown that a three step increase in activity, to a fully active species, could be obtained by adding residues B24—26 (-Phe-Phe-Tyr). Msc protection of porcine insulin at positions A1 and B29 (see Vol. 8, p. 283) has been used to prepare analogues in which the B-chain amino-terminal acid phenylalanine has been replaced by β-Ala[186] and 3,5-di-iodotyrosine.[187]

The synthesis and properties of insulin cross-linked at A1—B29 with carbonylbismethionine have now been fully described,[188] as have some A1—B1 cross-linked insulin species.[189] Two crystalline substituted bovine insulin derivatives have been described.[190, 191]

Crystalline semisynthetic ribonuclease S′ has been prepared by combination of a solid-phase synthetic peptide corresponding to residues 1—20 of ribonuclease A and native ribonuclease S (21—124).[192, 193] *X*-Ray studies gave diffraction patterns similar to that from ribonuclease S. The binding of His^4-S-peptide-(1—15) to ribonuclease S (21—124) has been studied by quantitative affinity chromatography.[194] S-peptide (1—14) and Ile^{13}-S-peptide (1—14) and the

[182] R. Obermeier and R. Geiger, *Z. physiol. Chem.*, 1976, **357**, 759.
[183] H. G. Gattner, E. W. Schmitt, and H. Zahn in ref. 2, p. 279.
[184] M. A. Ruttenberg, *Science*, 1972, **177**, 623.
[185] G. Weitzel, F.-U. Bauer, and K. Eisele, *Z. physiol. Chem.*, 1976, **357**, 187.
[186] R. Geiger, K. Geisen, G. Regitz, and H.-D. Summ, *Z. physiol. Chem.*, 1976, **357**, 1267.
[187] V. Teetz, H. G. Eckert, and R. Geiger, in ref. 2, p. 263.
[188] W.-D. Busse and F. H. Carpenter, *Biochemistry*, 1976, **15**, 1649.
[189] D. Brandenburg, W. Schermutzki, A. Wollmer, H. D. Vogt, and J. Gliemann, in ref. 1, p. 497.
[190] D. A. Sutton, B. R. D. Easter, and S. E. Drewes, *Z. physiol. Chem.*, 1976, **351**, 971.
[191] S. E. Drewes and D. A. Sutton, *Z. physiol. Chem.*, 1976, **357**, 1261.
[192] M. Pandid, E. A. Padlan, C. DiBiello, and I. M. Chaiken, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1844.
[193] M. Pandin, E. A. Padlan, C. DiBello, and I. M. Chaiken, in ref. 2, p. 319.
[194] I. M. Chaiken and H. C. Taylor, in ref. 1, p. 1013.

corresponding derivatives with homohistidine replacing histidine in position 12 have been synthesized by solid-phase techniques and their activities assessed.[195] During the solid-phase synthesis difficulty was encountered in coupling Boc-His(Tos). Addition of hydroxybenzotriazole caused loss of the tosyl group and internal lactam formation. The Dnp group was eventually employed for protection of the imidazole group of histidine in a successful synthesis. In another study on the effect of the first eight amino-terminal acids on the activity of ribonuclease S′, S-peptide (9—20) was obtained by treating native S-peptide with chymotrypsin and then coupling activated synthetic peptides to this natural fragment.[196] Alternatively, trifluoroacetylated S-peptide (7—20) was prepared by treatment of trifluoroacetylated S-peptide with elastase.[196]

3 Syntheses Achieved

In the year under review substantial progress has been made towards the synthesis of several large polypeptides by classical solution techniques; syntheses of some substantial molecules by the solid-phase procedure have also been described. In this section, attention is focused on syntheses of the above types. A comprehensive list of all reported syntheses is to be found in Appendix I.

Apolipoprotein C-1.—Two solid-phase syntheses of apolipoprotein C-1, a constituent of human plasma which may be important in cholesterol metabolism, have been reported.[91, 197] The protein consists of a single 57-residue chain lacking cysteine, tyrosine, and histidine. In one synthesis[91] the caesium salt of Boc-Ser(Bzl) was attached to the resin *via* a long spacer unit (see p. 336) to a substitution level of 0.095 mmol g^{-1}. The assembly was carried out using symmetrical anhydrides generated *in situ*; the carboxamides of asparagine and glutamine were protected by the benzhydryl group allowing them to be coupled in a similar manner. In cases where couplings were apparently difficult, dimethylformamide was used in the coupling medium. Boc-groups were removed by two treatments with 25% trifluoroacetic acid, in dichloromethane, with a shrink–swell procedure interposed. After each coupling the resin was treated with 0.1M acetic anhydride in pyridine. Removal of the peptide from the resin was accomplished by the usual HF treatment in the presence of anisole, which also removed all side-chain protecting groups apart from the *in*-formyl group of tryptophan. The latter was removed with 1M-Tris–HCl at pH 12 for 15 min. Gel-filtration on Bio-Gel P-10 in 6M-urea (to minimize aggregation) removed truncated impurities. Further purification by DEAE-cellulose chromatography, again in the presence of urea, gave a product judged to have similar biological characteristics to those of the natural protein.

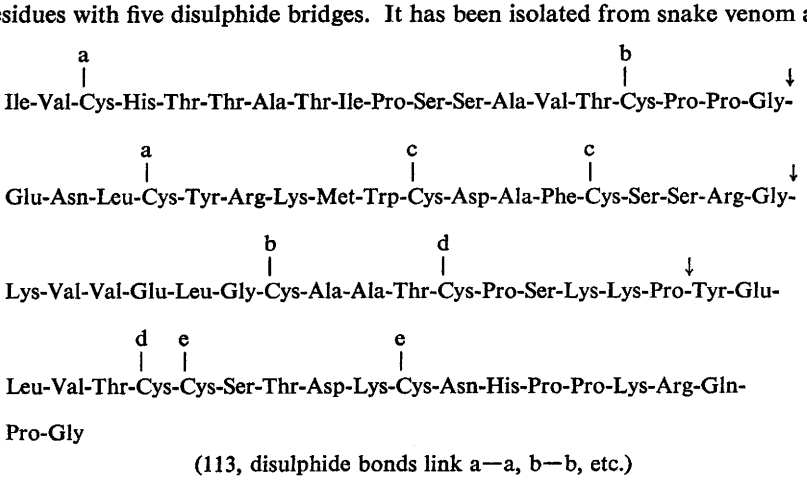
In the second synthesis[197] a conventional benzyl ester linkage to the resin was employed with a serine loading of 0.086 mmol g^{-1}. A lengthy shrink–swell protocol[198] was employed during the assembly. Repeated couplings with two-

[195] O. D. van Batenburg, J. Raap, K. E. T. Kerling, and E. Havinga, *Rec. Trav. Chem.*, 1976, **95**, 278.
[196] S. Levit and A. Berger, *J. Biol. Chem.*, 1976, **251**, 1333.
[197] D. R. K. Harding, J. E. Battersby, D. R. Husbands, and W. S. Hancock, *J. Amer. Chem. Soc.*, 1976, **98**, 2664.
[198] W. S. Hancock, D. J. Prescott, P. R. Vagelos, and G. R. Marshall, *J. Org. Chem.*, 1973, **38**, 774.

fold excesses of the appropriate symmetrical anhydride generated *in situ* were used, the second being carried out in a 1 : 1 mixture of dimethylformamide and dichloromethane. No carboxamide protection for the asparagine or glutamine residues was used but hydroxybenzotriazole (one equivalent) was added to accelerate the coupling reactions. Anhydrous HF was used to liberate the peptide from the resin, and the formyl group from the single tryptophan was removed subsequently under very mild conditions (pH 7.6, 3 days at 4 °C). Gel filtration on G-50 gave a single peak in a yield of 18.5% based on the substitution level of the starting resin. Further purification by ion-exchange chromatography on Sephadex SP-25 again gave one main peak, with recoveries varying between 20 and 50%, a problem associated with aggregation. The product gave single band on gel-electrophoresis, migrating in the same position as native material, and had significant apolipoprotein C-1 activity.

Both syntheses are characterized by a low degree of initial substitution on the polystyrene resin and this could be a contributing factor to the ultimate isolation of materials similar to natural apolipoprotein C-1. The shorter sequence of apolipoprotein C-III has also been synthesized using the resin with the spacer molecule described above.[89, 90]

α-Bungarotoxin.—α-Bungarotoxin (113) is a basic protein, containing 74 residues with five disulphide bridges. It has been isolated from snake venom and



(113, disulphide bonds link a—a, b—b, etc.)

exerts its biological response by blocking neuromuscular transmission. The fully protected peptide was assembled by a fragment condensation procedure from four fragments of approximately equal size (1—19, 20—37, 38—53, and 54—74) each terminating in either glycine or proline.[199] Maximum protection of side-chain functions by benzyl-based protecting groups was used during the synthesis and assembly of the fragments, with the Boc group for N_α protection. Apart from the carboxy-terminal glycine residue, which was protected as its benzyl ester, the *C*-terminal residues of the fragments were generally protected by salt formation during coupling reactions.

[199] V. T. Ivanov, I. I. Mikhaleva, O. M. Volpina, M. A. Myagkova, and V. I. Deigin, in ref. 2, p. 219.

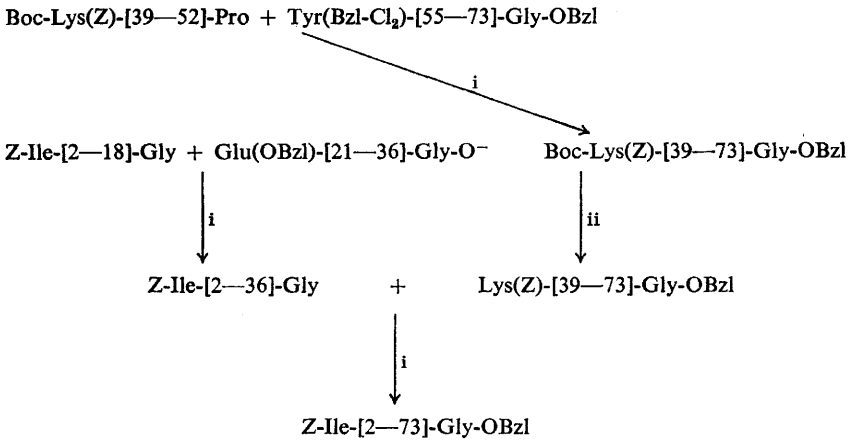
Assembly of the fragments did not proceed without complication. Chain elongation from *C*-terminal prolylglycine sequences proved to be troublseome owing to diketopiperazine formation and the lability of the amide bond in this dipeptide. Attempts to couple the peptide azide (114) to fragment (115) gave a

Boc-Asn(Bzh)-Leu-Cys(MBzl)-Tyr(Bzl-Cl_2)-Arg(Tos)-Lys(Z)-Met-N_3
(114)

Trp-Cys(MBzl)-Asp(OBzl)-Ala-Phe-Cys(MBzl)-Ser(Bzl)-Ser(Bzl)-Arg(Tos)-Gly-OH
(115)

very low yield (1%) in spite of previous examples of successful methionine azide acylations in the MSH and ACTH series. The tryptophan was ultimately added to (114) as a single residue. The *im*-2,4-dinitrophenyl group proved too labile for the protection of histidine; partial removal was observed during coupling reactions, and consequently the N_{tm}-tosyl derivative was employed.

The fully protected molecule was assembled from the four fragments as illustrated in Scheme 25. Fragments 1—19 and 20—37 were joined in 41%



Conditions: i, DCC–HOBt; ii, 25% TFA–CH_2Cl_2

Scheme 25

yield while 38—53 and 54—74 were coupled in 80% yield. The two halves were then combined to give fully protected α-bungarotoxin, which was isolated after gel-filtration, in an impressive yield of 75%. The general hydrophobic nature of the protein led to good solubility in organic solvents which greatly enhanced the fragment couplings. About 1 g of the fully protected 74-residue peptide was prepared. The protecting groups employed in this synthesis necessitate the use of anhydrous HF for final deprotection, and the problems involved in this step have not yet been overcome. Substantial aggregation was observed during HF treatment of other small protected basic proteins containing cysteine (see the discussion section of the paper).

Enkephalin, β-Endorphin, and Related Peptides.—The opiate-like activity of various peptides now recognized as part sequences of β-lipotropin (see Chapter 5, section 6) has caused a flurry of synthetic activity. The simplest biologically active natural molecules, the pentapeptide methionine- and leucine-enkephalins and their analogues have been synthesized in many laboratories both by solution [200–204] and solid-phase [205–209] methods. The latter has also been used to prepare the 16- and 17-residue α- and γ-endorphins,[210] as well as a series of β-lipotropin fragments up to the full sequence of β-endorphin (116) (31

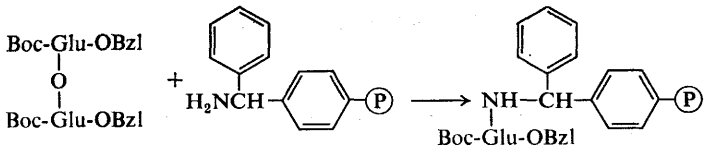
```
1                5                10                15
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-

   20               25               30
Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln
```

Ovine β-endorphin

(116)

residues).[211] Details of these syntheses are not yet available, but a fairly detailed paper from Li's laboratory describes an apparently rather painless solid-phase preparation of β-endorphin.[212] The *C*-terminal glutamine residue was introduced by symmetrical γ-anhydride acylation of benzhydrylamine resin with the α-benzyl ester of Boc-glutamic acid (Scheme 26). All subsequent residues were attached



Scheme 26

using similar preformed symmetrical anhydrides, and this use of identical coupling conditions from beginning to end of the synthesis may have contributed to its successful outcome. It avoids the disparity of reaction conditions associated

[200] J. D. Bower, K. P. Guest, and B. A. Morgan, *J.C.S. Perkin I*, 1976, 2488.
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[208] D. G. Coy, A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, and C. A. Sandman, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 632.
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[210] N. Ling, R. Burgus, and R. Guillemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3942.
[211] N. Ling and R. Guillemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3308.
[212] C. H. Li, S. Lemaire, D. Yamashiro, and B. A. Doneen, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 19.

with attachment of the first amino-acid residue to conventional chloromethyl resins. Couplings were aided by addition of 20% trifluoroethanol to the reaction medium (p.339). The terminal Boc-group was removed from the final peptide-resin before cleavage with hydrogen fluoride to avoid the danger of t-butyl-sulphonium salt formation at the methionine residue (p. 344). After purification by gel filtration and ion-exchange and partition chromatography, the final product was homogeneous by several analytical techniques and was obtained in overall yield of 20%. This was increased to 30% by including material from a second HF treatment of the resin. The synthetic β-endorphin was equiactive with the natural peptide in opiate receptor binding studies, and was also analgesic.

Δ^5-3-Ketosteroid Isomerase.—Ten protected fragments (117)—(126) spanning the entire sequence of the 125-residue protein Δ^5-3-ketosteroid isomerase have been prepared.[213] The pentafluorophenyl ester method was used almost exclusively in the fragment syntheses. It was intended to make the individual fragments by stepwise chain elongation, but in some cases this was frustrated by insolubility problems with increasing chain length. Thus intermediates in the preparation of (117) were soluble only up to the 12-residue stage, and three peptide fragments (1—4), (5—15), and (16—21) were used in its construction.

1
Z-Met-Asn-Thr(Bzl)-Pro-Glu[OBzl(4-Cl)]-His(Dnp)-Met-Thr(Bzl)-Ala-Val-Val-Gln-

21
Arg(Tos)-Tyr(Bzl)-Val-Ala-Ala-Leu-Asn-Ala-Gly-OH

(117)

22
Bpoc-Asn-Leu-Asn-Gly-Ile-Val-Ala-Leu-Phe-Ala-Asp[OBzl(4-Cl)]-Asn-Ala-Thr(Bzl)

39
Val-Glu[OBzl(4-Cl)]-Asn-Pro-OH

(118)

40 **47**
Bpoc-Val-Gly-Ser(Bzl)-Glu[OBzl(4-Cl)]-Pro-Arg(Tos)-Ser(Bzl)-Gly-OH

(119)

48
Bpoc-Thr(Bzl)-Ala-Ala-Ile-Arg(Tos)-Glu[OBzl(4-Cl)]-Phe-Tyr(Bzl)-Ala-Asn-Ser(Bzl)-

62
Leu-Lys[Z(3-Cl)]-Leu-Pro-Obu^t

(120)

63
Boc-Leu-Ala-Val-Glu[OBzl(4-Cl)]-Leu-Thr(Bzl)-Gln-Glu[OBzl(4-Cl)]-Val-Arg(Tos)-

75
Ala-Val-Ala-OH

(121)

[213] L. Kisfaludy, M. Löw, I. Schön, T. Szirtes, O. Nyéki, Cs. Vézer, P. Kereszttessy, F Korenczki, and M. Szirmai, in ref. 2, p. 239.

76
Boc-Asn-Gln-Ala-Ala-Phe-Ala-Phe-Thr(Bzl)-Val-Ser(Bzl)-Phe-Glu[OBzl(4-Cl)]-

90
Tyr(Bzl)-Gln-Gly-OH

(122)

91 **97**
Boc-Arg(Tos)-Lys[Z(3-Cl)]-Thr(Bzl)-Val-Val-Ala-Pro-OH

(123)

98 **107**
Boc-Ile-Asp[OBzl(4-Cl)]-His(Dnp)-Phe-Arg(Tos)-Phe-Asn-Gly-Ala-Gly-OH

(124)

108 **117**
Boc-Lys[Z(3-Cl)]-Val-Val-Ser(Bzl)-Met-Arg(Tos)-Ala-Leu-Phe-Gly-OH

(125)

118 **125**
Boc-Glu[OBzl(4-Cl)]-Lys[Z(3-Cl)]-Asn-Ile-His(Dnp)-Ala-Gly-Ala-[OBzl(4-Cl)]

(126)

Fragments (119), (121), (123), (125), and (126) were prepared by fully stepwise procedures. Fragment (125) was synthesized in an overall yield of 26%; as chain length increased solvents of increasing polarity were used for the coupling reaction. At the tri- and tetra-peptide stage *NN*-dimethylaminoethylamine was used to remove excess active ester. Most couplings were complete within 40 min, but formation of the Val-Val bond required 3 h and a three-fold excess of the pentafluorophenyl ester.

A Lysozyme Analogue.—The first reports of a major attempt to synthesize a lysozyme analogue (127) have appeared.[214, 215] In the target molecule 27 amino-acid substitutions were made from the sequence of hen egg-white lysozyme, five of these being common with the human sequence. Of the other changes, only four affected residues in the internal region of the protein (residues 12, 29, 55, and 105). The overall synthetic plan is to synthesize the 1—75 fragment and then couple it to the 76—129 portion of the analogue.

Maximum protection of side-chain functions was employed in the synthesis of the 1—75 sequence,[214] which was made by coupling the fragments 1—37 and 38—75. The adamantyloxycarbonyl group was used for the protection of the side-chains of ornithine and lysine, t-butyl ethers or esters for tyrosine, threonine, serine, aspartic, and glutamic acids, and the acetamidomethyl group for cysteine. Full use was made of phenyl esters for carboxyl protection. These are removed very rapidly by alkali in the presence of hydrogen peroxide minimizing racemization and rearrangement reactions, *e.g.* of -Asp(OBu^t)-Gly- sequences. Construction of the 1—37 peptide was accomplished from 10 fragments and was particularly difficult owing to the insoluble nature of the peptides in the 1—16

[214] I. J. Galpin, B. K. Handa, D. Hudson, A. G. Jackson, G. W. Kenner, S. R. Ohlsen, R. Ramage, B. Singh, and R. G. Tyson, in ref. 2, p. 247.
[215] G. W. Kenner, *Proc. Roy. Soc.*, 1977, **197**, 237.

```
                    5                  10                  15                  20
HLL                 Glu                Arg-Thr-Leu         Leu   Met   Gly
HEL  Lys-Val-Phe-Gly-Arg-Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-His-Gly-Leu-Asp-Asn-Tyr
Analogue            Orn                Nle   Ala Leu       Ala   Gly

                    25                  30                  35                  40
HLL           Ile       Ala       Met   Leu       Trp       Gly-Tyr
HEL  Arg-Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys-Phe-Glu-Ser-Asn-Phe-Asn-Thr
Analogue Orn               Nva                   Gly

                    45                  50                  55                  60
HLL  Arg            Tyr   Ala-Gly Arg                       Phe
HEL  Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser
Analogue            Orn   Glu                               Leu

                    65                  70                  75                  80
HLL       Tyr                 Lys             Ala-Val   Ala   His-Leu-Ser
HEL  Arg-Trp-Trp-Cys-Asn-Asp-Gly-Arg-Thr-Pro-Gly-Ser-Arg-Asn-Leu-Cys-Asn-Ile-Pro-Cys
Analogue Orn        Ala   Orn                 Ala       Gly

                    85                  90                  95                  100
HLL                 Gln-Asp-Asn   Ala-Asp-Ala   Ala         Arg                 Arg
HEL  Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Cys-Ala-Lys-Lys-Ile-Val-Ser
Analogue Ala  Nva   Gly                         Gly

                    105                 110                 115                 120
HLL       Pro-Gln   Ile-Arg                                 Gln Asn-Arg
HEL  Asp-Gly-Asn-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val
Analogue            Nle                       Orn                       Ser

                    125   129
HLL  Arg-Gln-Tyr-Val-Gln  Gly-Val
HEL  Gln-Ala-Trp-Ile-Arg-Gly-Cys-Arg-Leu
Analogue Ser      Val-Orn Gly
```

Amino-acid sequence of the synthetic target analogue (127) of lysozyme. HLL = human sequence, HEL = hen egg white lysozyme

region. Coupling of the fragment (128) to (129) by the DCC–HOSu technique in the solvent mixture DMF–HMPA gave the fully protected 1—37 polypeptide. It was purified by gel filtration on Enzacryl K2 using *N*-methyl-2-pyrrolidone as elutant.

Adoc-Lys(Adoc)-Val-Phe-Gly-Orn(Adoc)-Cys(Acm)-Glu(OBu^t)-Leu-Ala-Ala-Ala-Nle-Lys(Adoc)-Ala-Leu-Gly

(128)

Leu-Ala-Gly-Tyr(Bu^t)-Orn(Adoc)-Gly-Tyr(Bu^t)-Ser(Bu^t)-Leu-Gly-Asn-Trp-Nva-Cys(Acm)-Ala-Ala-Lys(Adoc)-Phe-Glu(OBu^t)-Ser(Bu^t)-Gly-OPh

(129)

No solubility problems were encountered in the synthesis of the 38—75 fully blocked polypeptide and its assembly proceeded relatively smoothly. After removal of the *C*-terminal phenyl ester of the 1—37 fragment and the N_α-Bpoc protecting group from the 38—75 sequence the two were joined, again by a DCC–HOSu coupling in the DMF–HMPA system. The 1—75 protected product was isolated again using Enzacryl K2, and its *C*-terminal phenyl ester was removed in readiness for coupling to the 76—129 sequence.

Further impressive progress towards the completion of this synthesis has been reported.[215]

Pancreatic Trypsin Inhibitor II (Kazal).—A series of papers [216–221] describing the synthesis of fragments of the 52-residue porcine pancreatic trypsin inhibitor II (Kazal) has appeared, as has a progress report on the joining of some of the fragments.[222] The six fragments (130)—(135) were constructed using a variety of coupling methods and protecting groups. The only fragment synthesized in a completely stepwise manner [217] was the short tetrapeptide (131); all the others required the condensation of shorter peptide sequences. An interesting feature is the use of thioacetamide to remove the Nps group from peptides containing cysteine protected by the acetamidomethyl group.

The overall strategy is one of minimal side-chain protection and coupling of the six fragments by the azide procedure of Rudinger. Progress on this part of the synthesis has extended to the joining of fragment (130) to (131) and (134) to (135).[222] Removal of the trityl group from (131) with acetic acid allowed condensation with the acyl azide prepared from (130). The resulting tetradecapeptide was purified on Bio-Rad AG-1 × 2 using an aqueous organic elutant and crystallized. Fragment (135) was partially deprotected by trifluoroacetic acid and then (134) condensed onto it *via* the pivalic mixed anhydride. The resultant

1 **10**
Z-Arg(NO_2)-Glu-Ala-Thr-Cys(Acm)-Thr-Ser-Glu(OBu^t)-Val-Ser-N_2H_2-Boc
(130)

11 **14**
Trt-Gly-Cys(Acm)-Pro-Lys(Tfa)-N_2H_2-Boc
(131)

15 **24**
Nps-Ile-Tyr(Bu^t)-Asn-Pro-Val-Cys(Acm)-Gly-Thr-Asp(OBu^t)-Gly-N_2H_2-Boc
(132)

25 **35**
Boc-Ile-Thr-Tyr-Ser-Asn-Glu(OBu^t)-Cys(Acm)-Val-Leu-Cys(Acm)-Ser-N_2H_3
(133)

36 **43**
Boc-Glu(OBu^t)-Asn-Lys(Tfa)-Lys(Tfa)-Arg-Gln(Mbh)-Thr-Pro-OH
(134)

44 **52**
Boc-Val-Leu-Ile-Gln-Lys(Tfa)-Ser-Gly-Pro-Cys(Acm)-OH
(135)

[216] R. Tomatis, A. Guggi, C. A. Benassi, S. Salvadori, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 65.
[217] A. Guggi, R. Tomatis, V. Periotto, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 79.
[218] R. Tomatis, A. Guggi, R. Ferroni, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 87.
[219] A. Guggi, R. Tomatis, C. A. Benassi, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 97.
[220] R. Tomatis, A. Guggi, E. Menegatti, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 107.
[221] A. Guggi, R. Tomatis, S. Salvadori, and R. Rocchi, *Internat. J. Peptide Protein Res.*, 1976, **8**, 115.
[222] R. Rocchi, C. A. Benassi, A. Guggi, and R. Tomatis, in ref. 1, p. 509.

polypeptide was very poorly soluble and eventually purified by prolonged stirring in glacial acetic acid followed by precipitation from DMF.

Basic Pancreatic Trypsin Inhibitor (Kunitz).—A new solid-phase synthesis of the 58-residue basic pancreatic trypsin inhibitor has been reported.[223] In contrast to syntheses reported previously [224, 225] this effort is distinguished by a careful evaluation of the synthetic material.

A conventional approach was adopted for the synthesis. Couplings were carried out using a 2.5-fold excess of the appropriately protected Boc-amino-acid derivative and completeness of reaction was assessed by the ninhydrin test. For all three asparagine residues which were added by the *p*-nitrophenyl ester method, couplings had to be repeated. In one case the ninhydrin test was still positive after a third coupling and the resin was acetylated at this stage. Removal of the peptide from the resin was accomplished by anhydrous HF treatment in the presence of anisole. Subsequent gel-filtration on Sephadex G-50 separated material of the correct molecular weight from aggregated and low molecular weight species. This material was subjected to reducing conditions and then rechromatographed on G-50. Air oxidation at pH 4.5 of a very dilute solution (0.01 mg ml^{-1}) gave a product with *ca.* 10% trypsin inhibitory activity. Affinity chromatography on trypsin-Sepharose increased this activity to 80—90%. Finally CM-Sephadex ion-exchange chromatography gave a fully active trypsin inhibitor.

The purity of the inhibitor was assessed by amino-acid analysis, peptide mapping, c.d. spectra, reaction with trypsin (100% inhibition at 1 : 1 stoicheiometry), and measurement of its binding constant with trypsin. The overall yield from the first amino-acid on the resin was 2.9%.

4 Appendix I: A List of Syntheses Reported during 1976

Natural Peptides, Proteins, Analogues, and Partial Sequences.—Syntheses are listed under the name of the peptide or protein to which they relate, as in previous years.

Peptide	*Ref.*
Acyl carrier protein	
Acyl carrier protein-(65—74)-peptide	1
Acyl carrier protein *N*-terminal hexapeptide	2
Adrenocorticotropic hormone (ACTH)	
Bovine ACTH	3
Porcine ACTH	4
Porcine ACTH-like intermediate lobe protein-(CLIP)-(18—39)-peptide	5

[223] N. H. Tan and E. T. Kaiser, *J. Org. Chem.*, 1976, **41**, 2787.
[224] Y. Kiso and H. Yajima, *Chem. amd Pharm. Bull. (Japan)*, 1974, **22**, 1087.
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[2] R. L. Prestidge, D. R. K. Harding, and W. S. Hancock, *J. Org. Chem.*, 1976, **41**, 2579.
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[4] H. Yajima, K. Koyama, Y. Kiso, A. Tanaka, and M. Nakamura, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 492.
[5] K. Koyama, Y. Mori, Y. Kiso, S. Hirabayashi, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 2301.

Peptide	*Ref.*
Human ACTH	6
ACTH-(1—27)- and -(1—26)-peptides	7
ACTH-(1—24)-peptide	8, 9
[Nar^{8}]-ACTH-(1—24)-peptide and -(1—18)-amide	10
[Gln^{5}, Phe^{9}]-, [Gln^{5}, Ala^{9}]-, and [Gln^{5}, Lys^{8}, Phe^{9}]-ACTH-(1—24)-peptides	11
[(3,5-$^{3}H_{2}$)Tyr^{2}]-β-Corticotropin-(1—24)-peptide and [D-Ser^{1}, (3,5-$^{3}H_{2}$)Tyr^{2}, Lys$^{17, 18}$]-β-corticotropin-(1—18)-amide	12
[(4-^{3}H)Phe^{7}]-β-Corticotropin-(1—24)-peptide	13
[D-Lys$^{15, 16}$, D-Arg$^{17, 18}$]-ACTH-(1—19)-peptide and an all-D retropeptide related to ACTH-(1—18)-peptide	14
ACTH-(1—18)-amide	15
[δ-Aminovaleric acid^{2}, Arg^{5}]-ACTH-(2—19)-peptide	16
ACTH-(1—19)-amide, ACTH-(1—18)-amide, and ACTH-(1—18)-peptide	17
[Lys(ε-trifluoroacetyl)$^{15, 16, 21}$]-ACTH-(15—24)-peptide	18
An analogue of corticotropin-(11—18)-peptide	19
α-ACTH-(5—10)-peptide	20
ACTH-(7—10)-peptide	21
Alamethicin (Ala-o)	
Ala-o-(1—19)-peptide	22
Angiotensin	
[Asn^{1}, Val^{5}]-Angiotensins I and II and the *C*-terminal hexapeptide of [Val^{5}]-angiotensin II	23
Eight analogues of [Phe(4-nitro)^{8}]-angiotensin I	24
Six analogues, substituted in positions 1 and 8, of [Ile^{5}]- and [Val^{5}]-angiotensins II	25
Two analogues of [His^{2}]-angiotensin II	26
[Asp^{1}, Ile^{5}]- and [Asn^{1}, Ile^{5}]-Angiotensins	27

[6] K. Koyama, H. Watanabe, H. Kawatani, J. Iwai, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 2558.
[7] K. Inouye, Y. Sumitomo, and M. Shin, *Bull. Chem. Soc. Japan*, 1976, **49**, 3620.
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[15] A. T. Gudkov, G. V. Shekhvatova, O. V. Gudkova, N. P. Zapevalora, and Yu. V. Mitin, *Tezisy Dokl.-Vses. Simp. Khim. Pept. Belkov 3rd*, 1974, 7 (*Chem. Abs.*, 1977, **86**, 5814).
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Peptide	*Ref.*
[Asn^1, Val^5, Ala^8]- and [Hydantoic acid^1, Val^5, Ala^8]-Angiotensins II	28
[Pro(4-F)^7]-Analogues of [Ile^5]- and [Val^5]-angiotensin II	29
Two analogues of angiotensin II amide and an antagonist, [Sar^1, Val^8]-angiotensin II	30
Angiotensin analogues with α-aza-amino-acids	31
Six angiotensin II analogues with vasopressin structural elements	32
Three angiotensin II analogues modified at position 7	33
Tritiated angiotensin II	34
Analogues with α-methyl groups	35
An antagonist, [Sar^1, Thr^8]-angiotensin II	36
Retro-enantiomeric analogues of angiotensin II	37, 38
Various analogues of angiotensin II, including *N*- and *O*-methylated and other amino-acids	39
Some partial sequences	40—42
Apamin	43
Apolipoprotein	
Apolipoprotein C-1-(1—57)-peptide	44, 45
Apolipoprotein C-III-(61—79)-peptide	46, 47
Basic trypsin inhibitor (BTI)	48
Some short BTI model peptides	49, 50
Bradykinin	51—53
Analogues with D-Pro and related fragments	53

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Peptide	*Ref.*
Analogues modified in positions 1, 6, and 9	54
A (1—15)-peptide yielding Lys-bradykinin on tryptic hydrolysis, and a related peptide	55
Six analogues	56
[Pro(4-F)^{2,3,7}]-bradykinin	57
Analogues with α-methyl groups	35
Three analogues and partial Tyr-containing sequences	58
Several analogues with norarginine and homoarginine at positions 1 and/or 9, and other substitutions	59—61
Bungarotoxin	
Protected α-bungarotoxin-(1—74)-peptide	62
Calf thymus, fraction F2a1	
A hexadecapeptide sequence corresponding to a histone of the above fraction	63
The [Gly^{10}]- and [Gly^{1}, Lys^{2}, Gly^{3}]-analogues of the histone	64
A decapeptide from the *N*-terminus of the histone	65
Carcinoembryonic antigen (CEA)	
CEA-(1—19)-peptide	66
Cardiotoxin	
A polypeptide corresponding to Taiwan cobra venom cardiotoxin II	67
The protected-(44—49)- and -(50—52)-peptides	68
The protected, resin-bound peptide	75
Cholecystokinin-pancreozymin (CCK)	69
CCK-(1—16)-peptide	70
CCK-(17—33)-amide	71
[Tyr^{27}]-CCK	72

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CCK-(17—23)-hydrazide, protected	73
A by-product from the last step in the synthesis of the above heptapeptide	74
Cobrotoxin	
The *C*-terminal hexapeptide	75
Cobrotoxin 1-(56—62)-peptide, protected	76
Cytochrome *c*	
Horse heart cytochrome *c*-(66—104)-peptide, synthetic approaches	77
Eledoisin	
Miscellaneous related peptides, from five to seven residues	78—81
Enkephalin	
[Leu^5]-Enkephalin	82, 83
Related peptides and analogues	84, 85
[Met^5]-Enkephalin	86—90
[(α,α-$^2H_2$)Gly^2, Met^5]-Enkephalin	86
Other analogues, including D-residues, *etc.*	85, 89, 91, 92
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Human fibrinopeptide A	93, 94
Human fibrinopeptide A-(11—16)-peptide	95
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Peptide	*Ref.*
Gastric inhibitory polypeptide (GIP)	96
GIP-(1—28)-peptide	97
GIP-(26—43)-peptide	98
Gastrin, including big gastrin	
Human big gastrin-1	99
Human gastrin	9
Gastrin-(11—17)-amide	9
Tetragastrin (the *C*-terminal tetrapeptide amide)	100
Analogues, including aza-analogues, of tetragastrin	101—103
A by-product (β-aspartyl linked) from a synthesis of gastrin-(13—17)-amide	104
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Two analogues	106
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A tetradecapeptide from human growth hormone	109
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β-Chain haemoglobin-(57—69)-, -(70—77)-, and -(78—100)-peptides	112
β-Haemoglobin S-(1—13)-peptide and four fragments of this	113
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β-Haemoglobin S-(1—13)-peptide	115
Some short peptide amides from haemoglobin S	116
Immunoglobulin	
A pentapeptide fragment of immunoglobulin E, and a dipeptide	117

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Peptide	*Ref.*
Insulin	
Human insulin	118
[Leu^{B9}]-human insulin	119
[$Iasn^{A21}$]- and [Sar^{A1}, $Iasn^{A21}$]-Insulin (Iasn = isoasparagine)	123
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Luteinizing hormone releasing hormone (LH-RH)	141, 142
The protected decapeptide amide	143
[D-Phe(4-NH_2)^6]-LH-RH	144

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[D-Phe², Phe³, D-Phe⁶]-LH-RH	145
[D-Phe², Leu³, D-Trp⁶]-, [D-Phe², Leu³, D-Phe⁶]-, and [D-Phe², Pro³, D-Trp⁶]-LH-RH	146
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A protected analogue of lysozyme-(1—75)-peptide	156
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Porcine β-MSH, [Arg⁸]-β_{c1}-MSH, [Gly¹⁰]- and [Phe¹²]-β_{c1}-MSH	160
Analogues of α-MSH-amide	161—163
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Peptide	*Ref.*
[Leu^{13}]- and [Nle^{13}]-Motilin	167
Various related fragments	168—170
Myelin protein	
An encephalitogenic fragment of the protein and its [Gly^{6}]-analogue (two nonapeptides)	171
Neurotensin	172—174
Acid and amide analogues	174
Oxytocin	175—178
[(2-^{13}C)Gly-NH$_2^9$]-Oxytocin	179, 180
[Hemi^1(2-^{13}C)Cys]-Oxytocin	180
[2-^{14}C(U)Tyr^2]-Oxytocin	181
[Hemi-(β,β-^{2}H$_2$)Cys^1]- and [hemi-(β,β-^{2}H$_2$)Cys^6]-oxytocins	182
[(1-^{14}C)Gly-NH$_2^9$]- and [(1-^{14}C)Gly^4]-Oxytocins	183
[Dopa^2]-Oxytocin	184
[His^4]-Oxytocin	185
[Trp^2]-Oxytocin	186
[Cys^5, Asn^6]-Oxytocin	187
[Dopa^2, Thr^4]-Oxytocin	188
[1-(L-2-Hydroxy-3-mercapto)propionic acid, Thr^4]-oxytocin ('hydroxy-[Thr^4]-oxytocin') and 'hydroxy-oxytocin'	189
Various analogues, including retro-analogues	191—195

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Related fragments	179, 196—200
Pancreatic secretory trypsin inhibitor II (PSTI II)	
PSTI II-(1—14)- and -(36—52)-peptides	201
PSTI II-(15—24)-peptide	202
PSTI II-(25—35)-peptide	203
Shorter fragments of the above	204—207
Parathyroid hormone (PTH)	
Two sequences corresponding to human PTH-(1—34)-peptide as proposed by two different sequence studies	208
Position 1 analogues of human PTH-(1—34)-peptide	209
[Nle^{8}, Nle^{18}]-, [Tyr^{34}]- and [Nle^{8}, Nle^{18}, Tyr^{34}]-Bovine PTH-(1—34)-peptide	210
Phyllomedusin	211
Physalaemin	
Some related hexa- to octa-peptides	212
Proinsulin	
Human proinsulin-(1—23)-, -(24—45)-, -(46—70)-, and -(71—86)-peptides	213
Two analogues with 43 and 46 residues	214
Proinsulin-(53—61)-peptide	215
Prolactin	
Ovine prolactin-(37—55)-peptide and a related heptapeptide	216
Pyruvate kinase	
Some short peptides related to part of rat liver pyruvate kinase	217

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Peptide	*Ref.*
Ribonuclease (RNase)	
A 63-residue analogue of RNase A	218, 219
The [Tyr^{41}]- and [Gln^{41}]-analogues of the above	219
RNase-(1—20)-peptide	220
Rat pancreatic RNase(1—17)- and -(5—17)-peptides	221
Barnase-[Gly^{94}]-(95—110)-peptide	1
Some tri- and tetra-peptides from the *C*-terminus	222
Ribosomal protein L-12	
Ribosomal protein L-12-(4—13)- and -(18—23)-peptides	223
Rubredoxin	
Rubredoxin-(20—27)-peptide	224
Scotophobin	
A series of tyrosine oligopeptides corresponding to the *C*-terminus of scotophobin	225
Secretin	226, 227
N^{α}-(Deamino-Tyr, β-Ala)-secretin	228
Secretin-(5—27)-peptide and the [Gln^{9}]-, [Asn^{15}]-, and [Gln^{9}, Asn^{15}]-analogues of this	229
Somatostatin	230—232
N^{1}-Tyr-Somatostatin	233
[D-Cys^{14}]-, [Ala^{2}, D-Cys^{14}]-, and [D-Trp^{8}, D-Cys^{14}]-Somatostatin	234
Analogues with Tyr substitution, D-Tyr and D-Trp	235
Various analogues	155
Some cyclic analogues	236—238
Somatostatin-(3—14)-peptide and related fragments	239

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Peptide	*Ref.*
Streptococcal proteinase	
Two fragments, of 23 and 19 residues	240
Smaller fragments	241—243
Substance P (substance II)	244, 247
N^1-Tyr-Substance P	245
[Tyr^8]-Substance P	246
Related short peptides	81, 247, 248
Thymopoietin	
Thymopoietin II-(29—41)-peptide	249
Thyrotropin releasing hormone (TRH)	141, 250—254
TRH analogues	155, 250, 252, 253, 255—261
Triose phosphate isomerase	
A hexapeptide from the active site	278
Tuftsin	52, 262—266
Analogues and related peptides	264—266, 267
Uperolein	211
Urogastrone	
Urogastrone-(1—47)-peptide	268

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Peptide	*Ref.*
Vasoactive intestinal peptide (VIP)	
Chicken VIP-(18—28)-peptide, [Nle[17]]-VIP-(14—28)-peptide, and [Leu[17]]-VIP-(14—18)-peptide	269
Vasopressin ([Arg[8]]-vasopressin)	270
[(2-[13]C)Gly-NH_2[9]]-Vasopressin	179
[(1-[14]C)Gly-NH_2[9]]-Vasopressin	183
[1-(3-Mercaptopropionic acid), Tyr(3,5-Br_2)[2], Lys[8]]-vasopressin	271
[1-Deamino, D-Har[8]]- and [D-Har[8]]-vasopressin	272
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Three analogues, substituted in positions 1 and 8	274
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[1-Deamino, Har[8]]- and [Har[8]]-vasotocins	277

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Poly-(β-Ala-Phe), poly-(Phe-Gly-Gly), poly-(β-Ala-Gly-Gly), and poly-(Gly-Gly-DL-Ala)	285
$(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}D\text{-}Pro\text{-}Gly)_{10}$ by solid phase, and two related sequential polypeptides	286
Poly-(Lys-X-Gly), where X = Ala, Nva, Val, or Leu	287
Poly-(Pro-Pro-Ala) and poly-(Pro-Pro-Val), collagen models	288
Poly-(Lys-Leu-Glu-Ser-Leu-Glu-Ser) and related polypeptides with shorter repeats as tropomyosin models	289
Poly-(Gly-Sar-Pro) and poly-(Gly-Pro-Sar)	290

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Peptide	*Ref.*
Some esters of *p*-alkoxybenzoylglycylglycine	399
Some dipeptides synthesized using triphenylphosphite and imidazole	400
Z-Nar(NO_2)-Pro-Pro-Gly-$NHNH_2$	59
Nps-D-Val-Pro-Sar-OC_6H_4-*o*-OBzl	401
Cysteine peptides synthesized using the benzamidomethyl protecting group, and related derivatives	402
Di- and tri-peptides made *via* diphenylphosphinothioyl protection	403
Z-Leu-Ala-Gln-Ser(Bu^t)-His-Ala-OMe and other histidine peptides	404
Resin-bound Ala-Pro-Ser(Bzl)-Leu-Val-Phe by a computer-controlled solid-phase synthesis	405
Two peptides related to a peptide hormone from *Saccharomyces*	406
N^{ω}-Z-Arg-X-Phe, where X = various amino-acids	407
Dipeptides containing alanine, norvaline, and valine	408
Some *C*-terminal glycine tetrapeptides	409
Tritiated HCO-Nle-Leu-Phe by solid phase	410
Protected Tyr-Gly, using a new phenolic protecting group	411
Peptides of 3- and 4-hydroxyprolines	412
Peptidic analogues of insect juvenile hormone, including *N*-methylamino-acids and aromatic amines, *etc.*	413—415
Boc-Cys(Bzl)-Lys(Z)-Asn-Phe-OMe and a dipeptide made using benzotriazolyloxytris(dimethylamino)phosphonium hexafluoro-phosphate (BOP)	231
Various short peptides, deprotected by catalytic transfer hydrogenation	417
Some dipeptides made *via* an insoluble carbodi-imide-functionalized polymer	418
Z-Ala-Ala-Phe-(4′-azido,3′,5′-$^3H_2$)	419
Dipeptides made using 5-dibenzosuberyl protection	420
Some peptides containing β-methylphenylalanine	421
Tripeptides containing aminomethylphosphonic acid	422

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Peptide	*Ref.*
Some aspartic acid–aminomalonic acid dipeptide esters	423
Stereoisomeric alanine peptides	424
Some peptides containing tryptophan	425
Leu-Phe-OBu^t *via* diphenylmethylphosphinylethoxycarbonyl protection	426
Some di- and tri-peptides of 3,5-di-iodotyrosine	427
Short peptides synthesized using base-labile amino protection (the 2-methylsulphonoethoxycarbonyl group)	428
Dipeptides made using the 2-triphenylphosphonioethoxycarbonyl group for amino-protection	429
Short peptides made using pentacarbonyl chromium- or tungsten-organocarbene ligands for amino protection	430
Some peptides synthesized *via* chiral α-ferrocenyl alkylamines	431
Various dipeptides synthesized *via* 4,6-diphenylthieno-[3,4-*d*] [1,3]dioxol-2-one 5,5-dioxide activation	432
Z-D-Cys(Me)-Pro-NHMe from a dehydropeptide	433
Phthaloyl-Gly-dehydroPhe-OEt	434
Copoly{(Glu[OMe]-Lys[Z]), (Glu[OBzl]-Lys[Z])}	435
Some random copolymers of glutamic acid and γ-*o*-nitrobenzyl glutamic acid	436

5 Appendix II: Amino-acid Derivatives Useful in Synthesis

As before, this list includes both new derivatives and known ones for which new physical data or preparations have been reported. The continuing search for new protecting groups was strongly in evidence during 1976, so that the list of abbreviations at the front of the book will be seen to contain some possibly unfamiliar combinations of letters. Abbreviations used for amino-acid residues and common solvents are the conventional ones.

Coded Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$*	*Conc.*	*Solvent*	*Ref.*
Alanine					
Adoc-Ala	145	−21.5 −25.6 [d]	2	MeOH	437

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* Values of $[\alpha]_D$ were recorded at 589 nm, except where otherwise indicated by the following superscripts, and were measured at 20—25 °C unless otherwise stated: [a] 365 nm, [b] 546 nm, [c] 436 nm, [d] 578 nm.

Coded Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Azoc-Ala,Dcha	146—149	−11	1	MeOH	438
Boc-Ala,Dcha *	144—145				439*
Boc-Ala-ONMa	136—137	−24.0	1	THF	357
Boc-Ala-ONsu	165 dec.	−44.2		Dioxan	230
Dpp-Ala-OMe	114—115	+12.2			100
Dpp-Ala	152—153	−21.4			100
Mtc-Ala,Dcha	146	−4.1	2	MeOH	428
Peoc-Ala	164	−15.8	2	MeOH	429
Ppt-Ala,Dcha	177—178	−3.7	1	EtOH	403
Sub-Ala-OMe,HCl	146—148	−0.3	5	MeOH	420
Sub-Ala,HCl	200	−3.0	5	DMF	420
Tfa-Ala	66	−61.0	1	H_2O	440
Z-Ala-NHMe	127—128	−13.0	1	MeOH	424
Z-Ala-NH$(CH_2)_2$OH	115—116	−11.8	1	MeOH	424
Arginine					
Arg(NO_2)-OBzl	59—62	−2.8	0.49	MeOH : H_2O, 1 : 1	263
Arg(Tos)	80—82	+3.0 [d]	1	DMF	158
Arg(Mbs)	144—146	−6.1	0.71	MeOH	52
Adoc-Arg(Adoc,Z)	144—145	+16.5 [b] +15.5 [d]	1	$CHCl_3$	407
Azoc-Arg(NO_2),Dcha	129—131	+2.8	1	MeOH	438
Azoc-Arg(Tos),Cha	131—133	−1.9	1	MeOH	438
Iboc-Arg(NO_2)	135—136	−28.7	1	EtOH	154
Z-Arg(Mbs),Dcha	110—112	+5.1	1.35	MeOH	52
Z-Arg(Mbs)-OBzl(NO_2)	90—110 dec.	−3.4	0.59	DMF	52
Z-Arg(NO_2)-NHNHBoc	85—87	−17.6	1	MeOH	206
Asparagine					
Asn-OBu^t,HCl	138—139	+11.1	1	MeOH	121
Adoc-Asn	181—182	−2.9 −3.7 [b]	2	MeOH	437
		−3.4 −9.4 [b]	2	80% AcOH	
Azoc-Asn	167—168	−2.8	1	MeOH	438
Boc-Asn(Mbh)	143—145	+3.7 +4.0 [d]	1 1	AcOH AcOH	270
Boc-Asn(Z[OMe])	132—133	+9.9	1	MeOH	441
Boc-Asn(Z[OMe])-OBzl	105—106				441
Ppt-Asn	163—164	−5.0	1	MeOH	403
Tfa-Asn	163—164	−22.8 [b]	1	MeOH	440
Aspartic acid					
Asp(OBzl[NO_2])	188—189	+15.7 †	1	AcOH	117
Asp(OPac)	155—157	−23.8 †	1	AcOH	117
Asp(ONp)-OMe,HBr	145—146				442
Adoc-Asp(OBzl)	95—97	+4.9 +5.7 [b]	2	80% AcOH	437

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* New procedure. This applies to all derivatives listed under this reference.
† At about 15 °C.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Azoc-Asp,2Dcha	158—160	+10.1	1	MeOH	438
Azoc-Asp(OBzl),Cha	156—157	+31.5	1	$CHCl_3$	438
Boc-Asp(OBzl)	100—101				439
Z-Asp(ONp)-OMe	100	−16.3	1	MeOH	442
Z(OMe)-Asp(OBzl-[NO_2]),Dcha	112—114	−2.2 *	0.7	DMF	117
Cysteine					
Cys(Ad)	215—216				444
Cys(Bzl[OMe])	205—207				443
Cys(CPh_2Py)	154—157	+51	2.1	1M HCl	336
Cys(Bzl[4-Cl])	213—215	+29	1	1M NaOH	446
Cys(Sub),HCl	206—208	+9.6	1	DMSO	420
Cys(Pr)-OBzl,HCl	138—140	−45	1	MeOH	445
Cys(Acm)-OMe	132—134	−11	1.3	MeOH	120
Cys(Trt)-OBzl,BF_3	115—117	−75	1	MeOH	445
Cys(Sub)-OMe,HCl	178—180	+11.2	1	MeOH	420
Cys(Pr)-OMe,HCl	128—130	−55	1	MeOH	445
Cys(Trt)-OMe,HCl	97—103	+20.9	1	DMF	230
Cys(Sub)-NH_2,HCl	180	+4.5	1	MeOH	420
Azoc-Cys(Acm),Cha	82—84	+18.8	1	$CHCl_3$	438
Boc-Cys(CPh_2Py),Dcha	202—204	+19			336
Boc-Cys(Acm)-ONp	98—99	−42.4	1	DMF	205
Boc-Cys(Pr^i)-ONSu	124—125	−117 [d]	1	MeOH	158
Boc-Cys(Npe)-OH †	131—133				105
Boc-Cys(Npe)-OH	74—77				105
Msc-Cys(Trt)	162	−73.2	1	EtOAc	121
Msc-Cys(Trt),Dcha	164	+24.8	1	MeOH	121
Ppt-Cys(Bzl),Dcha	170—171	+22.5	1	MeOH	403
Z-Cys(Sub)	73—75	−31.1	1	DMF	420
Z-Cys(Sub)-OTcp	139—140	−18.4	1	DMF	420
Z-Cys(Bzl[OMe])-OPcp	148—150	−45.1	1	$CHCl_3$	446
Z-Cys(Trt)-OPcp	178—179	+18.8	1	$CHCl_3$	446
Z-Cys(Me),Dcha	142—144	+6.3	1	$CHCl_3$	446
Z-Cys(Me)-OPcp	139—140	−28.0	1	$CHCl_3$	446
Z-Cys(Bzh)-OPcp	134—135	−22.1	1	$CHCl_3$	446
Z-Cys(Bzl[4-Cl]),Dcha	142—144	−12.7	1	$CHCl_3$	446
Z-Cys(Bzl[4-Cl])-OPcp	150—151	−41.5	1	$CHCl_3$	446
Z-Cys(Btm)-OPcp	136—137	−46.2	1	$CHCl_3$	446
Z-Cys(Bzl[4-CN])-OPcp	143—147	−42.4	1	$CHCl_3$	446
Z-Cys(Bzl[NO_2])-OPcp	139—141	−41.8	1.5	$CHCl_3$	446
Z-Cys(CPh_2Py),Dcha	170—172	+26	1	$CHCl_3$	336
Cystine					
(Cys-OBu^t)$_2$	Oil	−8.2	2	MeOH	332
(CysNCA)$_2$ ‡					447
Glutamic acid					
Glu(OBzl)-OPh	Oil				318

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* At about 15 °C.
† *N*-Methylmorpholine salt.
‡ New procedure.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Adoc-Glu(OBzl)	74—75	−6.2 −7.0 [b]	2	80% AcOH	437
Azoc-Glu,2Cha	193—196				438
Azoc-Glu(OBzl),Cha	156—157	+27.5	1	$CHCl_3$	438
Boc-Glu(OBzl)	138—138.5				439
Boc-Glu(OBzl[NO_2])	80—81				2
Boc-Glu-OPh	100—102	−42.2	1	MeOH	318
Boc-Glu(OBzl)-OPh	75—76	−30.5	1	MeOH	318
Nps-Glu(OBu^t)-ONSu	121—122	−66.6	1	MeOH	203
Z-Glu-OH	115—117	−7.6	3	AcOH	258
Z-Glu-O	88—91	−44	5	AcOH	258
Z-Glu-NHC_6H_4-*p*-NO_2	164—167	−19.7	1	MeOH	318
Glutamine					
Gln-OBu^t *	136—137	+10.4 +11.4 [b]	1	MeOH	170
Gln(OBzl[NO_2]),HCl	173	+6.7	1	H_2O	121
Gln(OMe),HCl	141—143	+29	1	MeOH	120
Adoc-Gln	152—154	−8.5 −10.0 [b]	2	MeOH	437
Boc-Gln-OBzl(NO_2)	141—142	−7.5	1	EtOAc	121
Boc-Gln(Z[OMe])	97—98	−2.7	1	MeOH	441
Boc-Gln(Z[OMe])-OBzl	108—109				441
Ppt-Gln,Dcha	172—174	+8.7	1	EtOH	403
Tfa-Gln	190	−20.5	1	H_2O	440
Z-Gln	128—130	−1.4 −8.7	4 1	AcOH MeOH	258
Z-Gln(Mbh)	114	−6.3 −6.6	1 2	DMF DMF	258
Z-Gln-OBu^t	92—93	−20.6 −24.4 [b]	1	EtOH	170
Glycine					
Gly-OPh,HBr	214—216				224
Gly-OCH_2CCl_3,HBr	Deliquescent solid				224
Gly-NHNHTroc	Deliquescent solid				224
Azoc-Gly	128—130				438
Boc-Gly-ONMa	134—135				357
Boc-Gly-ONSu	168—170				158
Bz-Gly-OCH_2CCl_3	125—126				448
Dpp-Gly	132				100
Dpp-Gly-OMe	118				100
Nps-Gly-ONSu	194—196				205
Peoc-Gly (bromide)	167 dec.				429
Peoc-Gly-OEt	87				429
Ppt-Gly	118—119				403
Sub-Gly,HCl	156				420
Sub-Gly-OMe,HCl	148—151				420
Tfa-Gly	118—119				440
Z-Gly-OCH_2CCl_3	Oil				224
Z-Gly-OPh	66—67				224

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* Bis(phenylsulphonyl)ammonium salt.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Z-Gly-NHNHTroc	119—120				224
Z-Gly-NHEt	99—100				154
Histidine					
His-OMe	212—214				449
His-OMe,2HCl	202	+16.5	1	MeOH	258
His(Trt)-OMe	122	+7.0 [d]	1	MeOH	158
Adoc-His(Adoc)	138	+13.7	2	MeOH	437
		+26.3	2	EtOAc	
Azoc-His	151—152	+1.8	1	MeOH	438
Azoc-His(Bzl)	200	+105.4	1	$CHCl_3$	438
Azoc-His(Dnp),Dcha	132—134				438
Boc-His	191	+24	1	MeOH	450
		−11	1	DMF	
Boc-His(CPh_2Py)-OMe	Oil	+2	1	MeOH	336
Nps-His(Tos)-ONSu					141
Z-His	167	−25	6	6M-HCl	404
	162	−25	6	6M-HCl	451
		−29 [b]			
Z-His(Adoc)	70	+39.7	2	EtOAc	437
		+47.9 [b]			
Z-His(Adoc),Dcha	123	+18.9	2	MeOH	451
		+19.8 [d]			
	153	+22.4 [d]	2	MeOH	451
Z-His(Boc)					451
Z-His(CPh_2Py)	108—111	+13	1	DMF	336
Z-His(CPh_2Py)-OMe					336
Z-His(Tos)	114	+13.7	2.2	MeOH	451
		+13.9 [d]			
		+17.4	2	DMF	451
		+18 [d]			
Z(OMe)-His(Tos)-ONp	93—95	+41	1	THF	141
Isoleucine					
Azoc-Ile,Cha	158—160	+2.9	1	$CHCl_3$	438
Boc-Ile-ONMa	69—70	−27.3	1	THF	357
Boc-Ile-ONSu	101—103	−42	1	MeOH	158
Boc-Ile-NHNHZ	114—116	−29.6	1	MeOH	201
Dpp-Ile	113—114	−7.2			100
Dpp-Ile-OBzl	105—108	−30.7			100
$MePh_2$Peoc-Ile	161				426
Mtc-Ile,Dcha	130	+0.8	2	MeOH	428
Sub-Ile,HCl	122—124	+32.7	5	DMF	420
		+51.6	5	MeOH	
Sub-Ile-OMe	Oil	+36.5	5	MeOH	420
Leucine					
Azoc-Leu,Cha	168—171	0	1	$CHCl_3$	438
Boc-Leu-ONMa	84—85	−22.2	1	THF	357
Dpp-Leu	131—134	−20			100
Dpp-Leu-OBzl	102	−27.8			100
$MePh_2$Peoc-Leu	164				426
Mtc-Leu,Dcha	144	−8.2	2	MeOH	428
Peoc-Leu-OMe	163—164	−17.5	10	MeOH	429

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[450] O. D. van Batenburg and K. E. T. Kerling, *Internat. J. Peptide Protein Res.*, 1976, **8**, 1.
[451] H. Eckstein, *Annalen*, 1976, 1289.

Coded Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Ppt-Leu,Dcha	137—138	−15.0	1	EtOH	403
Sub-Leu,HCl	206—210	+40.8	5	MeOH	420
		+13.4	5	DMF	
Sub-Leu-OEt,HCl	107—109	+17.6	5	MeOH	420
Tfa-Leu	72	−40	1	H_2O	440
Lysine					
Lys(Adoc)	213 dec.	+13.8	0.5	80% AcOH	437
		+15.9 [b]			
Lys(Tos)	212—215 dec.	+15.2	1.84	2M HCl	452
Lys(Z) *	252				453
Lys(Z)-OBzl,HCl	132—134	−2	1	MeOH	445
Lys(Tfa)-NCA †	49—51				344
Azoc-Lys(Boc),Cha	95—99	+1.4	1	MeOH	438
Azoc-Lys(Z),Dcha	65	+2.4	1	MeOH	438
Boc-Lys-NH_2	73—76	−53.7	2	MeOH	328
Boc-Lys-$NHNH_2$	127 dec.	−27.6	2.5	MeOH	328
Boc-Lys-NHNHBoc	Oil	−21	2.2	MeOH	328
Boc-Lys(Msc)-ONp	104.5—106	−20.9	1	EtOAc	161, 162
Boc-Lys(Tfa)	102—103				130
Boc-Lys(Z)-ONSu	94—95	−23 [d]	1	MeOH	158
Boc-Lys(Z)-NH_2	142	−27.8	2	MeOH	328
Boc-Lys(Z)-NHNHZ	Oil	−29.1	2.1	MeOH	328
Boc-Lys(Z)-NHNHBoc	Oil	−24.4	2	MeOH	328
Dpp-Lys(Dpp)-OMe	152—153	+5.8			100
Dpp-Lys(Z),Dcha	153—154	+13.3			100
Sub-Lys(Sub)-OMe,2HCl	158—161	+18.9	5	MeOH	420
Sub-Lys(Sub),2HCl	130	+26.5	5	DMF	420
Tfa-Lys(Tfa)	93—94	−6.4	1	EtOH	440
Tfa-Lys(Tfa),Dcha	144—145				440
Z-Lys(Boc)-ONSu	96—97	−17		Dioxan	230
Z-Lys(Mhoc)-OMe	Solid	−9.6	2	Acetone	7
Z(OMe)-Lys(Z)-ONSu	103—105				159
Methionine					
Met-OMe,HCl	144	+26.3	1	H_2O	87
Azoc-Met,Cha	140—141	+6.2	1	MeOH	438
Boc-Met-ODPtd	193—194	37.4 [b]†	1	CH_2Cl_2	432
Dpp-Met	141—142	−14			100
Dpp-Met-OMe	93—94	−35.8			100
Ppt-Met,Dcha	145—146	−1.2	1	MeOH	403
Tfa-Met	68	−24.3	1	H_2O	440
Phenylalanine					
Phe-NCA	95.5—96	−86.5	4.920	CH_2Cl_2	458
Phe-OMe,HCl	160—161	+27.3	1	AcOH	83

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* New procedure.
† And other derivatives.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Amoc-Phe-OMe					454
Azoc-Phe,Dcha,0.5Pr^{i}OH	185—186	+22.8	1	MeOH	438
Beoc-Phe	77				426
Boc-Phe	83—84	−4	1	AcOH	87
Boc-Phe-ODPtd	170	39 [b]*	1	$CHCl_2$	432
Boc-Phe-ONMa	139—140	−16.8	1	THF	357
Dpp-Phe	133	−40.1			100
Dpp-Phe-OBzl	158	−46.1			100
$MePh_2$Peoc-Phe					426
Mtc-Phe,Dcha	154	+25	1	MeOH	428
Peoc-Phe,bromide	160 dec.				429
Peoc-Phe-OMe	130 dec.	−24.9	5	MeOH	429
Ppt-Phe,Dcha	190—191	+8.7	1	EtOH	403
Sub-Phe,HCl	105—110	+33.4	5	DMF	420
Sub-Phe-OMe,HCl	143—146	+28.5	5	MeOH	420
Sub-Phe-OSub	111—112	+33.6	1	DMF	420
Z-Phe		−13.4	4.3	AcOH	417
Z-Phe-OMe	Oil	−15.6	1.02	MeOH	133
Z-Phe-NH_2		+19.9	4	EtOH	417
X-Phe, where X = some novel acid-labile protecting groups					455
Proline					
Pro-OBzl,HCl	145—148	−45 [d]	1	MeOH	445
Pro-ONp,HBr	192—193				286
Azoc-Pro,Cha	186—188	+4	1	MeOH	438
Boc-Pro	133—134	−64	1	AcOH	258
Boc-Pro-ODPtd	158—159	55.7 [b]*	1	CH_2Cl_2	432
Boc-Pro-ONMa	112—113	−57	1	THF	357
Dpp-Pro	170—173	−16.2			100
Dpp-Pro-OBzl	81—82	−43.3			100
Mtc-Pro,Dcha	138	−31.7	1	MeOH	428
Ppt-Pro,Dcha	194—195	−40	1	EtOH	403
Tfa-Pro	47—48	−60.9	1	C_6H_6	440
Z-Pro		−87	1	H_2O	417
Z-Pro-ODPtd	104—106	54.3 [b]*	1	CH_2Cl_2	432
Z-Pro-ONp	94				286
Serine					
Ser(Bzl)-OBzl,HCl	126—130	−29.8 †	1	EtOH	117
Azoc-Ser	126—128	−9	1	MeOH	438
Azoc-Ser(Bu^t),Cha	177—184	+17.8	1	MeOH	438
Azoc-Ser(Bzl),Cha	139—140	+32.3	1	$CHCl_3$	438
Boc-Ser(Bzl),Cha	159—160	+29.0	1	MeOH	456
Mtc-Ser,Dcha	141	+9.4	1	MeOH	428
Sub-Ser(Sub)	137	+29.9	5	DMF	420
Sub-Ser,HCl	125—137	+29.9	5	DMF	420
Sub-Ser(Sub),2HCl	108—119	+31.4	5	DMF	420
Tfa-Ser	Oil	−5.0	1	EtOH	440
Z-Ser(Bzl)-OPcp	125—126	+2.9	1	$CHCl_3$	446
Z-Ser-NHNHBoc	105—106	−7.8	1.03	DMF	204
		−25.5	1.08	MeOH	
Threonine					
Thr(Bu^t)-OBu^t	Oil				124

* Sign not specified.
† At 17 °C.

Coded Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Azoc-Thr(Bu^t),Cha	208—210	+0.7	1	MeOH	438
Azoc-Thr(Bzl),Cha	178—180	+27.2	1	MeOH	438
Tryptophan					
Trp-OBzl,HCl	218—220	+4.5 [a]	1	MeOH	445
Azoc-Trp,Cha	100—102	+4.4	1	MeOH	438
Beoc-Trp	92—97				426
Boc-Trp-OBzl	136—137	−9 [a]	1	MeOH	445
Dpp-Trp	165—169	−60.5			100
Dpp-Trp-OMe	147—149	−48.5			100
MePh$_2$Peoc-Trp	Oil				426
Mtc-Trp,Dcha	180	+6.1	1	MeOH	428
Tfa-Trp	160—161	+1.7	1	EtOH	440
Z-Trp	128	−7	1	MeOH	230
Z-Trp-OMe	Oil	−11.4	1.1	MeOH	133
Z-Trp-ONSu	74—76	−54.6	1	DMF	230
Tyrosine					
Tyr(Bzl[2-NO$_2$]),HBr	200—203 dec.	+4.76	1	DMF	411
Tyr(Bzl)-OBzl *					457
Tyr(Bzl)-OBzl,HCl	205—207	−17 [a]	1	MeOH	445
Azoc-Tyr,Dcha	112—114	+39.4	1	MeOH	438
Azoc-Tyr(Bu^t),Cha	158—161	+63	1	MeOH	438
Azoc-Tyr(Bzl),Cha	145—146	+45.4	1	MeOH	438
Boc-Tyr(Bzl)	109	5	1	AcOH	87
Boc-Tyr(Bzl)-Mea(Bzl)	125—126	−7.9	2	DMF	195
Boc-Tyr(Bzl[2,6-Cl$_2$]) *					39
Bpoc-Tyr(Bu^t)-OPcp	121—123	−31	1.2	$CHCl_3$	124
Mtc-Tyr,Dcha	96	+27.2	1	MeOH	428
Tfa-Tyr	189—190	+44.4	1	H_2O + 1 equivalent of NaOH	440
Z-Tyr(Bzl[2-NO$_2$])	117—119	−14.5	1	DMF	411
Z-Tyr-OEt	50—51	−7.6	1	MeOH	133
Z-Tyr-OMe		+21.9	2.2	Pyridine	417
Z-Tyr(Bzl[2-NO$_2$])-OMe	96—98	+0.5	1.05	DMF	411
Z-Tyr(Bu^t)-OTcp	98	−9.4	1	$CHCl_3$	121
Z(OMe)-Tyr-OMe	99—101	−16.5 †	0.9	MeOH	233
Z(OMe)-Tyr-NHNH$_2$	223—225	−12.8 †	1	DMF	233
Valine					
Adoc-Val,Dcha	164—165	−3.7 −4.4 [b]	2	CH_2Cl_2	437
Azoc-Val,Cha	162—163	+4	1	$CHCl_3$	438
Boc-Val,Dcha	135—136				439
Dpp-Val	103	−15.2			100
Dpp-Val-OMe	119—124	−32.7			100
Mtc-Val,Dcha	149	−2.0	2	MeOH	428
Ppt-Val	149—151	−10.0	1	EtOH	403
Tfa-Val	87	−16.1	1	H_2O	440
Z-Val-ODPtd	161—163	43.5 [b‡]	1	CH_2Cl_2	432
Z-(OMe)-Val-NHNHTroc	137—138	−11.8	0.9	DMF	96

* New procedure.
† At 26 °C.
‡ Sign not specified.

Other Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
D-Alanine					
D-Ala-OPh(SMe),HCl	171—172	−5.6	1	MeOH	163
Boc-D-Ala	82.5—83.5	+23.7	2	AcOH	14
	83—84	+21.5	17.8	AcOH	351
Boc-D-Ala-ONSu	166—167	+54.5	4	Dioxan	351
Boc-D-Ala-OPh(SMe)	88—89	53.7 *	1	MeOH	163
Z-D-Ala-NHMe	126—127	+11.9	1	MeOH	424
Z-D-Ala-NH$(CH_2)_2$OH	113—115	+11.3	1	MeOH	424
Other derivatives					163
2-Aminobutyric acid (Abu)					
Me-Abu-NMe_2	b.p. 109/ 3 mmHg				458
Tos-Abu(Boc)	149.5—150 dec.	−50.5	1	1M-NaOH	275
		−8.1	0.5	DMF	
		+15.3	1	MeOH	
Z-Abu	194.5—195 dec.	−9.25	1	H_2O	275
3- and 4-Aminocyclohexane-carboxylic acids					
Several derivatives, of the *cis* and *trans* forms					331
7-Aminoheptanoic acid					
The methyl ester					236
2-Amino-4-guanidinobutyric acid (Agb)					
Z-Agb(NO_2),Dcha	140—141	−7.6	0.7	MeOH	275
2-Amino-3-guanidino-propionic acid (Agp)					
Z-Agp(NO_2)	164.5—165.5	−4.3	4	1M-NaOH	275
		−9.7	1	MeOH	
		−34.4	1	DMF	
Z-D-Agp(NO_2)	164.5—165.5	+3.5	4	1M-NaOH	275
		+8.5	0.9	MeOH	
		+34.6	0.4	DMF	
Aminomalonic acid					
Several Z-diesters					423
4′-Aminophenylalanine					
The Boc-Z-derivative of the D-acid	158—159	−34.7 †	1	$CHCl_3$	144
The bis-Z-derivative	145—146	−5.45	2	Acetone	470
The 4′-Z,NCA derivative	205	−90.5	2	Dioxan	470
3-Aminopropionic acid					
The *N*-methyl dimethyl-amide	b.p. 83/ 3 mmHg				458
DL-Aminosuberic acid (Asu)					
Boc-Asu(OMe)-OBu^t					236
Boc-Asu-OBu^t	72—72.5				236
Boc-Asu(ONp)-OBu^t	Oil				236
D-Arginine					
Boc-D-Arg(Tos)	96—98	+3.0	3.5	DMF	14
7-Azatryptophan					
The *N*-acetyl derivative	240—243				148

* Sign not specified.
† At 26 °C.

Other Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
3-(3-Benzo[*b*]thienyl)alanine					
The *N*-acetyl derivative	154—156				148
The *N*-acetyl derivative of the *S*-dioxide	159—163				148
3-(4-Biphenyl)alanine (DL)					
The *N*-acetyl derivative	208—210.5				148
4-Bromophenylalanine					
The Boc-derivative	117—118	+9.6	2	EtOAc	13
γ-Carboxyglutamic acid					
Several derivatives					459, 460
γ-Carboxyglutamine					
Several derivatives					461
3-Chloroalanine					
The Z-,benzyl ester	90—92	−20.8	1	$CHCl_3$	460
D-Cysteine					
Threo-D-Cys(Me,Ac)-OMe	Oil	−66.1	5	EtOH	462
Dehydroamino-acids					
Dehydroalanine					
The Z-,benzyl ester	Oil				460
Dehydroaminobutyric acid					
The ethyl ester	b.p. 40—41/ 1.5 mmHg				463
Dehydroisoleucine					
The Z-,ethyl ester	Oil				465
Dehydroleucine					
The ethyl ester	b.p. 55—58/ 1.0 mmHg				463
The Z-,ethyl ester	Oil				465
Dehydronorleucine					
The ethyl ester	b.p. 58—60/ 0.3 mmHg				463
Dehydronorvaline					
The ethyl ester	b.p. 50—52/ 1.5 mmHg				463
Dehydrophenylanine					
The ethyl ester	b.p. 90—95/ 0.5 mmHg				463
The anilide	117—122				345
The *N*-benzoyl anilide	171.5—174.5				345
The Z-,ethyl ester	58, *Z*-isomer Oil, *E*-isomer				465
Dehydrovaline					
The methyl ester	b.p. 76/ 11 mmHg				464
The Boc-,methyl ester	70—72				464
The Z-,ethyl ester	37				465

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Other Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Some other Dehydroamino-acid Derivatives					345
Deoxystatin					
The Boc-derivative	91—92				326
Diaminobutyric acid (Dab)					
Dab(Z)-OMe,HCl	157—158	+15.5	1	MeOH	10
Boc-Dab(Z)	Oil	−14.7	2	MeOH	10
		−20.5	1	DMF	
		−12.5	1	AcOH	
Boc-Dab(Z),Dcha	99—102	0	1	MeOH or DMF	10
		−8.3	1	AcOH	
Diaminopropionic acid (Dap)					
Tos-Dap(Boc)	129—131	−71.2	4	1M-NaOH	275
	127—128	−72	3.9	1M-NaOH	466
Z-Dap	240—241	−7.4	0.4	1M-NaOH	275
	243—245	−14.2	1	AcOH	466
Z-Dap-OMe,HCl	163—166	−43.5	1.1	MeOH	466
	165—167	−42.5	1	MeOH	466
Z-Dap(Boc)	145—146	−20.1	4	1M-NaOH	275
		−9.5	1	MeOH	
	145—148	−9.5	1	MeOH	466
Z-Dap(Boc)-OMe	74—77	−6.5	1.1	MeOH	466
Derivatives of the D-acid					275, 467
2,5-Dibromophenylalanine					
The Boc-derivative	156—157	+4.1	1.5	EtOAc	13
3,5-Di-iodotyrosine (Dit)					
Dit(Bzl)	201—203	+5 [b]	2	70% AcOH	427
Dit-OMe,HCl	189—191	+10.5 [b]	2	MeOH	427
Dit(Bzl)-OMe,HCl	207—208	+8.8 [b]	1	MeOH	427
Boc-Dit	186—187	+21.1	1	EtOAc	12
Boc-Dit(Bzl)	131.5—132.5	+16.1 [b]	1	EtOAc	427
Z-Dit(Bzl)	164	+6.6 [b]	1	EtOAc	427
3′,4′-Dihydroxyphenylalanine (Dopa)					
Several derivatives					188
D-Glutamine					
Boc-D-Gln	114—117	−31	1	0.08M-NH_3 in EtOH	14
D-Histidine					
Boc-D-His(Boc),Dcha	155—158	−18	1.2	$CHCl_3$	14
Homoarginine (Har)					
Boc-Har(NO_2)	92—98	−17.2	2	Pyridine	131
	Amorphous				277
Boc-Har(NO_2)-ONp	125—127	−2.5 [d]	1	Acetone	131
		−3.0 [b]	1	Acetone	
Homocysteine (Hcy)					
Boc-Hcy(Bzl)	103—104	−13.3	1.2	MeOH	191
Homoserine (Hse)					
Z-DL-Hse-OBzl	60—61				468

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Other Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Other derivatives					468
3- and 4-Hydroxyprolines					
Various derivatives					412
4-Iodophenylalanine					
The Boc-derivative	120—121	+21.3	1.2	EtOAc	13
Isoasparagine					
The Z-,4-nitrobenzyl ester	137—141	−15.4 *	1	DMF	123
D-Lysine					
Boc-D-Lys(Z[2-Br])	Oil	−5.0	3	$CHCl_3$ + 1 equivalent of Dcha	14
D-Methionine					
Boc-D-Met,Dcha	138—140	−15	1	DMF	14
N-Methylalanine					
Boc-MeAla,Dcha	89—89.5				439
Dpp-MeAla	148—149	−29.6	1	MeOH	469
N-Methylaspartic acid					
Boc-MeAsp-NH_2	177—178	−1.1	1	MeOH	39
N-Methylisoleucine					
Dpp-MeIle	150—152	−12.3	1	MeOH	469
N-Methylleucine					
Dpp-MeLeu	161—163	−14.3	1	MeOH	469
N-Methylmethionine					
Dpp-MeMet	145—155	−24.0	1	MeOH	469
β-Methylphenylalanine					
Several derivatives					421
N-Methylphenylalanine					
Dpp-MePhe	182—184	−87.1	1	MeOH	469
N-Methyltyrosine					
Boc-MeTyr(Bzl[2,6-Cl_2])	84—85	−45.9	1	MeOH	39
Boc-MeTyr(Bzl[2,6-Cl_2])-OMe	Oil				39
N-Methylvaline					
Boc-MeVal,Dcha	107—110	−54.1	1	MeOH	466
Dpp-MeVal	150—151	−26.1	1	MeOH	469
3-(1-Naphthyl)alanine					
The acetyl derivative	186—188				148
3-(2-Naphthyl)alanine					
The acetyl derivative	171—172				148
	170—173 †				150
The Boc-derivative	93—95	+42	0.5	EtOH	150
4′-Nitrophenylalanine					
The benzyl ester, Tos salt	186	−10	1	MeOH	163
The Boc-derivative of the D-acid	113—114	−24.5	1	1M-$NaHCO_3$	144
(DL) 2-*o*-Nitrophenacylglycine					
The methyl ester hydrochloride	160.5—161				284
The Z-derivative	143—144				284

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* At 26 °C.

† For the DL-acid.

Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Ornithine					
Orn(Adoc)	210 dec.	+11.9 +14.5 [b]	1	80% AcOH	437
Orn(Pipoc)-OPic,2HBr	Solid	−0.2 +4.3 [a]	0.9	H_2O	54
Boc-Orn(Boc),Dcha	155—157	+10	1	DMF	54
Boc-Orn(Pipoc),Dcha	123—126	+12	1	EtOH	54
Boc-Orn(Pipoc)-OPic	119—120.5	−16	1	DMF	54
Boc-Orn(Pr^i)	203—207	+7.2	1.7	H_2O	150
Boc-Orn(Pr^i,Tos)	100—105	+1.8	2	EtOH	150
Azoc-Orn(Boc),Dcha	160—161	+19.8	1	MeOH	438
Z-Orn(Pipoc),Dcha	144—147	+9	1	EtOH	54
Z-Orn(Pipoc)-OPic	Oil	+3	1.2	$CHCl_3$	54
D-Phenylalanine					
Boc-D-Phe	85—88	−24	1	95% EtOH	14
D-Phe-NCA *	96—96.3	+85.1	4.880	CH_2Cl_2	458
Z-D-Phe-OMe	Oil	+15.2	0.64	MeOH	133
D-Proline					
Boc-D-Pro	133—135	+61	1	AcOH	14
Pyroglutamic acid					
Z-Glp	131—133	−28.2	1	MeOH	258
Z-Glp,Dcha	196—199	−16.5	3	$CHCl_3$	258
Z-Glp-ONSu	128—130	−20.5	1	DMF	258
Sarcosine					
Boc-Sar-ONMa	96—97				357
Boc-Sar-OTcp	74—76				25
Dpp-Sar	147—149				469
D-Thiamorpholine-2-carboxylic acid (Thm)					
Z-Thm-OPcp	128—130	+50.8	1	$CHCl_3$	446
Thiazolidine-4-carboxylic acid (Thz)					
Thz-NCA	83—85 dec.				471
Thz-NH_2	97—99	−112.6 †	1	H_2O	256
Ac-Thz-NH_2	201—203				471
Boc-Thz	132—135	−114.8	1	MeOH	256
Z-Thz	56—57	−102.0	0.5	$CHCl_3$	446
Z-Thz,Dcha	164—166	−89.2	1	$CHCl_3$	446
Z-Thz-OPcp	123—125	−69.7	1	$CHCl_3$	446
Thioasparagine					
Several derivatives					443
D-Threonine					
allo-Boc-D-Thr-OMe	Oil	+14.4	5	EtOH	462
allo-Boc-D-Thr(Tos)-OMe	Solid	+4.2	8	EtOH	462
D-Tryptophan					
Boc-D-Trp	140—144	+19	1	AcOH	14

[470] E. Peggion, A. Cosani, M. Palumbo, M. Terbojevich, and M. Goodman, *Biopolymers*, 1976, **15**, 2227.

[471] E. Benedetti, A. Christensen, C. Gilon, W. Fuller, and M. Goodman, *Biopolymers*, 1976, **15**, 2523.

[472] 'Pepttdes 1976', Proc. 14th European Peptide Symposium Université de Bruxelles, ed. A. Loffet, 1976.

* Also the DL-NCA.

† At 27 °C.

Other Amino-acids (*cont.*)

Compound	*m.p./*°C	$[\alpha]_D$*	*Conc.*	*Solvent*	*Ref.*
D-Tyrosine					
D-Tyr-OEt,HCl	163—165	−26.5	2	EtOH	133
Boc-D-Tyr(Bzl)-Mea(Bzl)	123—125	+7.4	2	DMF	195
D-Valine					
Boc-D-Val,Dcha	138—140	−3.2	2	EtOH	14

4

Peptides with Structural Features not Typical of Proteins

BY B. W. BYCROFT

1 Introduction

The size and complexity of many of the cyclic and linear peptides isolated and characterized over the past year demonstrate the efficacy of the physical and chemical methods employed in their structure determination. The intriguing question as to whether these large peptides are formed by ribosomal processes and subsequently undergo modification, or whether they are produced by the non-ribosomal mechanism, well understood for molecules such as gramicidin S, remains unanswered.

Many interesting smaller peptides have been isolated, predominantly from micro-organisms. The wealth of new highly active β-lactam antibiotics which are substantially different from penicillin and cephalosporin is noteworthy. The increasing use of cell-free systems for investigating biosynthetic processes is encouraging and the principle of directed biosynthesis offers an interesting alternative for producing potentially useful peptide antibiotics.

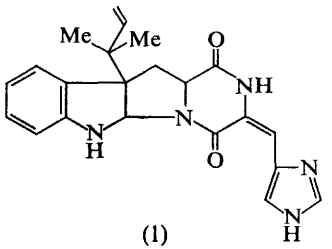
The main problems associated with synthesis in this area concern the construction of the modified peptide units. The smaller molecules, such as gliotoxin, penicillins *etc.*, provide excellent synthetic targets for the organic chemist. However, the larger molecules require the special skills of the peptide chemist, and several syntheses, notably that of tuberactinomycin, illustrate this point.

2 Cyclic Peptides (Homodetic Peptides)

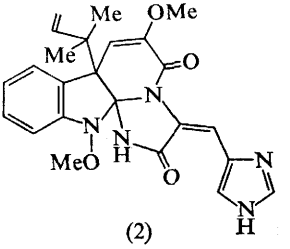
2,5-Dioxopiperazines (Cyclic Dipeptides).—A variety of new naturally occurring cyclic dipeptides have once again been reported. They have been isolated mainly from micro-organisms and belong predominantly to either the prenylated tryptophan or sulphur-bridged groups.

Roquefortine (1) from *Penicillium roqueforti* is only the second reported example of an indole natural product with an inverted C_5 unit at the C-3 position.[1] The other compound of this type is oxaline (2), the structure of which was established some time ago by an *X*-ray analysis. The detailed chemistry and spectral data on (2) have now been published [2] and although it is not formally a cyclodipeptide the structural similarities with (1) are noteworthy since both represent highly modified dipeptide entities.

[1] P. M. Scott, M. A. Merrien, and J. Polonsky, *Experientia*, 1976, **32**, 140.
[2] D. W. Nagel, K. G. R. Pachler, P. S. Steyn, R. Vleggaar, and P. L. Wessels, *Tetrahedron*, 1976, **32**, 2625.

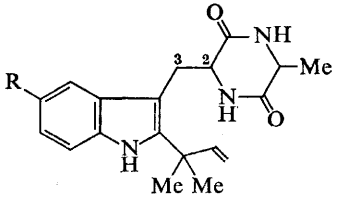


(1)

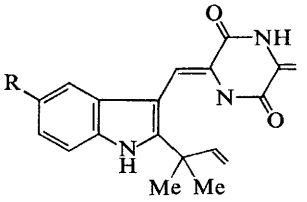


(2)

Several further examples of the more common metabolites with an inverted C_5 unit in the C-2 position have appeared. Echinulin and a new compound termed pre-echinulin (3) occur together in *Aspergillus chevalieri*,[3,4] while isoechinulin A, B, and C [(4)—(6)] have been isolated and characterized[5] from

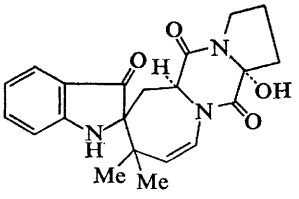


(3) R = H
(4) R = $CH_2CH{=}CMe_2$
2, 3 = dehydro

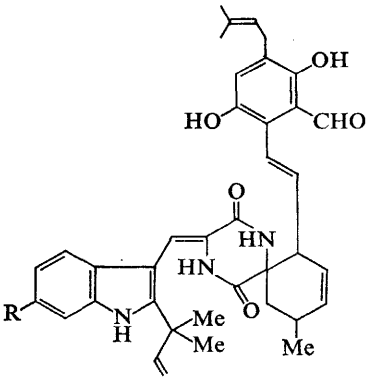


(5) R = $CH_2CH{=}CMe_2$
(6) R = $CH_2CH{-}CMe_2$ (O)

A. ruber. A previously assigned structure for isoechinulin A has been withdrawn. The compound (7), isolated from *A. ustus*, is a further member of the well-established austamide group.[6] The possibility that the novel substances (8) and (9), extracted from the mycelium of *A. amstelodamii*, are artefacts is supported



(7)

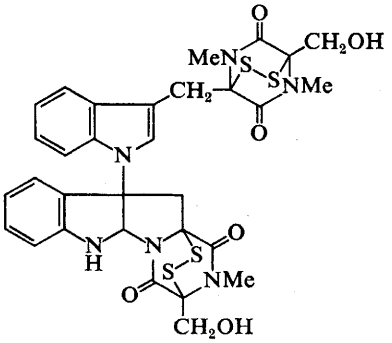


(8) R = H
(9) R = $CH_2CH{=}CMe_2$

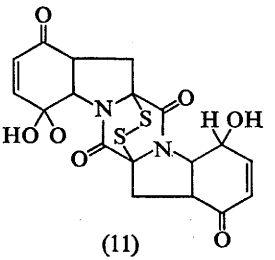
[3] T. Hamasaki, K. Nagayama, and Y. Hatsuda, *Agric. and Biol. Chem. (Japan)*, 1976, **40**, 203.
[4] R. D. Stipanovic and H. W. Schroeder, *Trans. Brit. Mycol. Soc.*, 1976, **66**, 178.
[5] H. Nagasawa, A. Isogai, A. Suzuki, and S. Tamura, *Tetrahedron Letters*, 1976, 1601.
[6] P. S. Steyn and R. Vleggaar, *Phytochemistry*, 1976, **15**, 355.

by the observation that they can be obtained by a thermal Diels–Alder condensation of auroglaucine with neoechinuline B and C, respectively, which are known metabolites of this fungus.[7]

Chetomin (10)[8] and epicorazine (11)[9] are two new epithiodioxopiperazine antibiotics with pronounced biological activity. Both structures rest on spectral



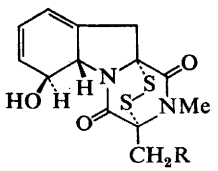
(10)



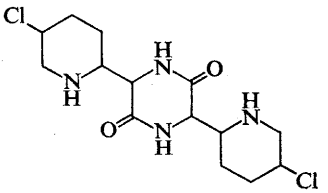
(11)

evidence, the former on an interesting application of ^{15}N and ^{13}C n.m.r. spectroscopy. Deoxygliotoxin (12; R = H) is not a usual metabolite of *Trichoderma viride*, but its formation can be directed by feeding *cyclo*-(L-Ala-L-Phe) to the organism.[10] This type of analogue biosynthesis could possibly be extended for other compounds of pharmacological interest.

The provisional structure (13) assigned to the chlorine containing piperazinedione from *Streptomyces griseoluteus*, which has shown encouraging clinical results against human lymphomas, has been confirmed by an *X*-ray analysis.[11]



(12)

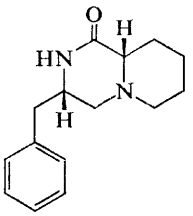


(13)

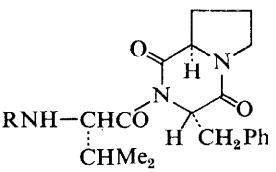
The toxic metabolite (14), isolated from *Penicillium verruculosum*, which is found on green peanuts can be considered to be a reduced cyclic dipeptide derived from L-Phe and pipecolinic acid.[12]

[7] G. Gatti, R. Cardillo, C. Fuganti, and D. Ghiringhelli, *J.C.S. Chem. Comm.*, 1976, 435.
[8] A. G. McInnes, A. Taylor, and J. A. Walter, *J. Amer. Chem. Soc.*, 1976, **98**, 6741. R. Baute, G. Deffieux, M.-A. Baute, M.-J. Filleau, and A. Neveu, *Tetrahedron Letters*, 1976, 3943.
[10] G. W. Kirby and D. J. Robins, *J.C.S. Chem. Comm.*, 1976, 354.
[11] G. R. Pettit, R. B. Von Dreele, D. L. Herald, M. T. Edgar, and H. B. Wood, *J. Amer. Chem. Soc.*, 1976, **98**, 6742.
[12] J. G. Macmillan, J. P. Springer, J. Clardy, R. J. Cole, J. W. Kirksey, *J. Amer. Chem. Soc.*, 1976, **98**, 246.

From field ergot which produces mainly ergocristine, the *cyclo*-(L-Phe-D-Pro) derivative (15) has also been isolated and characterized.[13] This same compound had previously been observed in the mycelium of *Claviceps purpurea*, and provides further circumstantial evidence that the biosynthesis of the cyclol moiety of the



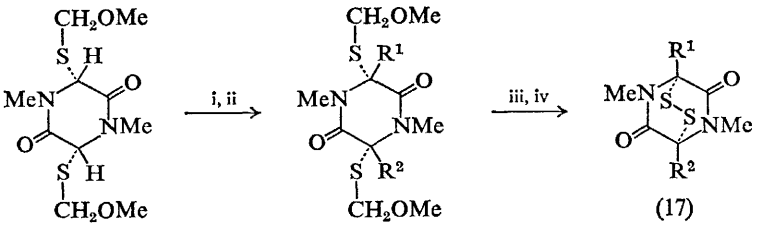
(14)



(15) R = lysergyl

ergot peptides proceeds through a linear intermediate. The current views on the biosynthesis of the ergot alkaloids have been well reviewed in a current article.[14] Interest continues in other aspects of ergot peptide chemistry, and a convenient synthesis of radiolabelled derivatives [15] as well as extensions of the original cyclol synthesis [16, 17] have been described.

The major synthetic effort has once again centred on the epidithiopiperazine-dione antibiotics. The first total synthesis of gliotoxin (12; R = OH) has further demonstrated the versatility and elegance of the general route already developed for the synthesis of this important group.[18] In addition the same workers have reported [19] a new general synthesis of the basic sulphur-bridged system which is illustrated in Scheme 1. The mono- or di-anion generated from (16) in both cases afford *cis*-products. Removal of the protecting groups followed by oxidation yields the sulphur-bridged cyclodipeptide (17). The efficacy of the



(16) (17)

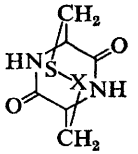
Reagents: i, $LiN(iPr)_2$–R^1Br; ii, $LiN(iPr)_2/R^2Br$; iii, BCl_3; iv, I_2

Scheme 1

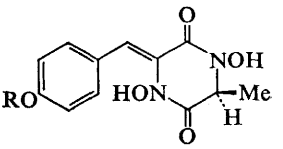
[13] A. Černý, A. Krajíček, J. Spačil, M. Beran, B. Kakač, and M. Semonský, *Coll. Czech. Chem. Comm.*, 1976, **41**, 3415.
[14] H. G. Floss, *Tetrahedron*, 1976, **32**, 873.
[15] E. Schreier, *Helv. Chim. Acta*, 1976, **59**, 585.
[16] D. E. Portlock, A. C. Ghosh, W. E. Schwarzel, R. R. Kurtz, E. Boger, H. C. Dalzell, and R. K. Razdan, *J. Heterocylic Chem.*, 1976, **13**, 781.
[17] P. Stutz and P. A. Stadler, *Monatsh.*, 1976, **107**, 763.
[18] T. Fukuyama and Y. Kishi, *J. Amer. Chem. Soc.*, 1976, **98**, 6723.
[19] T. Fukuyama, S. Nakatsuka, and Y. Kishi, *Tetrahedron Letters*, 1976, 3393.

method was demonstrated by a one-pot synthesis of the metabolite hyalodendrin (17; $R^1 = CH_2Ph$, $R^2 = CH_2OH$). An alternative way is available for the simple derivatives (17; $R^1 = R^2 =$ alkyl) which are formed in moderate yield on heating the dipotassium salt of 3,6-dicarboxydioxopiperazines in the presence of sulphur monochloride.[20]

Spectral data, particularly ^{13}C n.m.r., have been used to measure the strain imposed by the epithio bridge [21] and (18) has been synthesized in order to investigate the conformational effects of the isosteric substitution of sulphur for a methylene group.[22] In relation to general synthetic studies, the oxidation of dioxopiperazines under a variety of conditions has been explored.[23, 24] The biological oxidation (dehydrogenation) of the tyrosine residue in mycelianamide (19) occurs with the loss of the *pro*-S-methylene hydrogen from the labelled amino-acid.[25]



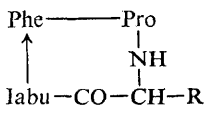
(18) X = CH_2



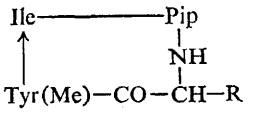
(19) R = geranyl

Other Naturally Occurring Cyclic Peptides.—This section deals with cyclic peptides other than dioxopiperazines or compounds related to the major groups listed below. Several new cyclic peptides with novel features have been described, and the size and complexity of many of them are particularly noteworthy.

Few naturally occurring tetrapeptides have so far been isolated. An *X*-ray analysis of a dihydro derivative of chlamydocin (20) has confirmed [26] the structure deduced from the chemical and spectra data. The original configurational assignments of the amino-acids were supported and the configuration about the epoxide established. This is significant as the pronounced cytostatic activity of the antibiotic is markedly reduced on alteration of either



(20)



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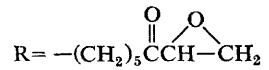
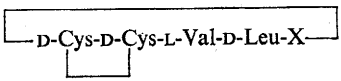


Iabu = 2-aminoisobutyric acid

[20] T. Sato and T. Hino, *Chem. and Pharm. Bull.*, 1976, **24**, 285.
[21] J. W. Ronaldson, *Austral. J. Chem.*, 1976, **29**, 2307.
[22] J.-L. Fauchere, N. Muthukumaraswamy, A. Tun-kyi, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2187.
[23] P. J. Machin and P. G. Sammes, *J.C.S. Perkin I*, 1976, 628.
[24] U. Schmidt and J. Häusler, *Angew. Chem. Internat. Edn.*, 1976, **15**, 497.
[25] G. W. Kirby and S. Narayanaswami, *J.C.S. Perkin I*, 1976, 1564.
[26] J. L. Flippen and I. L. Karle, *Biopolymers*, 1976, **15**, 1081.

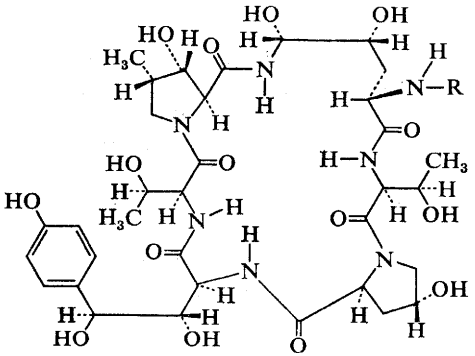
the epoxide or the adjacent ketone group. The analysis is also notable in that the structure represents the first cyclic tetrapeptide in which all four peptide units have been found in the *trans* geometry. The structure of a closely related compound (21), isolated from *Cylindrocladium scoparium*, rests predominantly on the interpretation of mass spectrometric data.[27]

The structure of malformin C (22), a new metabolite from a strain of *Aspergillus niger*, also stems essentially from the interpretation of mass spectral fragmentation patterns.[28] A detailed analysis of high resolution data on malformin A (23) and related cyclic peptides has been presented.[29]



(22) X = Leu
(23) X = Ile

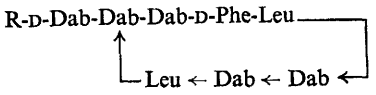
A cyclic hexapeptide with a fatty acid side-chain, a feature typical of many recently characterized peptides, has been isolated from *A. rugulosus*. Echinocardin B (24) is composed completely of L-amino-acids including the novel



(24) R = linoleyl

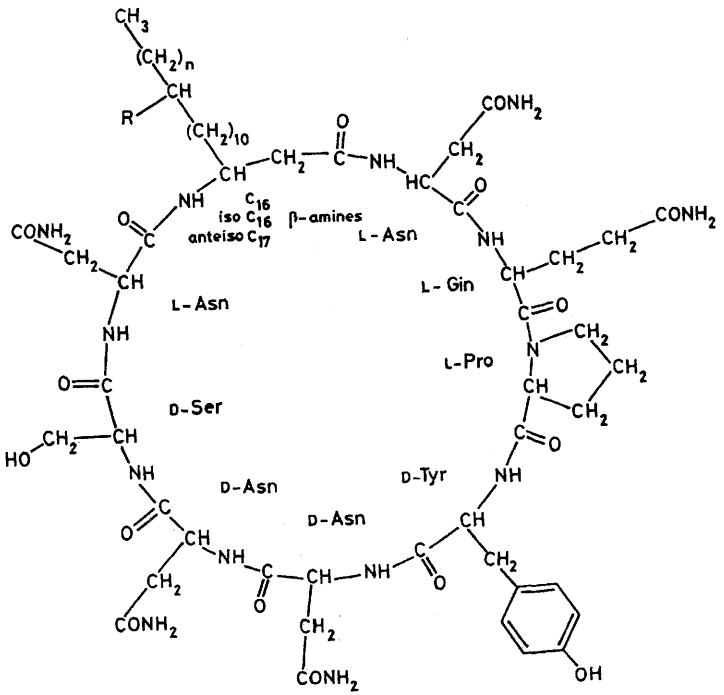
L-4,5-dihydroxyornithine and L-3,4-dihydroxyhomotyrosine. The structure was confirmed and full configurational assignments made on the basis of an *X*-ray analysis of a mercurial derivative.[30] The family of acylpeptides belonging to the EM49 group have now been designated octapeptins. The amino-acid sequence of octapeptin C (25), together with the chirality of the amino-acids, was determined by classical methods after removal of the acyl group.[31] A similar approach was adopted with mycosubtilin (26), an antifungal agent from *Bacillus subtilis*,

[27] A. Hirota, A. Suzuki, K. Aizawa, and S. Tamura, *Biomed. Mass Spectrom.*, 1974, **1**, 15.
[28] R. J. Anderegg, K. Biemann, G. Büchi, and M. Cushman, *J. Amer. Chem. Soc.*, 1976, **98**, 3365.
[29] M. Bodanszky, J. B. Henes, S. Natarajan, G. L. Stahl, and R. L. Foltz, *J. Antibiotics*, 1976, **29**, 549.
[30] C. Keller-Juslén, M. Kuhn, H. R. Loosli, T. J. Petcher, H. P. Weber, and A. von Wartburg, *Tetrahedron Letters*, 1976, 4147.
[31] T. Kato and J. Shoji, *J. Antibiotics*, 1976, **29**, 1339.



(25) R = β-hydroxyanteisononanoyl
Dab = α,γ-diaminobutyric acid

although, in this case, the lipid portion of the molecule is a β-amino-acid and part of the ring structure.[32] (26) is a mixture of homologous lipopeptides which differ only in the size of the β-amino-acid residue.

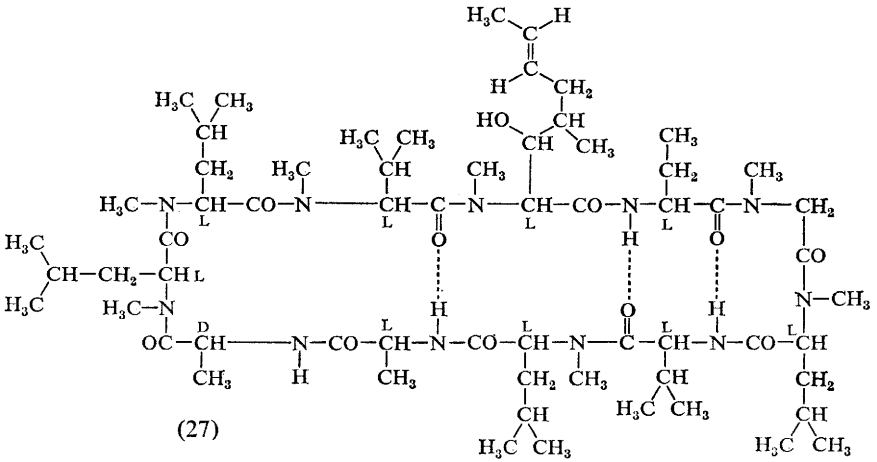


(26) R = H or Me

Cyclosporin A (27) is a larger peptide and does not contain a lipid residue. The structure was established by chemical and spectroscopic methods[33] and confirmed by an *X*-ray analysis of an iodo derivative.[34] As well as exhibiting marked antifungal activity, it also shows interesting immunosuppressive and antiphlogistic activity.

Although it is customary to include in this section only those peptides for which firm structural assignments have been made, samarosporin is included since it is a large cyclic peptide with broad antimicrobial activity. The tentative structure

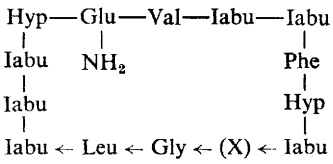
[32] F. Peypoux, G. Michel, and L. Delcambe, *European J. Biochem.*, 1976, **63**, 391.
[33] T. J. Petcher, H. P. Weber, and A. Ruegger, *Helv. Chim. Acta*, 1976, **59**, 1480.
[34] A. Ruegger, M. Kuhn, H. Lichti, H.-R. Loosli, R. Huguenin, C. Quiquerez, and A. von Wartburg, *Helv. Chim. Acta*, 1976, **59**, 1075.



(27)

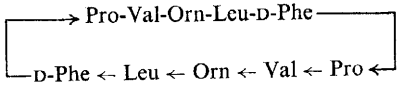
(28) has been assigned,[35] and the residue (X), observed in the hydrolysate as phenylalaninol probably as a result of decarboxylation, is undefined.

Gramicidin and Related Peptides.—The sustained interest in the gramicidin antibiotics is reflected in the publication of further review articles, covering the chemistry, synthesis,[36] topology and biological activity,[37] and the biosynthesis [38]



(28) Iabu = 2-aminoisobutyric acid

of gramicidins (29). Efforts continue towards the production of a cell-free system capable of synthesizing respectable quantities of gramicidin S. Further studies have been directed at better production and purification procedures of the synthetases from *Bacillus brevis*.[39–42]



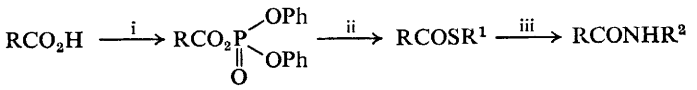
(29)

[35] N. Inoue, A. Inoue, M. Furukawa, and N. Kanda, *J. Antibiotics*, 1976, **29**, 618.
[36] N. Izumiya, *Tampakushitsu Kakusan Koso Bessatsu*, 1976, **76**, 166.
[37] L. K. Ramachandran, *Biochem. Rev.*, 1975, **46**, 1.
[38] H. Ito, K. Mori, and K. Kurahashi, *Seikagaku Jikken Koza*, 1975, **7**, 675.
[39] A. L. Demain, J. M. Piret, T. E. Friebel, E. J. Vandamme, and C. C. Matteo, *Microbiology*, 1976, 437.
[40] A. L. Demain and C. C. Matteo, *Antimicrob. Agents Chemother.*, 1976, **9**, 1000.
[41] H. Koischwitz and H. Kleinkauf, *Biochim. Biophys. Acta*, 1976, **429**, 1041.
[42] J. Vater and H. Kleinkauf, *Biochim. Biophys. Acta*, 1976, **429**, 1062.

The relationship between the topochemistry and biological activity of gramicidins appears to be more subtle than initially suspected. The mono and di-*N*-methyl-leucine derivatives of gramicidin both exhibit full biological activity and it was proposed [43] that the solution conformations (all *trans* peptide bonds) differed substantially in the backbone topology and the orientation of the phenylalanine and ornithine side-chains from that of the native antibiotic. A re-examination of the data has contradicted this claim and concluded that the *N*-methyl peptide bonds have the *cis*-geometry and that the conformation is essentially the same as in gramicidin itself.[44] A novel hydrogen bond arrangement is also proposed involving one carbonyl group interacting with two amide protons. These proposals find support from the *X*-ray data on ilamycin B which exhibits similar conformational features.[45]

The mono and di-*N*-methyl-valine derivatives of gramicidin have now been prepared [46] and they show only weak antibacterial activity. Initial o.r.d. data suggest that the molecular conformation of these compounds does differ substantially from that of gramicidin S.

Further syntheses of gramicidin analogues (see Table 2), designed to explore other aspects of the structure/activity relationship have been reported.[47–49] An interesting comparison between the use of alkylthiol esters in a synthetic sequence for the formation of a peptide bond (Scheme 2) and the non-ribosomal peptide biosynthesis has been drawn.[50] The synthesis proceeds in reasonable yield and the coupling step, catalysed by either pivalic acid or 2-hydroxypyridine, occurs without detectable racemization.

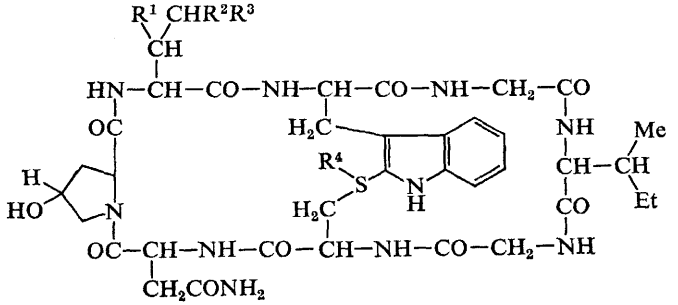


Reagents: i, $N_3PO(OPh)_2$; ii, R^1SH–Et_3N; iii, R^2NH_2–hydroxypyridine or pivalic acid

Scheme 2

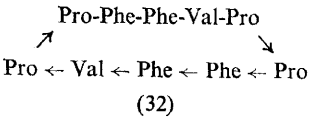
Cyclic Peptides from *Amanita Phalloides*.—The total syntheses of dinor-*S*-deoxoamaniamide (30) and the epimeric sulphoxides 6′-dehydroxyamanulline (31) have been reported.[51] The diastereoisomers were prepared from the corresponding sulphide by oxidation with hydrogen peroxide in acetic acid, the (*R*)-sulphoxide shows a stronger inhibitory effect on RNA polymerase than the (*S*)-isomer. Investigations on RNA polymerase with amatoxins have been facilitated by a convenient synthesis of tritium-labelled amanin.[52]

[43] N. G. Kumar, N. Izumiya, M. Migoshi, H. Sugano, and D. W. Urry, *J. Amer. Chem. Soc.*, 1975, **97**, 4104.
[44] D. J. Patel and A. E. Tonelli, *Biopolymers*, 1976, **15**, 1623.
[45] Y. Iitaka, H. Nakamura, K. Takada, and T. Takita, *Acta Cryst.*, 1974, **B30**, 2817.
[46] H. Abe, K. Sato, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1976, **49**, 3113.
[47] A. A. Akhrem, A. S. Tumash, N. A. Poddubnaya, and A. V. Otchik, *Vestsi Akad. Navuk B. SSR, Ser. Khim. Navuk*, 1976, 99.
[48] A. A. Akhrem, A. S. Tumash, and N. A. Poddubnaya, *Vestsi Akad. Navuk B. SSR, Ser. Khim. Navuk*, 1976, **95**.
[49] M. A. El-Maghraby, *J. Indian Chem. Soc.*, 1976, **53**, 496.
[50] S. Yamada, Y. Yokoyama, and T. Shioiri, *Experientia*, 1976, **32**, 967.
[51] A. Buku, R. Altmann, and T. Wieland, *Annalen*, 1976, 417.
[52] T. Wieland and O. G. Brodner, *Annalen*, 1976, 1412.

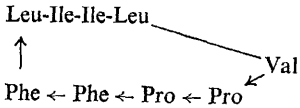


(30) R^1 = H, R^2 = OH, R^3 = H, R^4 = —
(31) R^1 = Me, R^2 = Me, R^3 = H, R^4 = O(*R*)O(*S*)

The crystal conformation of [Phe^4,Val^6]antamanide (32), a synthetic analogue of natural antamanide with biological activity, is completely different from any of the solution models previously proposed, and also differs from the crystal conformation of the metal complex. The molecule possesses a two-fold rotation axis, *cis* peptide linkages between Pro^2—Pro^3 and Pro^7—Pro^8, and one pair of intramolecular hydrogen bonds.[53] Cyclolinpeptide (33), the cyclic nonapeptide



(32)

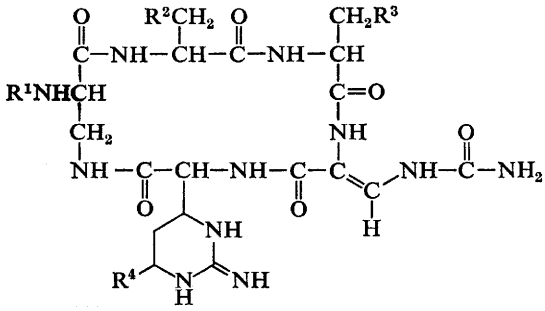


(33)

found in linseed, bears some superficial relationship with antamanide. However, a detailed analysis of its spectral properties has found that the molecule exhibits considerable conformational flexibility and possesses little ion-complexing ability.[54]

Viomycin and Related Peptides.—The major developments in this area have been dominated by the total syntheses of tuberactinomycin O (34) [55] and capreomycin 1A (36) and 1B (37).[56] On the basis of this work, the structures of the capreomycins have been revised and it is interesting to note that the attachment of the β-lysine residue to the cyclic pentapeptide is through an α-amino-group of a diaminopropionyl molecule for tuberactinomycin O (34) and viomycin (35), but to the β-amino position of a different diaminopropionyl residue in the capreomycins.

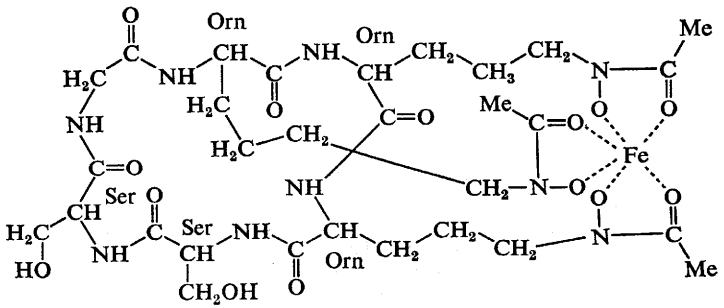
[53] I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, and B. Witkop, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1782.
[54] D. Balasubramanian, P. Chopra, F. Ardeshir, and S. Rajappa, *F.E.B.S. Letters*, 1976, **65**, 69.
[55] T. Teshima, S. Nomoto, T. Wakamiya, and T. Shiba, *Tetrahedron Letters*, 1976, 2343.
[56] T. Shiba, S. Nomoto, T. Teshima, and T. Wakamiya, *Tetrahedron Letters*, 1976, 3907.



(34) R^1 = β-Lys, R^2 = OH, R^3 = OH, R^4 = H
(35) R^1 = β-Lys, R^2 = OH, R^3 = OH, R^4 = OH
(36) R^1 = H, R^2 = OH, R^3 = NH—β-Lys, R^4 = H
(37) R^1 = H, R^2 = H, R^3 = NH—β-Lys, R^4 = H

The synthetic strategy (Scheme 3) involved the formation of a protected cyclic pentapeptide containing a diethoxyalanine residue. Deprotection followed by condensation of the masked aldehyde with urea afforded the antibiotics. Further studies on the derivatization of viomycin have aimed at preparing amino-acid derivatives.[57]

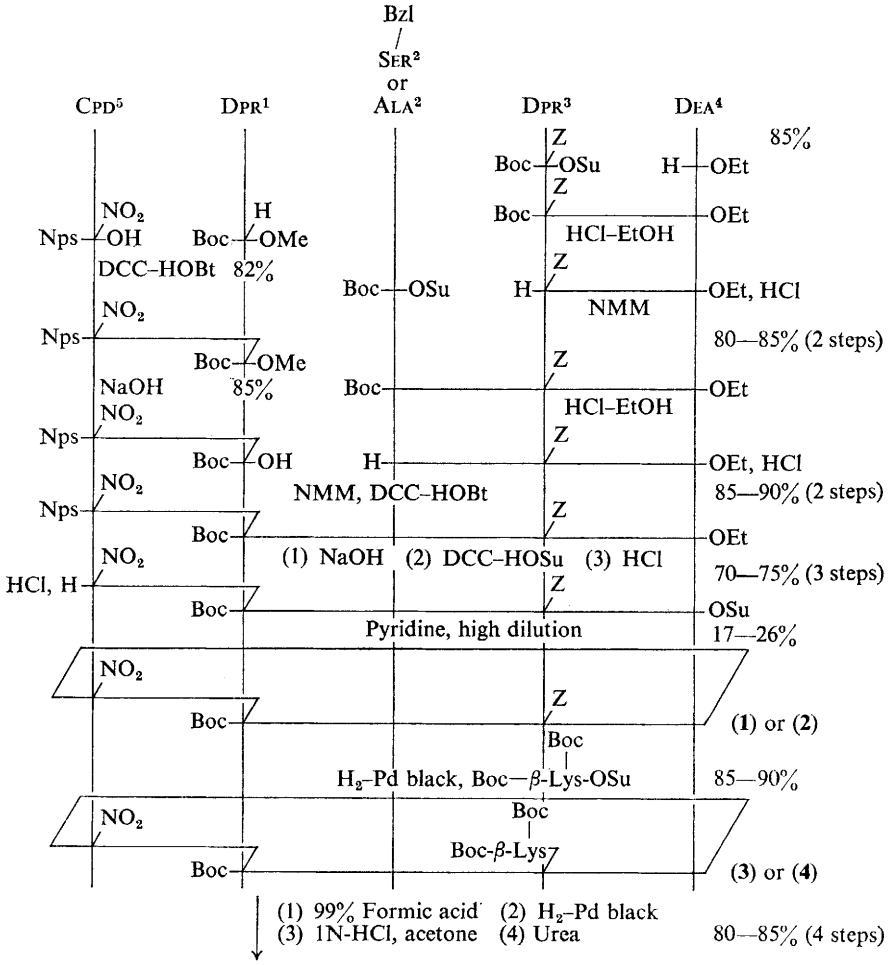
Iron-containing Cyclic Peptides.—This group of compounds has not previously been reviewed under a separate heading. However, because of their importance and the continuing interest in this area, the addition was considered warranted. During the past decade a number of compounds containing hydroxamic acid groups co-ordinated to a central ferric ion have been isolated, mainly from micro-organisms. One of the major groups is the ferrichrome family, all of which



(38)

possess the cyclic hexapeptide structure. Investigations have centred on conformational aspects; the *X*-ray analysis of ferrichrysin (38),[58] together with a detailed ^{13}C n.m.r. solution study on the metal-free compounds, deferriferrichrome,

[57] T. Kitagawa, T. Miura, C. Takaishi, and H. Taniyama, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 1324.
[58] R. Norrestam, B. Stensland, and C. I. Branden, *J. Mol. Biol.*, 1975, **99**, 501.



Abbreviations: Cpd, capreomycidine; Dpr, α,β-diaminopropionic acid; Dea, β,β-diethoxyalanine; β-Lys, β-lysine; Z, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; Nps, *o*-nitrophenylsulphenyl; DCC, *NN*-dicyclohexylcarbodi-imide; HODt, 1-hydroxybenztriazole; HOSu, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; 1,3-ser² analogues, 2,4-Ala² analogues

Scheme 3

deferriferricrocin, and deferrichrysin,[59] have been reported. The pronounced chemical shift differences induced by the metal binding are attributed to an overall change in the conformational state of the peptides. A similar investigation on alumichrome, the aluminium analogue of ferrichrome, using ^{15}N n.m.r. has shown that the chemical shifts of the amide ^{15}N resonances are also sensitive indicators of conformation changes.[60]

[59] M. Llinas, D. M. Wilson, M. P. Klein, and J. B. Neilands, *J. Mol. Biol.*, 1976, **104**, 853.
[60] M. Llinas, W. J. Horsley, and M. P. Klein, *J. Amer. Chem. Soc.*, 1976, **98**, 7554.

The crystal structure of ferrioxamine E has been determined.[61] Although this group are not formally cyclic peptides, the similarity with the ferrichromes, concerning the nature of the co-ordination of the three hydroxamate groups to the ferric ion, makes them of interest. The distribution of the iron-complexing metabolites in various *Aspergillus* species has been studied.[62]

Conformational Studies on Cyclic Homodetic Peptides.—The area is still attracting a considerable amount of attention. As well as the studies on the conformational states of the naturally occurring peptides which have been covered under the appropriate heading, there is an increasing number of papers on the application of new techniques, theoretical aspects, and conformational studies on synthetic peptides.

Several valuable reviews have appeared on all aspects of the subject,[63-67] a number of which review the methodology and stress the importance of integrating the conformational information from the various physical and spectroscopic methods.[63, 64] N.m.r. techniques still dominate as the most generally useful for defining solution conformations and recent advances have centred around more detailed interpretation of data. An analysis of the β and γ carbon ^{13}C chemical shifts of proline in cyclic peptides of known geometry has revealed that they are dependent not only on the *cis–trans* isomerism of the acyl residue attached to the nitrogen but also on the dihedral angle $C^{\beta}—C^{\alpha}—C=O$ of the proline residue.[68] The dependence of geminal proton–proton coupling constants on ϕ and ψ angles has been explored and offers to yield useful conformational data particularly with respect to glycine residues.[69] Magnetic resonance spectra of the ^{15}N nucleus are being used increasingly in the study of peptides [61, 70] and the possibility of useful information from $^{13}C—^{15}N$ coupling constants is under investigation.[71]

Table 1 *Crystal structures of cyclic dipeptides reported in* 1976

	Ref.
cyclo-(L-Val-Sar)	74, 75
cyclo-(Gly-L-3-thioPro)	76
cyclo-(L-Thr-L-His)	77
cyclo-(L-Pro-D-Phe)	78
cyclo-(*N*-Me-L-Ala-L-Ala)	79
cyclo-(*N*-Me-L-Ala)$_2$	80
rac-cyclo-(*N*-Me-Ala)$_2$	80
cyclo-(*N*-Me-L-Val)$_2$	80
cyclo-(*N*-Me-L-Val-*N*-Me-D-Val)	80
cyclo-(*N*-Me-L-Phe)$_2$	80
cyclo-(*N*-Me-L-Phe-*N*-Me-D-Phe)	80

[61] D. van der Helm and M. Poling, *J. Amer. Chem. Soc.*, 1976, **98**, 82.
[62] H. Diekmann and E. Krezdorn, *Arch. Microbiol.*, 1975, **106**, 191.
[63] L. C. Craig, D. Cowburn, and H. Bleich, *Ann. Rev. Biochem.*, 1975, **44**, 477.
[64] C. M. Deber, V. Madison, and E. R. Blout, *Accounts Chem. Res.*, 1976, **9**, 106.
[65] S. Kondo, T. Shiba, A. Suzuki, T. Takita, and K. Maeda, *Tampakushitsu Kakusan Koso Bessatsu*, 1976, **76**, 321.
[66] Y. Iitaka, *Tampakushitsu Kakusan Koso Bessatsu*, 1976, **76**, 299.
[67] Y. Imanishi, *Adv. Polymer Sci.*, 1976, **20**, 1.
[68] I. Z. Siemion, T. Wieland, and K.-H. Pook, *Angew. Chem. Internat. Edn.*, 1976, **14**, 702.
[69] M. Barfield, V. J. Hruby, and J.-P. Meraldi, *J. Amer. Chem. Soc.*, 1976, **98**, 1308.
[70] D. Gattegno, G. E. Hawkes, and E. W. Randall, *J.C.S. Perkin II*, 1976, 1527.
[71] J. M. Schulman and T. Venanzi, *J. Amer. Chem. Soc.*, 1976, **98**, 6739.

Theoretical calculations on the barrier to rotation about the N—C$^{\alpha}$(ϕ) and C$^{\alpha}$—CO(ψ) bonds appear to be at odds with experimental observations, particularly the calculated ψ values.[72] An alternative type of representation of peptide conformation has been presented and is claimed to be useful in recognizing unfavourable conformations.[73]

An unusual number of crystal structures of cyclic dipeptides have been reported [74–80] during the year, and these are listed in Table 1. They extend substantially the knowledge of substituent effects and ring conformation, as well as side-chain interactions.

Semi-empirical calculations on *cyclo*-(L-Thr-L-His) indicate that the threonine side-chain is folded above the dioxopiperazine ring owing to specific interaction with the imidazole of the histidine,[81] a conclusion supported by the *X*-ray analysis.[77] The equilibrium between the boat and planar forms of several proline-containing cyclic dipeptides has been calculated from ^{13}C n.m.r. data.[82] The side-chains of the non-prolyl residues have a number of exceptable rotameric positions which depend largely on solvent, and these can be deduced from c.d. and n.m.r. spectral data.[83] The ^{13}C chemical shifts and spin–lattice relaxation times for *cyclo*-(L-Pro-L-Leu) and *cyclo*-(L-Pro-D-Leu) have been determined and the differences, not suprisingly, reflect a difference in the conformations of the two dioxopiperazine rings.[84] The folding of the ring in a series of glycine- and sarcosine-containing cyclic dipeptides appears to increase with the bulkiness of the amino-acid side-chain in water and DMSO.[85]

Ab initio molecular orbital calculations on *cyclo*-(Gly-Gly), *cyclo*-(D-Ala-L-Ala), and *cyclo*-(L-Ala-L-Ala) have yielded total energy values for the different configurations and conformations and reflect reasonably the observed differences.[86]

The crystal structure of *cyclo*-(tri-L-prolyl) confirms the suspected *cis* geometry for the three peptide bonds. All the amide bonds in the molecule also show appreciable deviation from planarity.[87] An extension of an already important systematic study of sarcosine-containing peptides has concentrated on a series of cyclic pentapeptides (see Table 2). N.m.r. data have revealed that, in the main, the crystal conformations are not the same as those adopted in solution. The only compound which retains the same conformation in both is *cyclo*-(Sar)$_5$. Replacement of one or more sarcosine units with primary amino-acids such as

[72] A. T. Hagler, L. Leiscrowitz, and M. Tuval, *J. Amer. Chem. Soc.*, 1976, **98**, 4600.
[73] R. Balasubramanian, *Biochem. J.*, 1976, **157**, 769.
[74] V. I. Smirnova, L. N. Zeibot, N. E. Zhukhlistova, G. N. Tishchenko, and V. I. Andrianov, *Kristallografiya*, 1976, **21**, 525.
[75] P. A. Timmins, *Acta Cryst.*, 1975, **B31**, 2561.
[76] W. Haar, S. Fermandjian, F. Robert, O. Lefebvre-Soubeyran, and S. Savrda, *Tetrahedron*, 1976, **32**, 2811.
[77] M. Cotrait, M. Ptak, B. Busetta, and A. Heitz, *J. Amer. Chem. Soc.*, 1976, **98**, 1073.
[78] R. Ramani, K. Venkatesan, R. E. Marsh, and W.-J. Kung, *Acta Cryst.*, 1976, **B32**, 1051.
[79] A. Filhol and P. A. Timmins, *Acta Cryst.*, 1976, **B32**, 3116.
[80] E. Benedetti, R. E. Marsh, and M. Goodman, *J. Amer. Chem. Soc.*, 1976, **98**, 6676.
[81] M. Genest and M. Ptak, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 1174.
[82] I. Z. Siemion, *Org. Magnetic Resonance*, 1976, **8**, 432.
[83] V. Madison, P. F. Young, and E. R. Blout, *J. Amer. Chem. Soc.*, 1976, **98**, 5358 & 5365.
[84] R. Deslauriers, Z. Grzonka, and R. Walter, *Biopolymers*, 1976, **15**, 1677.
[85] D. B. Davies and M. A. Khaled, *J.C.S. Perkin II*, 1976, 1238.
[86] A. Støgard, *Biopolymers*, 1976, **15**, 2295.
[87] M. E. Druyan, C. L. Coulter, R. Walter, G. Kartha, and G. K. Ambady, *J. Amer. Chem. Soc.*, 1976, **98**, 5496.

glycine or alanine leads to a mixture of conformations with the crystal conformation hardly present.[88] In order to assign conformational features such as hydrogen bonding and shielding of individual peptide bonds, a series of glycine-containing cyclic pentapeptides have been prepared with one or more (α-$^2H_2$)-glycine residues.[89] *Cyclo*-(Pro-Phe-Gly-Phe-Gly) is claimed on the basis of n.m.r. data to adopt one predominant solution conformer with all *trans* peptide bonds.[90]

The sarcosine- and glycine-containing cyclic hexapeptides *cyclo*-(Sar-Sar-Gly)$_2$, *cyclo*-(Sar)$_6$, and *cyclo*-(Sar-Gly-Gly)$_2$ have been shown to assume a variety of conformations in dimethyl sulphoxide. Evidence is also presented that the complexes formed with alkali thiocyanates exist in one favoured conformation.[91] The same group has also investigated, using n.m.r. techniques, the conformations of the hexapeptides *cyclo*-(Gly-X-Gly)$_2$ where X is a glutamic or aspartic acid residue.[92]

Cyclic hexapeptides with C_2 symmetry such as *cyclo*-(L-X-L-Pro-D-Phe)$_2$ are predicted to exhibit two solution conformations which are distinguished by *cis* and *trans* proline residues. The crystal structure of *cyclo*-(L-Ala-L-Pro-D-Phe)$_2$ shows C_2 symmetry and the conformation resembles one of those predicted. Both proline residues are *trans* and in the 2-position of β-turns.[93] The crystal lattice conformation of the chymotrypsin inhibitor *cyclo*-(L-Leu-L-Try-Avaler-Avaler) (Avaler = 5-amino-n-valeric acid) is similar to that adopted by some cyclic hexapeptides.[94]

Synthesis of Homodetic Cyclic Peptides.—Only those aspects of synthesi swhich relate directly to cyclic peptides or to structural features not typical of protein therein are covered in this section. Other aspects of synthesis are covered elsewhere in this report (Chapter 3). Table 2 summarizes the details of the syntheses of cyclic peptides reported in 1976. The list includes those synthesized for the first time and those for which experimental detail is presented.

Perhaps the most notable achievement, already reviewed, has been the total syntheses of the capreomycins and tuberactinomycin (see Scheme 3). The principal aims continue to be the provision of models for conformational studies and structure–activity relationships. The conformational models have been reviewed in the previous section and are listed, as are the analogues of gramicidin S.

A further synthesis of *enantio*-[1-Val]malformin has been reported and confirms the reduced biological activity of this palindrome.[95] The bicyclic system (39) has been synthesized by coupling the two respective cyclic tetrapeptides.[96–98] The molecule is predicted to adopt the cylindrical arrangement

[88] K. Titlestad, *Acta Chem. Scand.*, 1976, **B30**, 753.
[89] H. J. Moschler, A. Tun-kyi, J.-P. Meraldi, K. Wüthrich, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2197.
[90] D. Demel and H. Kessler, *Tetrahedron Letters*, 1976, 2801.
[91] T. Sugihara, Y. Imanishi, and T. Higashimura, *Biopolymers*, 1976, **15**, 1529.
[92] T. Sugihara, Y. Imanishi, T. Higashimura, and Y. Shimonishi, *J. Org. Chem.*, 1976, **41**, 2584.
[93] J. N. Brown and R. G. Teller, *J. Amer. Chem. Soc.*, 1976, **98**, 7565.
[94] I. L. Karle, *Macromolecules*, 1976, **9**, 61.
[95] P. Kurath, *Helv. Chim. Acta*, 1976, **59**, 1127.
[96] C. H. Hassall, R. G. Tyson, and K. K. Chexal, *J.C.S. Perkin I*, 1976, 2010.
[97] B. K. Handa and C. H. Hassall, *J.C.S. Perkin I*, 1976, 2014.
[98] K. K. Chexal, B. K. Handa, C. H. Hassall, and W. A. Thomas, *J.C.S. Perkin I*, 1976, 2019.

Table 2 *Syntheses of cyclic peptides in* 1976 *(derived from linear precursors unless otherwise stated)*

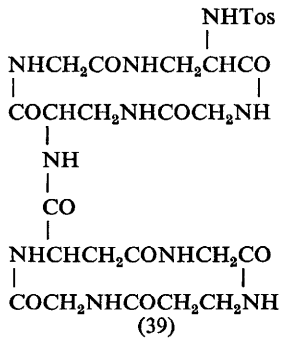
Peptide	*Bond formed in cyclization step*	*Method for cyclization*	*Ref.*
[1-MeVal]-gramicidin S	Leu-Phe	*a*	46
[1-MeVal][1′-MeVal]-gramicidin S	Leu-Phe	*a*	46
cyclo-(Sar-Ser-Orn-Ser-D-Phe)$_2$	—	*b*	47
cyclo-(Sar-Ser-Gly-Ser-D-Phe)$_2$	—	*b*	47
cyclo-(Pro-Ser-Gly-Ser-D-Phe)$_2$	—	*b*	47
cyclo-(Pro-Ser-Orn-Ser-D-Phe)$_2$	—	*b*	48
cyclo-(Val-Dab-Leu-D-Phe-Pro)$_2$	Pro-Dab	*a*	49
Dinor-*S*-desoxyamaninamide (30)	Cys-Asn, Gly-Cys	*c*	51
Tuberactinomycin O (34)	Dea-Cpd	*a*	55
Capreomycin IA (36)	Dea-Cpd	*a*	56
Capreomycin IB (37)	Dea-Cpd	*a*	56
cyclo-(Ala-Sar$_4$)	Sar-Ala	*d*	88
cyclo-(D-Ala-Ala-Sar$_3$)	Sar-Sar	*d*	88
cyclo-(Ala-Ala-Sar$_3$)	Ala-Sar	*d*	88
cyclo-(Ala-Sar-D-Ala-Sar$_2$)	Ala-Sar	*d*	88
cyclo-(Ala-D-Ala-Ala-Sar$_2$)	Sar-Ala	*d*	88
cyclo-(D-Ala-Ala-Sar-D-Ala-Sar)	Ala-Sar	*d*	88
cyclo-(Gly-Sar$_4$)	Sar-Gly	*d*	88
cyclo-(Gly$_2$-Sar$_3$)	Sar-Sar	*d*	88
cyclo-(Gly-Sar-Gly-Sar$_2$)	Sar-Sar	*d*	88
cyclo-(Gly-Cys(DPM)-Gly-Gly*-Pro), Gly* = α-[$^2H_2$]-Gly	Pro-Gly	*c*	89
cyclo-(Gly*-Cys(DPM)-Gly-Gly*-Pro)	Pro-Gly	*c*	89
cyclo-(Gly-Cys(DPM)-Gly-Gly*-D-Pro)	Pro-Gly	*c*	89
cyclo-(Gly*-Cys(DPM)-Gly-Gly*-D-Pro)	Pro-Gly	*c*	89
cyclo-(Gly-Ala-Gly-Gly*-Pro)	Pro-Gly	*c*	89
cyclo-(Gly*-Ala-Gly-Gly*-Pro)	Pro-Gly	*c*	89
cyclo-(Gly-Ala-Gly-Gly*-D-Pro)	Pro-Gly	*c*	89
cyclo-(Gly*-Ala-Gly-Gly*-D-Pro)	Pro-Gly	*c*	89
cyclo-(Sar-Gly-Gly)$_2$	Gly-Sar	*c*	91
cyclo-(Gly-Asp-Gly)$_2$	Gly-Gly	*c*	92
cyclo-(Gly-Glu-Gly)$_2$	Gly-Gly	*c*	92
enantio-[1-Val]-malformin	Leu-Val	*b*	95
cyclo-(β-Ala(α-NH_2)-Gly-D-β-Ala(α-NH_2)-Gly)	Gly-A_2pr	*e*	96
cyclo-(Ser(OBu^t)-β-Ala-Gly-Asp(OMe))	β-Ala-Gly	*e*	97
cyclo-(Lys-Asn-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Asp(α-CO_2H)) (40)	Asp-Lys	*f*	99

[a] DCC–*N*-hydroxysuccinimide. [b] Azide method. [c] *p*-Nitrophenyl ester. [d] 2,4,5-Trichlorophenyl ester. [e] *o*-Phenylene chlorophosphite. [f] DCC–hydroxybenzotriazole

indicated in Figure 1; as yet the evidence for this conformation is not conclusive, but each of the cyclic tetrapeptides has been shown to possess the required geometry. The investigation is part of a larger study concerned with the design of systems containing histidine, serine, and aspartic acid residues in a similar spatial relationship to that observed in the serine proteinases.

The cyclic undecapeptide (40), which has been synthesized by a combination of solid-phase and conventional methods, is an analogue of somatostatin, and exhibits good growth hormone and insulin release inhibiting activity *in vivo*.[99]

[99] D. Sarantakis, J. Teichman, E. L. Lien, and R. L. Fenichel, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 336.



(39)

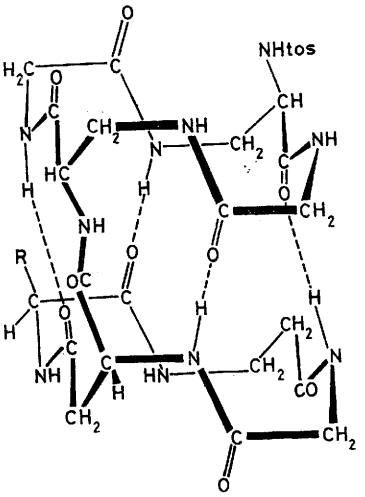
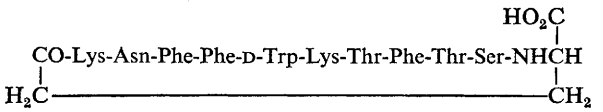


Figure 1



(40)

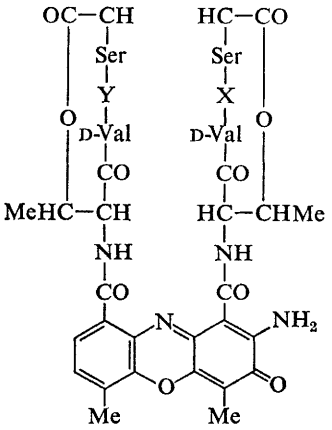
Solid-phase methods using *N*-2-nitrophenylsulphenylamino-acid *N*-carboxy-anhydrides have also been employed for the syntheses of two decapeptides which were subsequently cyclized through the azides to antamanide and (Gly^5)(Gly^{10})-gramicidin S.[100] The base-catalysed polymerization of amino-acid *N*-carboxy-anhydrides yields mixtures of macrocyclic peptides.[101] Identification of the smaller compounds was achieved by direct comparison with authentic samples; for the larger peptides the ring size has been deduced from gel chromatograms.

[100] J. Halstrøm, T. Christensen, and K. Brunfeldt, *Z. physiol. Chem.*, 1976, **357**, 999.
[101] M. Rothe and D. Mulhäusen, *Angew. Chem. Internat. Edn.*, 1976, **15**, 307.

3 Depsipeptides and other Cyclic Heterodetic Peptides

An excellent review article by the Russian group, specifically on heterodetic peptides, has appeared.[102] Unfortunately the literature is covered only up to the end of 1974 and it is therefore already out of date, nevertheless it offers an important source of information on this significant group of compounds.

Actinomycins.—The principle of directed biosynthesis has been used to produce new actinomycins. When cultured in the presence of azetidine-2-carboxylic acid, *Streptomyces antibioticus* synthesizes (41) and (42) which have been termed azetomycins.[103]



(41) X = Y = azetidine-2-carboxylic acid
(42) X, Y = proline, azetidine-2-carboxylic acid

The clinical application of actinomycin D is limited to certain types of tumours and the possibility that modified actinomycins may have broader activity continues to stimulate research. The analogue [di(1′-L-Dpr)] actinomycin in which both lactone functions have been replaced by lactams has been prepared.[104] Although active against bacteria, it exhibited little anti-tumor activity.

A detailed ^{13}C n.m.r. analysis and signal assignment for actinomycin D and related model peptides has been facilitated by biosynthetically produced ^{13}C enriched material. The results conflict seriously with assignments made in an earlier investigation.[105] The phenoxazone chromophore of actinomycin D has been subjected to available electronegativity self-consistent field calculations and the results compared with experimental data.[106] Further studies on the interaction between actinomycin D and DNA or nucleotides, using n.m.r.,[107, 108] c.d.,[109] and phase partition [110] techniques, have been published.

[102] Yu. A. Ovchinnikov and V. T. Ivanov, in 'Heterodetic Peptides', IRS Review of Science, Organic Chemistry, Series Two, Vol. 6, 1976, p. 183.
[103] J. V. Formica and M. A. Apple, *Antimicrob. Agents Chemother.*, 1976, **9**, 214.
[104] S. Moore, R. P. Patel, E. Atherton, M. Kondo, J. Meienhofer, L. Blau, R. Bittman, and R. K. Johnson, *J. Medicin. Chem.*, 1976, **18**, 766.
[105] H. Booth, A. B. Mauger, and W. J. Rzeszotarski ,*Org. Magnetic Resonance*, 1976, **8**, 219.
[106] J. C. Tai, W. S. Craig, and B. P. Gaber, *J. Amer. Chem. Soc.*, 1976, **98**, 7925.
[107] F. Quadrifoglio, A. Ciana, and V. Crescenzi, *Biopolymers*, 1976, **15**, 595.
[108] D. J. Patel, *Biochim. Biophys. Acta*, 1976, **112**, 98.
[109] F. S. Allen, R. P. Moen, and U. Hollstein, *J. Amer. Chem. Soc.*, 1976, **98**, 864.
[110] P. Davanloo and D. M. Crothers, *Biochemistry*, 1976, **15**, 4433.

Valinomycin.—A general review on ionophores from the laboratory that first noted the ion-transporting properties of depsipeptides has served to link the valinomycin and enniatin groups with the other natural and synthetic ionophores.[111] The first accurate *X*-ray analysis of a member of the enniatin group has provided structural parameters. These correspond well with those predicted from theoretical calculations and solution techniques for the predominant solution conformer of the molecule and its alkali ion complexes [112] (see Figure 2). A crystal structure on the non-peptide enniatin analogue *cyclo*-(L-HyIv-D-HyIv) reveals a similar P-form conformation.[113]

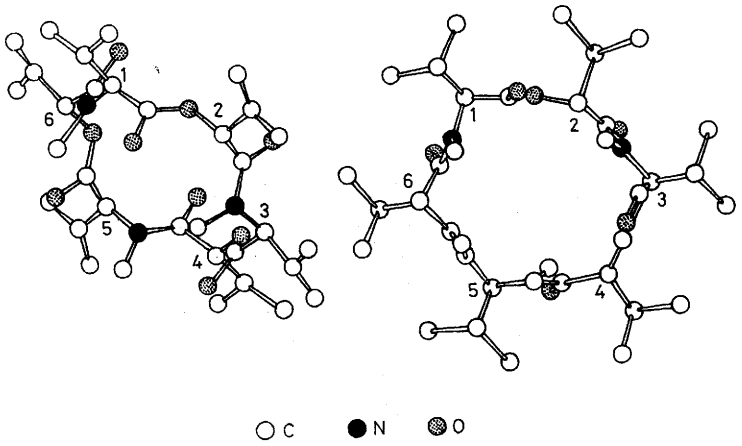


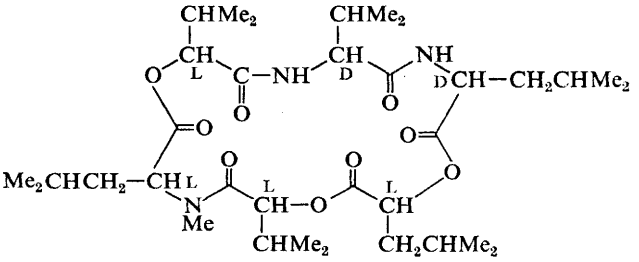
Figure 2 *The crystalline conformation of enniatin B.* (*a*) *Projection on the XZ-plane.* (*b*) *View down the pseudo-three-fold axis*
(Reproduced by permission from *FEBS Letters*, 1976, **65**, 315)

A further ^{1}H n.m.r. study on valinomycin and the alkali metal complexes is viewed in terms of the recent crystal data.[114] A more interesting investigation which makes use of the existing ^{1}H n.m.r. assignments has evaluated the potential of nuclear Overhauser effects (NOE) for conformational studies. The information obtained is claimed to set specific constraints on any model proposed and offers considerable promise for more general conformational studies of peptides.[115]

An analogue of valinomycin in which the D-hydroxyisovalerate and L-lactate residues are replaced by D- and L-proline respectively was prepared some time ago. A detailed conformational study has concluded that the cation complexes of this molecule have S_6 symmetry and are essentially isostructural with the potassium complex of valinomycin.[116]

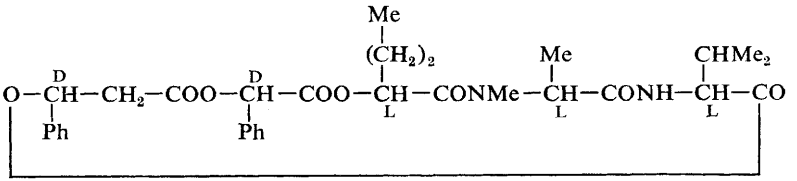
[111] B. C. Pressman, *Ann. Rev. Biochem.*, 1976, **45**, 501.
[112] G. N. Tishenko, Z. Karimov, B. K. Vainshtein, A. V. Evstratov, V. T. Ivanov, and Yu. A. Ovchinnikov, *F.E.B.S. Letters*, 1976, **65**, 315.
[113] T. G. Shishova, V. L. Simonov, V. T. Ivanov, A. V. Evstratov, I. I. Mikhaleva, T. A. Balashova, and Yu. A. Ovchinnikov, *Bio-org. Khim.*, 1975, **1**, 1689.
[114] D. B. Davies and M. A. Khaled, *J.C.S. Perkin II*, 1976, 1327.
[115] T. P. Pitner, R. Walter, and J. D. Glickson, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 746.
[116] D. G. Davis, B. F. Gisin, and D. C. Tosteson, *Biochemistry*, 1976, **15**, 768.

Other Naturally Occurring Cyclic Depsipeptides.—Strains of *Pithomyces*, normally employed for the production of sporidesmins, have yielded two new depsipeptides. Pimaydolide from *P. maydicus* has been assigned the structure (43) on the basis of spectral and chemical data.[117] While pithomycolide (44)

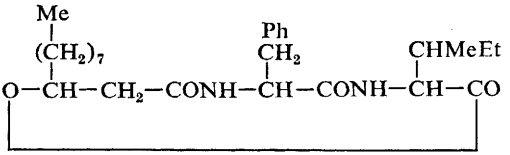


(43)

from *P. chartarum* is interesting in that it contains three lactone linkages the structure of this compound rests largely on the interpretation of the mass spectral fragmentation.[118] Similar mass spectral evidence on the methyl ester of beauvellide, a metabolite of *Beauveria tenella*, has led to the structure (45). It is surprising, in view of the current interest in the topology of depsipeptides, that no stereochemical assignments were made to this molecule.[119]



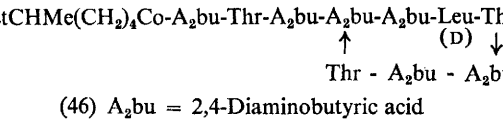
(44)



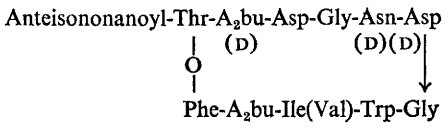
(45)

The appearance of a review[120] on the polymyxin family, really homodetic peptides, but conveniently included at this point, coincides with the structure determination of an additional member of the group,[121] polymixin M (46). Brevistin (47), produced by *Bacillus brevis*, is clearly closely-related to the

[117] D. W. Russell, W. D. Jamieson, A. Taylor, and B. C. Das, *Canad. J. Chem.*, 1976, **54**, 1355.
[118] R. Rahman, A. Taylor, B. C. Das, and J. A. Verpoorte, *Canad. J. Chem.*, 1976, **54**, 1360.
[119] F. Frappier, P. Ferron, and M. Pais, *Phytochemistry*, 1975, **14**, 2703.
[120] T. Suzuki and Y. Kimura, *Tampakushitsu Kakusan Koso Bessatsu*, 1976, **76**, 114.
[121] A. B. Silaev, S. N. Maevskaya, Zh. P. Trifonova, E. P. Yulikova, N. M. Vasil'eva, and G. S. Katrukha, *Zhur. obshchei Khim.*, 1975, **45**, 2331.



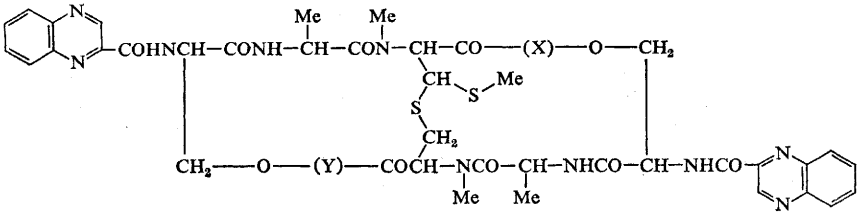
(46) A_2bu = 2,4-Diaminobutyric acid



(47)

polymyxins; indeed brevistinic acid, prepared by opening the lactone with dilute alkali, is deacylated with the enzyme preparation polymyxin acylase.[122]

The echinomycin group has received considerable attention since the revision of the general structure some two years ago to the present formulation (48). Most members of the group differ only in the amino-acid residues X and Y and



(48)

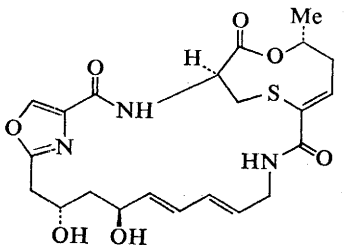
since the revised structure is unsymmetrical there are two possible positional isomers for compounds originally considered to be homogeneous. These have been detected for quinomycin E (48; X, Y = L-β-methylalloisoleucine and L-*N*-methyl-β-methylalloisoleucine).[123] The triostin group differs from the echinomycins in that the thio-acetal cross-link is replaced by a disulphide bridge, and further confirmatory evidence for these structures has been presented.[124]

The quinoxaline antibiotics are known to bind strongly with double helical DNA and more recent studies have centred on the interaction with synthetic polynucleotides.[125]

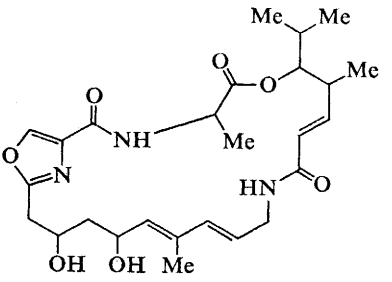
The structure of the highly modified cyclic depsipeptide griseoviridin has been revised to (49) by two independent *X*-ray analyses,[126, 127] as well as by a detailed chemical and spectroscopic investigation.[127] Madumycin (50), recently isolated and characterized, appears to be an additional member of this interesting group.[128]

Biosynthetic studies have not figured very prominently during the year. Etamycin, not unexpectedly, is formed by a non-ribosomal mechanism.[129] The

[122] J. Shoji and T. Kato, *J. Antibiotics*, 1976, **29**, 380.
[123] J. Shoji, R. Konaka, K. Kawano, N. Higuchi, and Y. Kyogoku, *J. Antibiotics*, 1976, **29**, 1246.
[124] H. Otsuka, J. Shoji, K. Kawano, and Y. Kyogoku, *J. Antibiotics*, 1976, **29**, 109.
[125] L. P. G. Wakelin and M. J. Waring, *Biochem. J.*, 1976, **157**, 721.
[126] G. I. Birnbaum and S. R. Hall, *J. Amer. Chem. Soc.*, 1976, **98**, 1926.
[127] B. W. Bycroft and T. J. King, *J.C.S. Perkin I*, 1976, 1996.
[128] M. G. Brazhnikova, M. K. Kabinova, N. P. Pomanova, T. M. Filippova, E. Borovskiji, Y. Zelinskij, and Y. Golik, *Bio-org. Khim.*, 1975, **1**, 1383.
[129] F. Kamal and E. Katz, *J. Antibiotics*, 1976, **29**, 944.



(49)



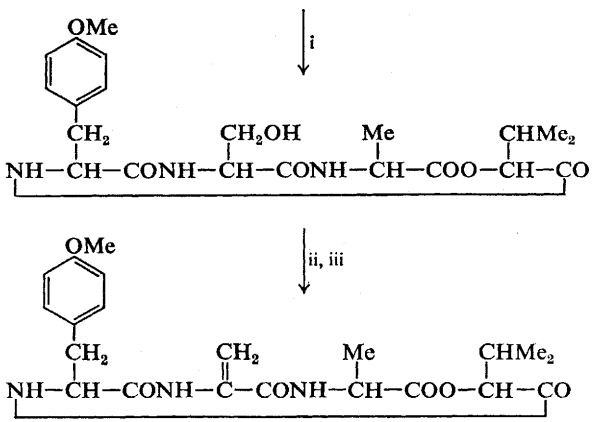
(50)

trans-4-methylproline residue in the cyclohexadepsipeptide monamycin appears to be derived from leucine, possibly by oxidative cyclization. While the *N*-methylation of the leucine residue in the same antibiotic most likely occurs after the incorporation of the amino-acid into a peptide intermediate.[130]

Other Synthetic and Conformational Studies on Cyclic Depsipeptides.—The synthesis of (51), an analogue of a natural toxin, is indicative of the problems encountered in the synthesis of these compounds containing unusual features. Six alternative routes were explored and the one subsequently employed is outlined in Scheme 4.[131]

Surfactin, a haemolytic, anticoagulant cyclodepsipeptide, has proved a different synthetic challenge. A route has been developed and used to synthesize nor-surfactin (53). Cyclization of the linear precursor (52) using DCC–*N*-hydroxy-succinimide gave (53) which displayed the same biological activity as the parent compound.[132]

Boc-Ser-Ala-Hyv-Tyr(Me)-OSu

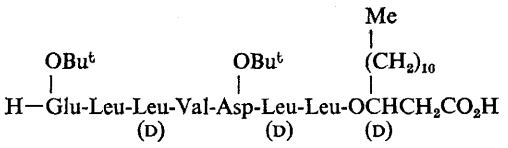


(51)

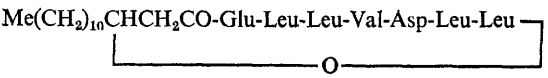
Reagents: i, CF_3CO_2H; ii, Py–TsCl; iii, Et_3NH–DMF

Scheme 4

[130] V. Arroyo, M. J. Hall, C. H. Hassall, and K. Yamasaki, *J.C.S. Chem. Comm.*, 1976, 845.
[131] S. Lee, H. Aoyagi, Y. Shimohigashi, N. Izumiya, T. Ueno, and H. Fukami, *Tetrahedron Letters*, 1976, 843.
[132] J. D. Morrison, T. L. Ciardelli, and J. R. Husman, *Tetrahedron Letters*, 1976, 1773.

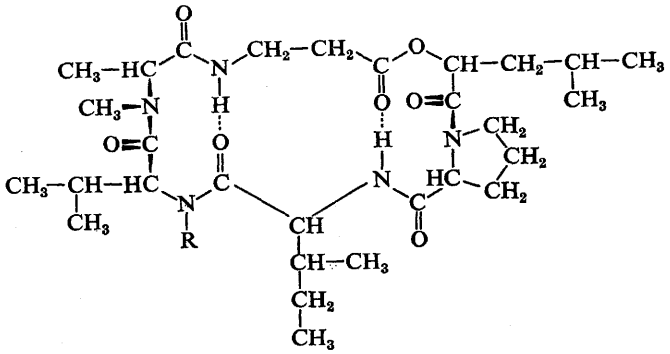


(52)



(53)

A crystal structure on (L-MeVal-L-Hyv-D-MeVal-D-Hyv) has shown that the molecule possesses all *trans* amide and ester bonds with the side-chains in pseudo-equatorial positions.[133] An extensive spectroscopic investigation on the insecticidal destruxins has led to the proposal that (54) is the most probable solution con-



(54)

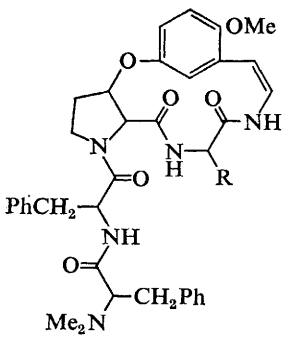
formation.[134] A similar study on the virginiamycins indicates considerable conformational mobility for these molecules.[135]

4 Peptide Alkaloids

The considerable activity in this area over the past five years has abated and recent efforts can be regarded as consolidation. Two authoritative reviews by the German group [136, 137] lend perspective to the field, while the full details of the *X*-ray analysis on tri-*N*-methylfrangulanine methiodide [138] add solidarity. The

[133] G. N. Tischenko, V. I. Smirnova, L. N. Zeibot, N. V. Nazimova, and V. I. Andrianov, *Bio-org. Khim.*, 1976, **2**, 885.
[134] H. Naganawa, T. Takita, A. Suzuki, S. Tamura, S. Lee, and N. Izumiya, *Agric. Biol. Chem. (Japan)*, 1976, **40**, 2223.
[135] M. J. O. Anteunis, R. E. A. Callens, D. K. Tavernier, *European J. Biochem.*, 1975, **58**, 259.
[136] R. Tschesche and E. U. Kaussmann, in 'The Alkaloids', Academic Press, New York, 1975, Vol. 15, p. 165.
[137] R. Tschesche, *Heterocycles*, 1976, **1**, 131.
[138] M. Takai, Y. Ogihara, Y. Iitaka, and S. Shibata, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 2181.

only new development has been the isolation and characterization of two new alkaloids jubanine A (55) and B (56) from the stem bark on *Ziziphus jujuba*.[139]

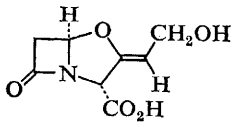


(55) R = CHMeEt
(56) R = CH_2Ph

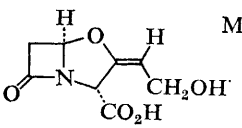
5 Penicillin, Cephalosporins, and Related β-Lactam Antibiotics

Once again the volume of publications in this area has increased substantially and in accord with previous years an exhaustive coverage has not been attempted. For a valuable summary of the present state of this field, readers are referred to the Proceedings of a recent International Symposium on β-lactams.[140] In addition, two useful review articles dealing predominantly with chemical modifications have appeared.[141, 142]

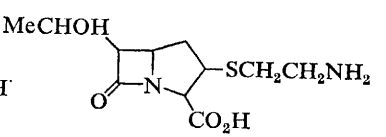
Perhaps the most interesting developments have been the isolation and characterization of new β-lactam antibiotics which are novel both in their chemical structures and in their biological activity. Clavulanic acid (57) is a potent β-lactamase inhibitor, produced by *Streptomyces clavuligerus*, the structure of which has been established by an *X*-ray analysis.[143] The geometrical isomer (58)



(57)



(58)

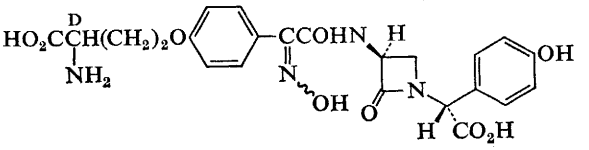


(59)

has also been prepared by a photochemical isomerization of the benzyl ester of (57).[144] Thienomycin (59) is a further example of a novel bicyclic β-lactam

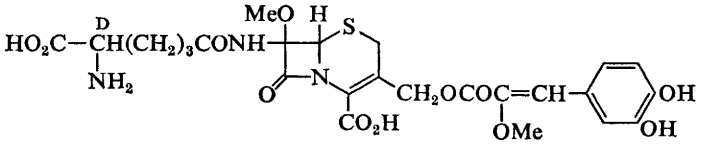
[139] R. Tschesche, I. Khokhar, H. Wilhelm, and G. Eckhardt, *Phytochemistry*, 1976, **15**, 541.
[140] 'Recent Advances in the Chemistry of β-Lactam Antibiotics', ed. J. Elks (Special Publication No. 28), The Chemical Society, London, 1977.
[141] P. G. Sammes, *Chem. Rev.*, 1976, **76**, 113.
[142] P. G. Sammes, 'Penicillins and Cephalosporins', IRS Review of Science, Organic Chemistry, Series Two, Vol. 6, 1976, p. 253.
[143] T. T. Howarth, A. G. Brown, and T. J. King, *J.C.S. Chem. Comm.*, 1976, 266.
[144] A. G. Brown, T. T. Howarth, I. Stirling, and T. J. King, *Tetrahedron Letters*, 1976, 4203.

antibiotic with high biological activity.[145] Nocardicin A (60) and B (61) from a strain of *Nocardia* are unique monocyclic antibiotics.[146, 147] The structures and stereochemistry have been established by a thorough chemical and spectroscopic study.[148] It is noteworthy that the *syn*-isomer, *i.e.* (60), possesses enhanced biological activity over that of the *anti*-isomer.

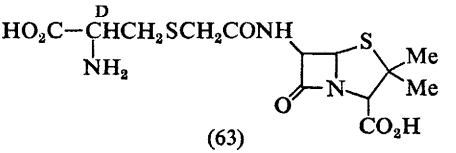


(60) = N—OH *syn* to amide
(61) = N—OH *anti* to amide

An additional member (62) of the now well established cephamycin group has been isolated and the C-3 side-chain identified both by spectroscopic analysis and synthesis.[149] A further example of directed biosynthesis has led to a new penicillin. An auxotroph of *Acremonium chrysogenum* which yields cephalosporin C and penicillin N only on media supplemented with DL-α-aminoadipic acid, when grown in the presence of L-*S*-carboxymethylcysteine, produces the sulphur analogue of penicillin N (63).[150]



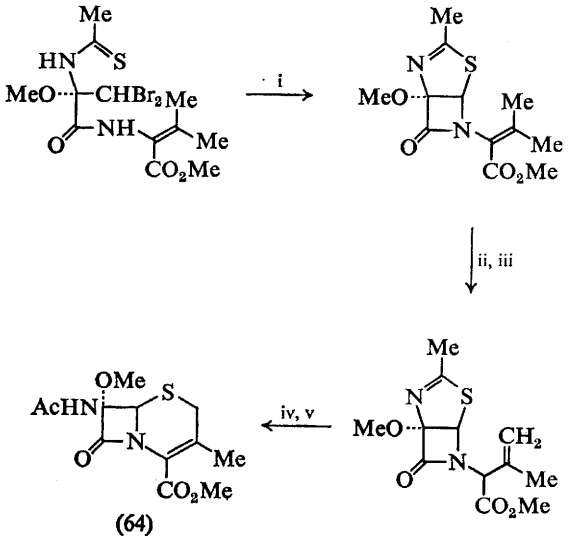
(62)



(63)

A number of important synthetic routes to the penam and cephem ring systems have been designed along claimed biomimetic lines. The first is based on the proposed tripeptide precursor and has been elegantly employed to synthesize a number of methoxy and methyl derivatives of penicillin and cephalosporin.[151, 152] The synthesis of (64), outlined in Scheme 5, serves to illustrate the method.

[145] J. S. Kahan, F. M. Kahan, E. O. Stapley, R. T. Coegelman, and S. Hernandez, U.S.P. 395357.
[146] M. Hashimoto, T. Komori, and T. Kamiya, *J. Antibiotics*, 1976, **29**, 890.
[147] M. Kurita, K. Jomon, T. Komori, N. Miyani, H. Aoki, S. Kuge, T. Kamiya, and H. Imanaka, *J. Antibiotics*, 1976, **29**, 1243.
[148] M. Hashimoto, T. Komori, and T. Kamiya, *J. Amer. Chem. Soc.*, 1976, **98**, 3023.
[149] H. Fukase and H. Iwasaki, *Bull. Chem. Soc. Japan*, 1976, **49**, 767.
[150] H. Troonen, P. Roelants, and B. Boon, *J. Antibiotics*, 1976, **29**, 1258.
[151] S. Nakatsuka, H. Tanino, and Y. Kishi, *J. Amer. Chem. Soc.*, 1975, **79**. 5008.
[152] H. Tanino, S. Nakatsuka, and Y. Kishi, *Tetrahedron Letters*, 1976, 185,

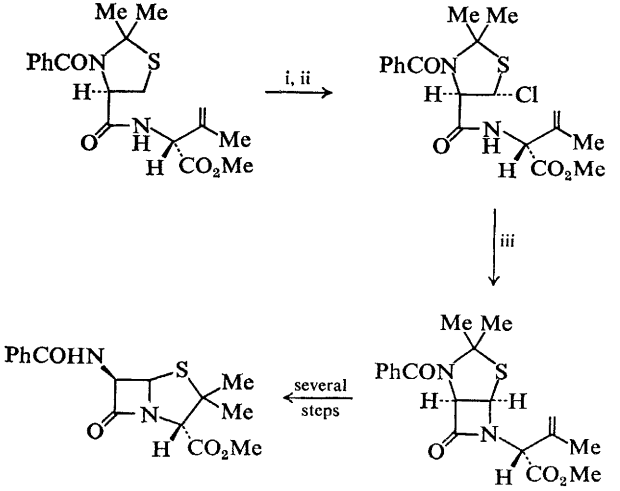


(64)

Reagents: i, NaH; ii, NBS; iii, Zn/HAc; iv, $PhCO_3H/CF_3CO_2H$; v, PCl_3.

Scheme 5

A further independent conversion of a peptide precursor into penicillins uses a thiazolidine unit to protect the cysteine moiety (Scheme 6).[153, 154]



Reagents: i, $(PhCOO)_2$; ii, HCl; iii, NaH

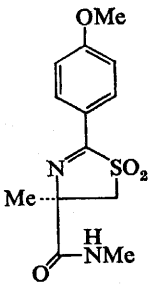
Scheme 6

[153] J. E. Baldwin, A. Au, M. A. Christie, S. B. Haber, and D. Hesson, *J. Amer. Chem. Soc.*, 1975, **97**, 5957.

[154] J. E. Baldwin, M. A. Christie, S. B. Haber, and L. I. Kruse, *J. Amer. Chem. Soc.*, 1976, **98**, 3045.

Both of the methods described above rely on the nucleophilic attack of an amide nitrogen to displace a substituent at the β-position of the cysteine residue for the formation of the β-lactam ring. An alternative approach which may also have biosynthetic implications requires the oxidation of the amide nitrogen followed by the nucleophilic displacement by an anion generated at the β-centre of the cysteine. A model for this proposal has been developed; the sulphone (65) on treating with base affords the β-lactam (66) in reasonable yield.[155]

An interesting potential route based not on biosynthetic considerations but on the well documented cycloaddition reaction has surprisingly proved unsuccessful. The intramolecular cycloaddition reaction for compounds such as (67) failed to give azetidinones.[156]



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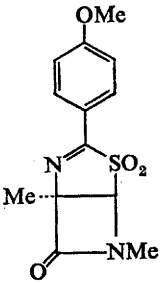

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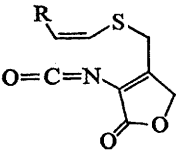
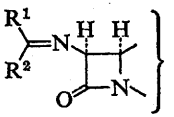


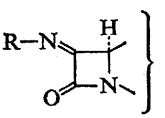
(67)

Semi-synthetic routes to 6- and 7-substituted penicillins and cephalosporins continue to attract attention. The majority of the syntheses are based on the formation of a suitable intermediate such as (68) or (69). The anions generated from (68) with strong base can be quenched with either an alkyl halide or hypohalide. Alternatively, the imines of general structure (69) readily add nucleophiles to give the required products. The latter has proved the most popular and a number of interesting modifications have been reported.[157–160] A completely different route involving 1,3-dipolar reactions has been developed.[161]

Other structural modifications of note carried out on the cephem skeleton include the formation of 2-methyl-3-cephem,[162] episulphide,[163] and carboxylic



(68)

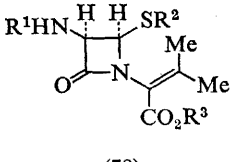


(69)

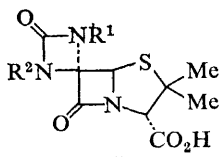
[155] A. I. Scott, S. E. Yoo, S.-K. Chung, and J. A. Lacadie, *Tetrahedron Letters*, 1976, 1137.
[156] T. Petrzilka, C. Fehr, and G. Schmid, *Helv. Chim. Acta*, 1976, **59**, 1988.
[157] H. Yanagisawa, M. Fukushima, A. Ando, and H. Nakao, *Tetrahedron Letters*, 1976, 259.
[158] H. Yanagisawa and H. Nakao, *Tetrahedron Letters*, 1976, 1811 and 1815.
[159] Y. Sugimura, K. Iino, Y. Iwano, T. Saito, and T. Hiraoka, *Tetrahedron Letters*, 1976, 1307.
[160] Y. Sugimura, K. Iino, Y. Iwano, T. Saito, and T. Hiraoka, *J.C.S. Chem. Comm.*, 1976, 516.
[161] K. Hirai, Y. Iwano, T. Saito, T. Hiraoka, and Y. Kishida, *Tetrahedron Letters*, 1976, 1303.
[162] T. Kamiya, T. Teraji, M. Hashimoto, O. Nakaguchi, and T. Oku, *J. Amer. Chem. Soc.*, 1976, **98**, 2343.
[163] J. E. Baldwin and D. P. Hesson, *J.C.S. Chem. Comm.*, 1976, 667.

acid [164] derivatives, as well as functionalization at the C-2 and C-4 positions.[165] The reactions of the carbamoyl group of the cephamycins have also been studied.[166]

A diversity of penicillin derivatives have once again been prepared. 1,2-Secopenicillins (70) provide useful starting materials [167] and have been employed for the synthesis of 5,6-dehydropenicillins.[168] 6-Spiropenicillanates (71) have been synthesized by the action of phosgene on the corresponding



(70)

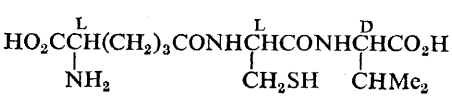


(71)

6-acylamino-substituted penicillin.[169] The importance of the broad-spectrum semi-synthetic antibiotics ampicillin and cephaloglycin has led to the synthesis of the corresponding hydrazine and hydroxylamine derivatives.[170, 171]

Spectroscopic studies have included ^{13}C n.m.r. data on penicillin *S*-oxides [172] and a c.d. investigation on the configuration of benzyl penicilloates.[173]

Endeavour in the area of biosynthesis has centred predominantly on the role of peptide precursors. The tripeptide (72) with the chirality indicated is formed from L-α-aminoadipic acid, L-cysteine, and L-valine in *Penicillum chrysogenum*.[174]



(72)

The stereoisomers of (72) labelled with tritium in the methyl groups or the α-position of the valine have been prepared. The labels were incorporated into a compound that behaved like penicillin N only when the LLD isomer was incubated with a cell-free system derived from *Cephalosporium acremonium*.[175] As expected, the label from isopenicillin N and not penicillin N was incorporated into the solvent-soluble penicillins produced by *P. chrysogenum*.[176] The inversion

[164] A. J. Bose, S. G. Amin, J. C. Kapur, and M. S. Manhas, *J.C.S. Perkin I*, 1976, 2193.
[165] D. H. Bremner and M. M. Campbell, *J.C.S. Chem. Comm.*, 1976, 538.
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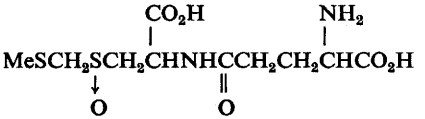
[168] A. Brandt, L. Bassignani, and L. Re, *Tetrahedron Letters*, 1976, 3975.
[169] D. H. Bremner, M. M. Campbell, and G. Johnson, *J.C.S. Chem. Comm.*, 1976, 293.
[170] P. H. Bentley and E. Hunt, *Tetrahedron Letters*, 1976, 3731.
[171] P. H. Bentley and G. Brooks, *Tetrahedron Letters*, 1976, 3735.
[172] C. R. Harrison and P. Hodge, *J.C.S. Perkin I*, 1976, 1772.
[173] R. Busson, P. J. Claes, and H. Vanderhaeghe, *J. Org. Chem.*, 1976, **41**, 2556 & 2561.
[174] J. A. Chan, F.-C. Huang, and C. J. Sih, *Biochemistry*, 1976, **15**, 177.
[175] P. A. Fawcett, J. J. Usher, J. A. Huddleston, R. C. Bleaney, J. J. Nisbet, and E. P. Abraham, *Biochem. J.*, 1976, **157**, 651.
[176] P. A. Fawcett, J. J. Usher, and E. P. Abraham, *Biochem. J.*, 1976, **151**, 741.

of configuration at the α-carbon of the L-valine during the incorporation into penicillin G occurs without loss of the nitrogen atom. This has been confirmed by a novel application of ^{15}N n.m.r. spectroscopy.[177]

6 Linear Peptides Containing Unusual Structural Features

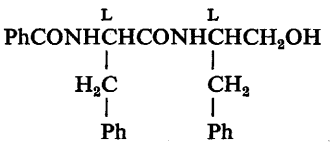
An excellent review on the ubiquitous glutathione and related γ-glutamyl compounds offers a comprehensive coverage of these important peptides.[178]



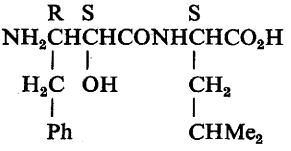
(73)

γ-Glutamylmarasmine (73) is the natural precursor of the garlic-like odour of certain species of Basidiomyceteous mushrooms.[179]

It is interesting to note that the modified dipeptide (74) has been isolated from both an *Aspergillus* species [180] and a marine algae.[181] The structure of the molecule

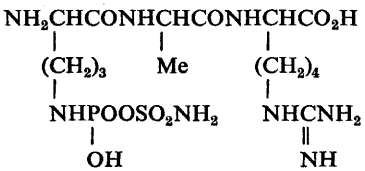


(74)



(75)

and the chirality have been confirmed by synthesis.[180] Bestatin (75), a specific inhibitor of aminopeptidases, contains a novel β-amino-acid, the structure and stereochemistry of which have also been proved by synthesis.[182]

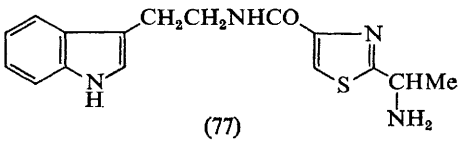


(76)

The toxin supposedly responsible for the symptoms of bean halo-blight has been isolated and tentatively assigned [183] the unusual tripeptide structure (76). The basic compound (77) present in a strain of *Thermoactinomyces* can be regarded as a modified tripeptide derived from tryptophan, cysteine, and

[177] H. Booth, B. W. Bycroft, C. M. Wels, K. Corbett, and A. P. Maloney, *J.C.S. Chem. Comm.*, 1976, 110.
[178] A. Meister and S. S. Tate, *Ann. Rev. Biochem.*, 1976, **45**, 559.
[179] R. Gmelin, H.-H. Luxa, K. Roth, and G. Hofle, *Phytochemistry*, 1976, **15**, 1717.
[180] R. E. Cox, K. K. Chexal, and J. S. E. Holker, *J.C.S. Perkin I*, 1976, 578.
[181] B. C. Maiti and R. H. Thomson, *Experientia*, 1976, **32**, 1106.
[182] H. Suda, T. Takita, T. Aoyagi, and H. Umezawa, *J. Antibiotics*, 1976, **29**, 600.
[183] R. E. Mitchell, *Nature*, 1976, **260**, 75.

alanine.[184] Feldamycin (78) is a novel antibiotic containing two histidine residues linked formally through the carboxy-group and β-position of a threonine.[185]



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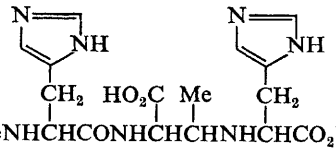


(78)

Two new antibiotics, cerexin A (79)[186] and stenothricin (80),[187] both of which possess fatty acid residues as well as a significant number of D-amino-acid residues, have been characterized by spectral and chemical procedures.

FA-Asn-Val-Val-Asn-Asn-γHyl-*a*Thr-Ser-Trp-*a*Ile
D D D D D D D

(79) FA = β-hydroxylsoundecanoyl; γHyl = L-threo-γ-hydroxylysine

The details of the investigation which led to the revision of the structure of alamethicin have appeared.[188] The related peptide suzukacillin which possesses similar biological properties to those of alamethicin has been assigned the

β-Ketoacyl-CySO$_3$H-Val-Ser-Lys-Sar-*a*Thr-Ser-Dpr
D D D

(80) β-Ketoacyl = several isomers

structure (81), largely on the basis of mass spectral data.[189] All the amino-acids and the phenylalaninol have the L-configuration and tentative proposals concerning the conformation of the molecule have been made. An empirical procedure for

Ac-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-
Pro-Val-Aib-Aib-Glu(Pheol)-Gln-OH

(81) Pheol = NHCHPhCH$_2$OH; Aib = α-aminoisobutyric acid

predicting secondary peptide structure has been applied to alamethicin.[190] Unfortunately the investigation was based on the original incorrect structure.

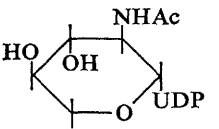
[184] Y. Konda, Y. Suzuki, S. Omura, and M. Onda, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 92.
[185] A. D. Argoudelis, S. A. Mizsak, L. Baczynskyj, and R. J. Wnuk, *J. Antibiotics*, 1976, **29**, 1117.
[186] J. Shoji, T. Kato, and R. Sakazaki, *J. Antibiotics*, 1976, **29**, 1268.
[187] W. A. Konig, C. Engelfried, H. Hagenmaier, and H. Kneifel, *Annalen*, 1976, 2011.
[188] D. R. Martin and R. J. P. Williams, *Biochem. J.*, 1976, **153**, 181.
[189] G. Jung, W. A. Konig, D. Leibfritz, T. Ooka, K. Janko, and G. Boheim, *Biochim. Biophys. Acta*, 1976, **433**, 164.
[190] M. E. Johnson and T. T. Wu, *J. Theoretical Biol.*, 1976, **60**, 183.

The structures of the highly modified peptides thiostrepton and siomycin A have been the subject of an interesting ^{13}C n.m.r. study.[191]

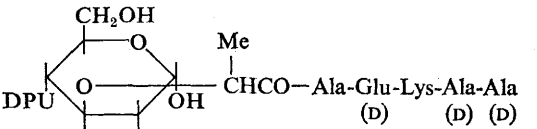
7 Peptides Linked to Carbohydrate

In order to keep this chapter within bounds, only those peptides which contain unusual features and are linked to carbohydrates are covered and consequently this section is essentially limited to cell-wall peptides and glycopeptide antibiotics.

Glycopeptides from Bacterial Cell Walls.—The biosynthesis of peptidoglycan, the major structural polymer of cell wall, requires two nucleotide-activated precursors, UDP-*N*-acetylglucosamine and UDP-MurNAc-pentapeptide. Enzymes in the membrane utilize these precursors in a cycle of reactions with undecaprenylphosphate as carrier. The cross-linking of the glycan product of this process by penicillin-sensitive transpeptidases results in the formation of the glycan network. Enzyme preparations from *Goffkya homari* have been used to investigate the formation of the peptidoglycan from (82) and (83).[192] In addition



(82)



(83)

analogues of (83) with residues 4 and 5 replaced singly by D-aminobutyric, D-norvaline, or D-valine were biosynthesized by cell-free systems from the same organism and used to study the steric effects on glycopeptide synthesis.[193]

The oligomeric intermediate in the biosynthetic pathway of *Bacillus megaterium* has been identified as a [disaccharide(pentapeptide)]$_{12}$ pyrophosphoryl undecaprenol.[194] More details relating to the kinetics of the transpeptidases from streptomyces R39 [195] and a thermosensitive mutant of *E. coli* [196] have been reported.

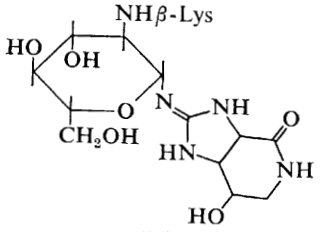
The original conclusion that the peptidoglycan of *Pseudomonas aeruginosa* is very similar to that of *E. coli* has been confirmed.[197] The nature of the linkage of the peptidoglycan to teichoic acid in *Staphylococcus aureus* and other Gram-positive bacteria has been investigated and a general structure proposed.[198]

Several *N*-acetylmuramyl peptides of varying lengths corresponding to the partial structure of the cell wall of *S. aureus* have been chemically synthesized, as part of a programme to investigate the minimal structural requirements for

[191] K. Tori, K. Tokura, K. Okabe, M. Ebata, and H. Otsuka, *Tetrahedron Letters*, 1976, 185.
[192] W. P. Hammes and O. Kandler, *European J. Biochem.*, 1976, **70**, 107.
[193] C. V. Carpenter, S. Goyer, and F. C. Neuhaus, *Biochemistry*, 1976, **15**, 3146.
[194] E. Fuchs-Cleveland and C. Gilvarg, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4200.
[195] N. Fuad, J.-M. Frere, J.-M. Ghuysen, C. Duez, and M. Iwatsubo, *Biochem. J.*, 1976, **155**, 623.
[196] D. Mirelman, Y. Yasheriv-Gan, and U. Schwarz, *Biochemistry*, 1976, **15**, 1781.
[197] A. Yanai, K. Kato, T. Beppu, and K. Arima, *Agric. and Biol. Chem. (Japan)*, 1976, **40**, 1505.
[198] J. Coley, A. R. Archibald, and J. Baddeley, *F.E.B.S. Letters*, 1976, **61**, 240.

immunoadjuvant activity.[199, 200] The monomer and dimer peptide fragments from *Micrococcus lysodeikticus* have been synthesized by homogeneous methods and shown to be identical with the natural peptides.[201]

Glycopeptide Antibiotics.—Raceomycin A (84) is the simplest member of the streptothricin group, all of which possess broad-spectrum antibacterial activity. Initial biosynthetic studies on (84) have revealed that the β-lysine residue is



(84)

derived from lysine. Surprisingly the streptolidine unit does not appear to be derived from arginine.[202] The value of ^{13}C n.m.r. as a tool for investigating complex structures is exemplified with an interesting application to the glycopeptide antibiotic vancomycin.[203]

8 Miscellaneous

Biosynthetic studies relating to the poloxyin nucleoside peptide antibiotics[204] and syntheses of some tripeptides containing the nucleic acid bases in the side-chains[205] have been described. Peptides containing dehydroamino-acid residues continue to attract attention[206–208] and have been used in a claimed biomimetic synthesis of cysteine.[207]

The molecular features common to sweet-tasting aspartyl dipeptide esters and amino-malonic diesters are of interest and several, considerably sweeter than sucrose, have been synthesized.[209, 210] Other topics which may be of specific interest include the synthesis of peptides containing aza amino-acid[211, 212] or N^{ω}-linked arginine[213] residues, the synthesis of model kynurenine peptides,[214] and a new method for linking tryptophan to thiol residues.[215]

[199] S. Kusumoto, Y. Tarumi, K. Ikenata, and T. Shiba, *Bull. Chem. Soc. Japan*, 1976, **49**, 533.
[200] S. Kusumoto, S. Okada, T. Shiba, I. Azuma, and Y. Yamamura, *Tetrahedron Letters*, 1976, 4287.
[201] M. Mulliez, D. Blanot, J. Savrda, and E. Bricas, *Biochimie*, 1976, **58**, 131.
[202] Y. Sawada, T. Kubo, and H. Taniyama, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 2163.
[203] D. H. Williams and J. R. Kalman, *Tetrahedron Letters*, 1976, 4829.
[204] S. Funayama and K. Isono, *Agric. and Biol. Chem. Japan*, 1976, **40**, 1039.
[205] Yu. P. Shvachkin and E. N. Olsufeva, *Zhur. obshchei Khim.*, 1975, **45**, 2351.
[206] E. G. Breitholle and C. H. Stammer, *J. Org. Chem.*, 1976, **41**, 1344.
[207] U. Schmidt and E. Ohler, *Angew. Chem. Internat. Edn.*, 1976, **15**, 42.
[208] H. Poisel and U. Schmidt, *Angew. Chem. Internat. Edn.*, 1976, **15**, 294.
[209] M. Fujino, M. Wakimasu, M. Mano, K. Tanaka, N. Nakajima, and H. Aoki, *Chem. and Pharm. Bull. (Japan)*, 1976, **24**, 2112.
[210] Y. Ariyoshi, *Agric. and Biol. Chem. (Japan)*, 1976, **40**, 983.
[211] A. S. Datta and M. B. Giles, *J.C.S. Perkin I*, 1976, 244.
[212] K. V. Rao and B. Ravindranath, *J. Heterocylic Chem.*, 1975, **12**, 147.
[213] I. Photaki and A. Yiotakis, *J.C.S. Perkin I*, 1976, 259.
[214] D. E. Rivett and F. H. C. Stewart, *Austral. J. Chem.*, 1976, **29**, 2095.
[215] W. E. Savige and A. Fontana, *J.C.S. Chem. Comm.*, 1976, 600.

5

Chemical Structure and Biological Activity of Hormones and Related Compounds

With contributions by
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1 Introduction

A major change in organization of this chapter has become necessary. The subject of chemical structure and biological activity was re-introduced into the series in Volume 5 with an article by Law. Although this dealt largely with peptide hormones, some material pertaining to enzymes was included, as it was in the chapter in the following volume by Camble and Dutta. However, it was already apparent that the combined fields of hormone and enzyme chemistry were too great to be encompassed in single review articles, and subsequent chapters dealt exclusively with enzymes (Fersht, Volume 7), or hormones and related compounds (Schafer and Szelke, Volume 8). This alternation necessitated two-year coverage which, in both fields again provided too much important material for review within the permitted volume length and compressed time-scale necessary for rapid publication. Indeed, it has proved difficult to locate authors willing to cover comprehensively either peptide hormone or enzyme structure–activity relationships, even for one-year periods. Accordingly, this chapter covers only hormones and is sub-divided into several individually authored sections. Inevitably this has led to some disparity of style and selectivity of coverage, and some topics are regrettably omitted. On the other hand, all the sections have been contributed by specialists active in the fields covered. The division of subject matter has been largely on the basis of that in previous chapters. The enkephalins and endorphins, which have contributed the major growth area in the year under review, have merited separate coverage.

2 Hypothalamic Releasing Hormones

Contributed by U. Ludescher, R. O. Studer, and D. Gillessen

The clinical applications of TRH and LH-RH [1, 2] have been reviewed, as has the physiological and clinical significance of somatostatin.[1–3] The distribution

[1] R. Hall and A. Gomez-Pan, in 'Advances in Clinical Chemistry', ed. O. Bodansky and A. L. Latner, Academic Press, New York, 1976, Vol. 18, p. 173.
[2] R. Hall and A. Gomez-Pan, in 'Advanced Medicine', ed. A. F. Lant, Beckman Publishers, London, 1975, p. 243.
[3] R. Guillemin and J. E. Gerich, in 'Annual Review of Medicine', ed. W. P. Creger, Annual Reviews Inc., Palo Alto, 1976, Vol. 27, p. 379.

and effects in the central nervous system,[4] the behavioural influences,[5–7] and the effects on single neurons[4] have been reviewed. Their mechanism of action on the anterior pituitary has been discussed.[8] A short review will be found in ref. 9.

Thyrotropin Releasing Hormone (TRH, Thyroliberin).—The scaling up of the solid-phase synthesis of TRH has been described in two reports.[10, 11] In addition to the previously reported crystalline TRH tartrate,[12] whose physical[13, 14] and biological[15–19] properties have been extensively investigated, two further crystalline salts have been obtained: TRH pyroglutamate[20] and TRH oxalate.[21]

The distribution of TRH in various parts of the brain and in the spinal cord, especially at nerve terminals, has been well established through further investigations using radioimmunoassay[22–25] and immunohistochemistry[24, 26] techniques, and gives evidence for a separate neurophysiological role of TRH in addition to its hypophysiotropic one.

An inhibitory activity of TSH-release has been found in hypothalamic extracts of *Rana temporaria* collected during certain seasons.[27] A seasonal variation of

[4] J. F. Wilber, E. Montoya, N. P. Plotnikoff, W. F. White, R. Gendrich, L. Renaud, and J. B. Martin, in 'Recent Progress in Hormone Research', ed. R. O. Greep, Academic Press, New York, 1976, Vol. 32, p. 117.
[5] A. J. Prange, G. R. Breese, I. C. Wilson, and M. A. Lipton, in 'Anatomical Neuroendocrinology', ed. W. E. Stumpf and L. D. Grant, S. Karger, Basel, 1975, p. 357.
[6] N. P. Plotnikoff, *Biochem. Pharm.*, 1976, **25**, 363.
[7] N. P. Plotnikoff and A. J. Kastin, in 'Peptides', ed. R. Walter and J. Meienhofer, Ann Arbor Science Publishers, Ann Arbor, 1975, p. 645.
[8] F. Labrie, A. De Léan, P. Borgeat, N. Barden, G. Poirier, and J. Drouin, in 'Anatomical Neuroendocrinology', ed. W. E. Stumpf and L. D. Grant, S. Karger, Basel, 1975, p. 62.
[9] W. F. White, in 'Peptides', ed. R. Walter and J. Meienhofer, Ann Arbor Science Publishers, Ann Arbor, 1975, p. 841.
[10] J. K. Chang, R. C. Myers, D. Winkler, and R. C. Orlowski, in 'Peptides', ed. R. Walter and J. Meienhofer, Ann Arbor Science Publishers, Ann Arbor, 1975, p. 469.
[11] A. Loffet, *Ann. Endocrinol.*, 1976, **37**, 213.
[12] C. Hatanaka, M. Obayashi, O. Nishimura, N. Toukai, and M. Fujino, *Biochem. Biophys. Res. Comm.*, 1974, **60**, 1345.
[13] C. Hatanaka, H. Nakamachi, M. Kaneko, H. Yonezawa, N. Toukai, and M. Fujino, *Takeda Kenkyusho Ho*, 1976, **35**, 16 (*Chem. Abs.*, 1976, **85**, 193 116).
[14] N. Nakajima, K. Yoshida, and Y. Asahi, *Takeda Kenkyusho Ho*, 1976, **35**, 23 (*Chem. Abs.*, 1976, **85**, 112 708).
[15] Y. Saji, N. Matsumoto, M. Ando, and N. Fukuda, *Yakuri To Chiryo*, 1976, **4**, 515 (*Chem. Abs.*, 1976, **85**, 104 443).
[16] R. Shikata, T. Masaki, S. Akashi, and T. Nakayama, *Yakuri To Chiryo*, 1976, **4**, 1076 (*Chem. Abs.*, 1976, **85**, 87 829).
[17] T. Nakamura, S. Oda, H. Yamada, Y. Murata, T. Ando, A. Kobayashi, Y. Kimura, and M. Namura, *Yakuri To Chiryo*, 1976, **4**, 1085 (*Chem. Abs.*, 1976, **85**, 104 431).
[18] S. Wanibe, K. Kataoka, Y. Hirooka, K. Yamauchi, Y. Imai, T. Mitsuma, A. Nagasaka, and N. Nihei, *Nippon Naibumpi Gakkai Zasshi*, 1976, **52**, 701 (*Chem. Abs.*, 1976, **85**, 154 406).
[19] S. Kondo, S. Yamasaki, N. Okita, and M. Kurata, *Kurume Igakkai Zasshi*, 1976, **39**, 373 (*Chem. Abs.*, 1976, **85**, 154 412).
[20] M. Fujino and C. Hatanaka, Japan Kokai, 76 52 170 (*Chem. Abs.*, 1976, **85**, 193 122).
[21] M. Fujino and C. Hatanaka, Japan Kokai, 76 32 717 (*Chem. Abs.*, 1977, **86**, 55 700).
[22] A. Winokur and R. D. Utiger, in 'Anatomical Neuroendocrinology', ed. W. E. Stumpf and L. D. Grant, S. Karger, Basel, 1975, p. 328.
[23] E. Okon and Y. Koch, *Nature*, 1976, **263**, 345.
[24] S. L. Jeffcoate, N. White, T. Hokfelt, K. Fuxe, and O. Johansson, *J. Endocrinol.*, 1976, **69**, 9P.
[25] J. S. Kizer, M. Palkovits, and M. J. Brownstein, *Endocrinology*, 1976, **98**, 311.
[26] T. Hokfelt, K. Fuxe, O. Johansson, S. Jeffcoate, and N. White, *European J. Pharmacol.*, 1975, **34**, 389.
[27] E. R. Kuhn and H. Engelen, *Gen. Comp. Endocrinol.*, 1976, **28**, 277.

the content of TRH in the hypothalamus and the pineal gland of frogs has been observed.[28]

Biosynthesis of TRH is still a controversial subject. Some authors[29] were unable to detect any *de novo* synthesized TRH in homogenates of freeze-dried hypothalamic tissue, whereas others[30] found that *in vitro* biosynthesis in newt brain homogenates occurs as long as intact RNA is present. Biosynthesis and degradation are dealt with in a recent review.[31]

The rapid inactivation of TRH by plasma and tissue homogenates has been studied further. The enzymatic degrading activity in plasma is increased by administration of thyroxine and tri-iodothyronine, and it has been speculated that this effect plays an important role in the regulation of the hypothalamo-hypophyseal-thyroid axis.[32, 33] This enzymatic activity is lower in portal than in peripheral blood,[34] and is affected by freezing and thawing of the blood samples.[35] Pyroglutamyl-histidine and proline[29, 36] seem to be the main degradation products, and histidine appears at a later stage.[37] Enzymatic activity for the degradation of TRH was also found in tissue homogenates from hypothalamus, as well as from other parts of the brain.[38–40] Different enzyme activities have been observed depending on the species, the pretreatment of the tissue material, and the fractionation procedure of the homogenates, yielding either predominantly deamidated TRH;[29, 40–42] or deamidated TRH, prolinamide, and proline;[29] or histidylprolinamide.[41] The influence of various compounds to inhibit these activities was also studied throughout these investigations.

When TRH is used to stimulate prolactin secretion *in vitro* from GH-3 cells no structural modification has been observed after release from the receptor.[43] The receptor mechanism and the involvement of cAMP in the releasing process of TSH from the anterior pituitary were studied,[8, 44, 45] but not all groups found an increase of cAMP concentration after stimulation with TRH.

Various reviews report on the clinical use[1, 2, 46] of TRH and its effects on the central nervous system.[4, 7]

[28] I. M. D. Jackson, R. Saperstein, and S. Reichlin, *Endocrinology*, 1977, **100**, 97.
[29] K. Bauer and F. Lipmann, *Endocrinology*, 1976, **99**, 230.
[30] Y. Grimm-Joergensen and J. F. McKelvy, *Brain Res. Bull.*, 1976, **1**, 171.
[31] S. Reichlin, in 'Subcellular Mechanisms in Reproductive Neuroendocrinology', ed. F. Naftolin, K. J. Ryan, and I. J. Davies, Elsevier, Amsterdam, 1976, p. 109.
[32] A. Dupont, F. Labrie, L. Levasseur, J. H. Dussault, and A. V. Schally, *Clin. Endocrinol.*, 1976, **5**, 323.
[33] K. Bauer, *Nature*, 1976, **259**, 591.
[34] K. M. Knigge, D. Schock, and M. Ching, *Acta Endrocrinol.*, 1976, **83**, 449.
[35] R. L. Eskay, C. Oliver, J. Warberg, and J. C. Porter, *Endocrinology*, 1976, **98**, 269.
[36] K. M. Knigge and D. Schock, *Neuroendocrinology*, 1975, **19**, 277.
[37] M. Benuck and N. Marks, *Life Sci.*, 1976, **19**, 1271.
[38] E. C. Griffiths, K. C. Hooper, D. Hutson, S. L. Jeffcoate, and N. White, *Mol. Cell. Endocrinol.*, 1976, **4**, 215.
[39] E. C. Griffiths, K. C. Hooper, S. L. Jeffcoate, and N. White, *Brain Res.* 1976, **105**, 376.
[40] W. L. Taylor and J. F. Dixon, *Biochim. Biophys. Acta*, 1976, **444**, 428.
[41] C. Prasad and A. Peterkofsky, *J. Biol. Chem.*, 1976, **251**, 3229.
[42] J. F. McKelvy, P. Leblanc, C. Laudes, S. Perrie, Y. Grimm-Jorgensen, and C. Kordon, *Biochim. Biophys. Res. Comm.*, 1976, **73**, 507.
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[44] C. Y. Bowers, B. Wu, and K. Folkers, in 'Anatomical Neuroendocrinology', ed. W. E. Stumpf and L. D. Grant, S. Karger, Basel, 1975, p. 333.
[45] D. K. Sundberg, C. P. Fawcett, and S. M. McCann, *Proc. Soc. Exp. Biol. Med.*, 1976, **151**, 149.
[46] C. T. Swain and J. M. Hershman, *Pharmacol. Therap. Part C*, 1976, **1**, 351.

The TRH analogues reported during the period of the review are listed in Table 1. Unfortunately no biological data are available for most compounds. The biological activity of analogues (2) and (3) is surprisingly high considering that glutamyl-histidyl-prolinamide shows only 0.04%.[60] Compound (6) is resistant to plasma degradation[48] like the unmethylated analogue but shows a four-fold increase in potency as might be expected from the N^{τ}-methylation of

Table 1 *Analogues of TRH* (Glp-His-Pro-NH_2)

Compound number	*Structure* [b]	*Biological activity* [a] *TSH*	*CNS*	*Test*	*Ref.*
(1)	Glu-Ala-Pro-NH_2	++		A	47
(2)	Glu-Phe-Pro-NH_2	+++		A	47
(3)	Glu-Phe-Pro-OMe	+++		A	47
(4)	Glu-Gly-Pro-NH_2	−		A	47
(5)	Glu-Gly-Ser-NH_2	−		A	47
(6)	HCO-Pro-His(N^{τ}Me)-Pro-NH_2	+++		A	48
(7)	oro-His-Pro-NH_2			B	49
(8)	kic-His-Pro-NH_2			B	49
		+++	++++	A, C	50
		+++	+++	A, D	51
(9)	ktmc-His-Pro-NH_2			B	49
(10)	Glp-His(di-CH_2CO_2H)-Pro-NH_2	−	−	A, D	51
(11)	Glp-Ser-Pro-NH_2			E	52
(12)	Glp-Ser(Bzl)-Pro-NH_2			E	52
(13)	Glp-His(N^{τ}Me)-Pro(3-t-Me)-NH_2			F	53
(14)	Glp-His-Pro(3-t-Me)-NH_2			F	53
(15)	Glp-His-DL-Pro(3-t-Et)-NH_2			F	53
(16)	Glp-His-DL-Pro(3,3-di-Me)-NH_2			F	53
(17)	Glp-His-Pro(3-t-Me)-NH$(CH_2)_5$Me			F	53
(18)	Glp-His-Pro(3-t-Me)-NH-cyclohexyl			F	53
(19)	Glp-His-Hypro-NH_2				54
(20)	Glp-His-pc-NH_2				54
		++++	++++	A, C	50
		++++	+++	A, D	51
(21)	Glp-His-tc-NH_2	+++		A	55
(22)	Glp-His-Pro-NHPr[i]	−		A	56
(23)	Glp-His-Pro-NHBu[n]				57
(24)	Glp-His-Pro-NHBu[t]	−		A	56, 57
(25)	Glp-His-Pro-NH$(CH_2)_4$Me				57, 58
(26)	Glp-His-Pro-NH$(CH_2)_6$Me	−		A	56

[47] L. G. Morozova, A. P. Smirnova, Y. P. Shvachkin, and V. P. Fedotov, *Byull. Eksp. Biol. Med.*, 1976, **81**, 745 (*Chem. Abs.*, 1976, **85**, 104 292).

[48] J. E. Rivier, M. Brown, C. Rivier, N. C. Ling, and W. W. Vale, in 'Peptides', ed. A. Loffet, Editions de l'Université de Bruxelles, Bruxelles, 1976, p. 427.

[49] E. Schwertner and S. Herrling, *Ger. Offen.* 2 449 167.

[50] D. F. Veber, F. W. Holly, R. F. Nutt, and S. L. Varga, *Ger. Offen.* 2 514 381.

[51] D. F. Veber, F. W. Holly, S. L. Varga, R. Hirschmann, R. F. Nutt, V. J. Lotti, and C. C. Porter, in 'Peptides', ed. A. Loffet, Editions de l'Université de Bruxelles, Bruxelles, 1976, p. 453.

[52] W. Voelter, K. Zech, and H. G. Menge, *Ger. Offen.* 2 639 393.

[53] B. A. Morgan and D. J. Schafer, *Ger. Offen.* 2 609 154.

[54] K. Bankowski and S. Drabarek, *Acta Pol. Pharm.*, 1976, **33**, 81 (*Chem. Abs.*, 1977, **86**, 55 688).

[55] C. G. Willson, C. Gilon, B. Donzel, and M. Goodman, *Biopolymers*, 1976, **15**, 2317.

[56] W. Klingler, H. Kalbacher, P. Göbel, F. J. Seif, K. Zech, and W. Voelter, *Z. physiol. Chem.*, 1976, **357**, 269.

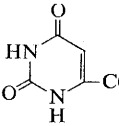
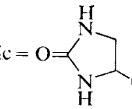
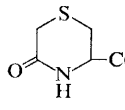
[57] W. Voelter, H. Kalbacher, and K. P. Zeller, *Chem.-Ztg.*, 1976, **100**, 238.

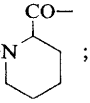
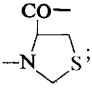
[58] Belg. P. 819 198.

Table 1 (*cont.*)

Compound number	*Structure* [b]	*Biological activity* [a] *TSH*	*CNS*	*Test*	*Ref.*
(27)	Glp-His-Pro-NH$(CH_2)_4$-NH_2				59
(28)	Glp-His-Pro-NH$(CH_2)_6$-NH_2	+++		A	48
(29)	Glp-His-Pro-NH$(CH_2)_7$-NH_2				59
(30)	Glp-His-Pro-NH$(CH_2)_{10}$-NH_2				59

[a] Activity range: —, below the test limit or < 0.1%; +, 0.1—0.99%; ++, 1—9.99%; +++, 10.0—99.9%; ++++, 100—999%; A = TSH release *in vivo* (TRH = 100%); B = TSH release (test unspecified); C = restoration of the anticonvulsant activity of methazolamide in picolinic acid treated animals (TRH = 100%); D = restoration of the anticonvulsant activity of methazolamide in reserpine-treated animals (TRH = 100%); E = antidepressant (test unspecified); F = antagonism of reserpine induced hypothermia.

[b] oro =  ; kic =  ; ktmc =  ;

Pro(t-3-Me) = *trans*-3-methyl-L-proline; DL-Pro(t-3-Et) = *trans*-3-ethyl-DL-proline; DL-Pro(3,3-di-Me) = 3,3-dimethyl-DL-proline; pc =  ; tc =  ; Hypro = hydroxyproline.

the imidazole moiety. Analogues (7)—(9) are claimed to have a potency and activity profile similar to TRH but to possess, in addition, prolonged action. Compounds (11)—(18) have been reported to act on the central nervous system. From a series of analogues, including also compounds (22)—(26), it was concluded that the biological activity increases with the elongation of the straight alkyl chain from the methyl up to the pentyl residue but that it is drastically reduced or abolished if branched alkyl residues are introduced.[56] Analogue (28) was prepared for coupling to proteins or polymers to raise and purify antibodies.[48]

Special efforts have been made to prepare a sample of [D-His^2]-TRH free of its L-isomer. The resulting product had an activity of less than 0.1%,[48] instead of the 80—100% reported previously.[61–63]

[Im-5-iodo-His^2]-TRH and [Im-2,5-di-iodo-His^2]-TRH previously used as tracers in radioimmunoassays for TRH, have been physicochemically extensively characterized and found to be biologically inactive.[64]

When a number of TRH-analogues were tested for their effects on prolactin release and synthesis in a GH cell system it was observed that some analogues, *e.g.* [cSer^1]-TRH, [cThr^1]-TRH, [Pro^1]-TRH, [D-His^2]-TRH, [His(N^τ-Me)^2]-TRH, and analogue (28), stimulated synthesis much more strongly than release

[59] S. Fuchs and W. Voelter, *Z. Naturforsch.*, 1976, **31b**, 1410.
[60] R. Guillemin, R. Burgus, and W. Vale, in 'Vitamins and Hormones', ed. R. S. Harris, P. L. Munson, E. Diczfalusy, and J. Glover, Academic Press, New York, 1971, Vol. 29, p.1.
[61] S. Fuchs, P. Göbel, H. Horn, W. Klingler, G. Kölle, F. Seif, W. Voelter, and K. Zech, *Acta Endocrinol.*, 1975, **78** (Suppl. 193), 2.
[62] W. Voelter, S. Fuchs, and K. Zech, *Tetrahedron Letters*, 1974, 3975.
[63] K. von Puttkamer, W. Klemm, D. Gupta, K. Zech, S. Fuchs, and W. Voelter, *J. Endocrinol.*, 1975, **65**, 16P.
[64] N. Ling, J. Leppäluoto, and W. Vale, *Anal. Biochem.*, 1976, **76**, 125.

as compared with TRH itself. From these results it was concluded that release and synthesis are mediated through different pathways.[65]

N.m.r. studies on [His(N^{τ}-Me)^2]-TRH revealed for this analogue a conformation in solution very similar to TRH. The enhanced potency of this compound can therefore not be explained on the basis of conformational changes.[66] Similar studies on the conformation of [β-thienyl(2)ala^2]-TRH showed that the thienyl side-chain is preferentially in a '*trans*' position to the prolinamide residue, whereas the imidazolyl side-chain in TRH occupies the 'gauche' conformation. Since this analogue possesses still considerable activity (30% *in vivo*, 75% *in vitro*) it has to be concluded that the conformation of the side-chain of the second residue plays no important role in the first step of receptor recognition.[67]

Low-energy structures have been calculated for TRH[68] and found to be in good agreement with experimental data previously obtained by n.m.r. studies. In addition, a calculation of the TRH conformation by the CNDO/2 MO method was started using suitable model fragments.[69]

Luteinizing Hormone Releasing Hormone (LH-RH, Gonadoliberin, Luliberin).—Several specialist LH-RH reviews have appeared,[48, 70–73] and LH-RH is also dealt with in some general reviews on hypothalamic hormones mentioned earlier.[1, 2, 7, 9] The variety of synthetic approaches to LH-RH has been enlarged by the use of the REMA method.[74] The number of new LH-RH analogues reported is decreasing but still considerable (Table 2). Several authors have investigated the structure–activity relationship of LH-RH analogues modified

Table 2 *Analogues of LH-RH*

Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

Compound number	*Structure* [a]	*Biological activity* [b] *in vitro*	*in vivo*	*Test* [d]	*Ref.*
(31)	[pAad^1]		+++	H	75
(32)	[Leu^1]	–		S	76
(33)	[Pro^1, D-Ala^6]	–		S	76

[65] P. S. Dannies and A. H. Tasjian, jun., *Nature*, 1976, **261**, 707.
[66] A. M. Bellocq and M. Dubien, *Biochim. Biophys. Acta*, 1976, **420**, 1.
[67] A. M. Bellocq, S. Castensson, and H. Sievertsson, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 577.
[68] A. W. Burgess, F. A. Momany, and H. A. Scheraga, *Biopolymers*, 1975, **14**, 2645.
[69] A. S. Abdulnur, R. L. Flurry, jun., and J. M. Bopp, jun., *J. Theor. Biol.*, 1976, **59**, 189.
[70] D. H. Coy, A. V. Schally, J. A. Vilchez-Martinez, E. J. Coy, and A. Arimura, in 'Hypothalamic Hormones', ed. M. Motta, P. G. Crosignani, and L. Martini, Academic Press, New York, 1975, p. 1.
[71] D. Gillessen, H. Künzi, A. Trzeciak, G. Roncari, and R. O. Studer, in 'Hypothalamic Hormones', ed. D. Gupta and W. Voelter, Verlag Chemie, Weinheim, 1975, p. 107.
[72] A. V. Schally, A. J. Kastin, and D. H. Coy, *Internat. J. Fertil.*, 1976, **21**, 1.
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[74] H. C. Beyerman, T. Rammeloo, J. F. M. Renirie, J. L. M. Syrier, and A. van Zon, *Rec. Trav. chim. P*, 1976, **95**, 143.
[75] K. U. Prasad, R. W. Roeske, and F. L. Weitl, *J. Medicin. Chem.*, 1976, **19**, 492.
[76] F. Labrie, M. Savary, D. H. Coy, E. J. Coy, and A. V. Schally, *Endocrinology*, 1976, **98**, 289.

Table 2 (*cont.*)

Compound number	*Structure* [a]	*Biological activity* [b] *in vitro*	*in vivo*	*Test* [d]	*Ref.*
(34)	[D-Glp[1], des-His[2]]-(1—9)NHEt	—		S	76
(35)	[D-Glp[1], des-His[2], D-Trp[3], D-Ala[6]]-(1—9)NHEt	*		S	77
(36)	[β-Ala[2]]	+++		A	78
(37)	[D-Glu[2]]	+++		A	78
(38)	[Lys[2]]	*c*		A	78
(39)	[D-Lys[2]]	*c*		A	78
(40)	[D-Met[2]]	*c*		A	78
(41)	[4-Nitro-Phe[2]]	—		A	77
		*		S	77
(42)	[D-Pro[2]]	*c*		A	78
(43)	[D-Tyr[2]]	*c*		A	78
(44)	[D-Phe[2], 2-Me-Ala[6]]		*	P, R	79
(45)	[D-Phe[2,6], Leu[3]]		**	P	80
			*	U	80
(46)	[D-Phe[2], Leu[3], D-Trp[6]]		*	P	80
			**	U	80, 81
(47)	[D-Phe[2], Pro[3], D-Trp[6]]		***	P	80
			***	U	80, 81
(48)	[D-Phe[2,6], Phe[7]]	—		A	77
		**		S	77
(49)	[Val[2], Leu[3], D-Leu[6]]	—		A	77
		*		S	77
			**	T	82
(50)	[Leu[2,3], D-Ala[6]]-(1—9)NHEt	*		S	76
(51)	des-His[2]-[D-Lys(lauryl)[6]]	***		S	83
(52)	des-His[2][D-Orn(lauryl)[6]]	***		S	83
(53)	des-His[2]-[D-Lys(lauryl)[6]]-(1—9)NHEt	***		S	83
(54)	des-His[2]-[D-Orn[6]]-(1—9)NHEt	**		S	83
(55)	des-His[2]-[D-Orn(lauryl)[6]]-(1—9)NHEt	***		S	83
(56)	des-His[2]-(1—9)-$NHCH_2CF_3$		*	T	82
(57)	des-His[2]-[D-Orn[6]]	***		S	83
(58)	des-His[2]-[D-Lys[6]]-(1—9)NHEt			S	83
(59)	[Nap(2)Ala[2]]		+++	H	75
			+++	K	75
(60)	[Leu[3]]	—		S	76
(61)	[γ-Abu[6]]	++		A	78

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Table 2 (*cont.*)

Compound number	*Structure* [a]	*Biological activity* [b] *in vitro*	*in vivo*	*Test* [d]	*Ref.*
(62)	[D-MeAla^6]		−	H	84
(63)	[D-Glu^6]	++++		A	78
			++++	K	84
(64)	[Lys^6]	++		A	78
(65)	[D-Lys(Ac)^6]	++++		A	78
(66)	[D-Lys(lauryl)^6]	++++		A	78
(67)	[D-Lys(Me)$_2^6$]	++++		A	78
(68)	[D-Lys(lauryl)^6]-(1—9)NHEt	+++++		A	83
(69)	[D-Orn^6]	++++		A	78
(70)	[D-Orn(Ac)^6]	++++		A	78
(71)	[D-Orn(Bzl)^6]	+++		A	78
(72)	[D-Orn(lauryl)^6]	++++		A	78
(73)	[D-Orn^6]-(1—9)NHEt	++++		A	83
(74)	[D-Orn(lauryl)^6]-(1—9)NHEt	++++		A	83
(75)	[4-Amino-D-Phe^6]	++++			85
(76)	[D-Ser(Bu^t)^7]-(1—9)NHEt		+++++	J	86, 87
(77)	*Cyclo*-[Leu^6]	+++		A	78
(78)	[D-Lys(Boc)^6]-(1—9)NHEt		+++++	G	87
(79)	[D-Glu(OBu^t)^6]-(1—9)NHEt		+++++	G	87
(80)	[D-Glu^6]-(1—9)NHEt		+++++	G	87
(81)	[D-Asp(OBu^t)^6]-(1—9)NHEt		+++++	G	87
(82)	[D-Cys(Bu^t)^6]-(1—9)NHEt		+++++	G	87
(83)	[D-Ser(Bu^t)^6]-(1—9)NHEt		++++++	G	87
(84)	[D-Ser^6]-(1—9)NHEt		+++++	G	87
(85)	[D-Thr(Bu^t)^6]-(1—9)NHEt		+++++	G	87
(86)	[D-t-Leu^6]-(1—9)NHEt		++++	G	87
(87)	[D-Pen(Bu^t)^6]-(1—9)NHEt		++++	G	87
(88)	[D-Pen(Me)^6]-(1—9)NHEt		++++	G	87
(89)	[D-Leu^6, Ser(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(90)	[D-Leu^6, Cys(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(91)	[D-Ala^6, Cys(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(92)	[D-Ser(Bu^t)^6, Cys(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(93)	[D-Ser(Bu^t)^6, Ser(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(94)	[D-Cys(Bu^t)^6, Ala^7]-(1—9)NHEt		+++	G	87
(95)	[Asp(OBu^t)^7]-(1—9)NHEt		++++	G	87
(96)	[Cys(Bu^t)^7]-(1—9)NHEt		+++++	G	87
(97)	[Cys(SBu^t)^7]-(1—9)NHEt		+++	G	87
(98)	[Glu(OBu^t)^7]-(1—9)NHEt		++++	G	87
(99)	[Arg^7]		++	G	87
(100)	[Lys^7]		++	G	87
(101)	[Lys(Boc)^7]		+++	G	87
(102)	[Lys(Boc)^7]-(1—9)NHEt		++++	G	87
(103)	[Orn(Boc)^7]-(1—9)NHEt		++++	G	87
(104)	[Ser(Bu^t)^7]-(1—9)NHEt		++++	G	87
(105)	[Thr(Bu^t)^7]-(1—9)NHEt		++	G	87

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Table 2 (*cont.*)

Compound number	*Structure* [a]	*Biological activity* [b] *in vitro*	*in vivo*	*Test* [d]	*Ref.*
(106)	[Arg(NO_2)^8]	+			88
(107)	[Orn(Pr^i)^8]		++	H	75

[a] pAad = L-pyro-(2-aminoadipic acid); Nap(2)ala = 3-(naphthyl-2)alanine; Pen = penicillamine = β,β-Dimethylcysteine. [b] LH release only: The symbols for potency are used as in Table 1; *, **, and *** indicate inhibition (of increasing potency). [c] < 5%. [d] The following assay systems are referred to (same symbols as in Volume 8): *in vitro*: A, radioimmunoassayable LH release from rat pituitary cell culture. *in vivo*: G, induction of ovulation in dioestrous rats; H, radioimmunoassayable LH release in ovariectomized, oestrogen/progesterone pretreated rats; J, radioimmunoassayable LH release in man; K, LH release in immature rats. Inhibitory: P, ovulation inhibition in the proestrous rat; R, antagonism of LH release by LH-RH in proestrous rats; S, antagonism of LH release by LH-RH in cell culture; T, antagonism of LH release by LH-RH in immature rats; U, antagonism of LH release in isolated rat hemipituitaries.

in positions 1, 2, 3, 6, 7, and 8.[75–80, 82–84, 87, 88] In principle, the results confirm the earlier observations that replacement of the *C*-terminal glycinamide by ethylamido or related groups and the introduction of D-amino-acids in position 6 lead to increased activity, whereas elimination or modification of histidine in position 2 leads to inactive respiratory inhibitory compounds. A combination of these features leads very often but not always to a potentiated agonistic or antagonistic activity. Replacement of glycine at position 6 by D-Lys and D-Orn (69) results in increased activity. Subsequent introduction of an acetyl (65) or (70) or lauryl residue (66) or (72) into the ε- or δ-amino-group leads to a slight reduction of the activity, which can again be potentiated by the additional replacement of glycinamide by the ethylamido-group (68) or (74).[78, 83] [4-Amino-D-Phe^6]-LH-RH (75), which has about half the activity of the corresponding [D-Phe^6]-LH-RH, lends itself to affinity labelling or attachment to insoluble carriers for possible purification of receptors.[85] By introduction of another positive charge in position 7 the intrinsic activity is diminished to a great extent. [Arg^7]-LH-RH (99) and [Lys^7]-LH-RH (100) have *ca.* 1% of the biological activity of LH-RH,[87] whereas the intermediate product [Lys(Boc)^7]-LH-RH (101) still has a considerable activity of 32%.[87]

Several analogues with protected amino-acids in position 6 or 7 show extraordinarily high activities: [D-Ser(Bu^t)^6]-LH-RH(1—9)NHEt [76] has almost twice the activity of [D-Leu^6]-LH-RH(1—9)NHEt, which is already one of the most active analogues, and [Cys(Bu^t)^7]-LH-RH(1—9)NHEt [96] shows the six-fold activity of LH-RH(1—9)NHEt.[87] [D-Leu^6]-LH-RH(1—9)NHEt, administered by intranasal spray to 18 healthy men, led to a significant release of LH which was sustained for as long as 12 h.[89] After subcutaneous injection of [D-Ser(Bu^t)^6]-LH-RH(1—9)NHEt (76) in 7 healthy men a marked increase of the LH content in serum occurred which still persisted 6 h later.[86] The analogue [D-Phe^{2,6}]-LH-RH was able to inhibit the response to exogenous LH-RH for up to 6 h after its injection.[82]

Long-term administration of [D-Leu^6]-LH-RH(1—9)NHEt caused regression of neoplastic tissue in a rat bearing a spontaneous mammary adenocarcinoma

[88] M. Shinitzky, E. Hazum, and M. Fridkin, *Biochim. Biophys. Acta*, 1976, **453**, 553.
[89] D. Gonzalez-Barcena, A. J. Kastin, D. S. Schalch, D. H. Coy, and A. V. Schally, *Fertility and Sterility*, 1976, **27**, 1246.

and in rats in which tumours had been induced by treatment with dimethylbenzanthracene. The observations made suggested that the tumour was endocrine dependent and that [D-Leu^6]-LH-RH(1—9)NHEt acted through a suppression of ovarian steroid function because the same regression resulted after bilateral ovariectomy.[90]

Complete inhibition of uterine implantation sites in rats occurred when a total dose of 7 mg LH-RH per rat was subcutaneously administered at a daily dose of 1 mg over days 1—7 (of pregnancy). LH-RH was also found to be effective as an 'interceptive', since pregnancy was terminated in all rats when it was administered from days 7—12 of pregnancy at a daily dose of 1 mg per rat, s.c. or p.o. Similar results were obtained in rabbits. Also, LH-RH was found to prevent effectively conception when administered before ovulation.[91] Furthermore, it has been shown that LH-RH has a post-coital contraceptive effect.[92]

The search continues for more potent inhibitors having high affinity to LH-RH receptors but no intrinsic activity and being resistant to degradation. In principle, two pathways were followed stemming from the earlier observation that either omission of histidine in the 2 position or its replacement by D-phenylalanine results in antagonistic compounds. The general potentiating effect of the replacement of glycine in the 6 position by a D-amino-acid has been observed also for [D-Lys^6],[93] [N^ε-lauryl-D-Lys^6] (51), [D-Orn^6] (57), and the [N^δ-lauryl-D-Orn^6] (52) analogues in the des-His^2-series.[83] Contrary to the enhancement of the activity in the agonist series the replacement of the *C*-terminal glycinamide by the ethylamido-group fails to improve the antagonistic activity.[83] This may partially be due to an increase in the inherent gonadotropin-releasing activity, thus masking an increase in inhibitory activity.[76] There are indications that the replacement of histidine in position 2 by D-phenylalanine instead of its omission leads to compounds with higher inhibitory activity. An additional substitution in the 3 position is beneficial in the sense that it reduces any inherent agonistic activity. This is exemplified in the analogues [D-Phe^2, Leu^3, D-Phe^6]-LH-RH (45), [D-Phe^2, Leu^3, D-Trp^6]-LH-RH (46), and [D-Phe^2, Pro^3, D-Trp^6]-LH-RH (47), which are under the most powerful *in vitro* inhibitors.[80] [D-Phe^2, D-Ala^6]-LH-RH and [D-Phe^2, 2-Me-Ala^6]-LH-RH (44) inhibit ovulation under certain conditions.[79] [D-Phe^2, D-Phe^6]-LH-RH showed significant inhibition of LH and FSH release up to 6 and 8 h after injection. Prolonged action may be related to the lipophilicity of the D-amino-acid in position 6.[82]

A number of conformational studies have been published.[88, 94–98] Based on results obtained by fluorescence measurements it was suggested that the side-chains of His^2, Tyr^5, and Arg^8 interact to form a unit possibly playing a role in the hormone action. Trp^3 seems to be at maximal distance from this unit.[88, 94] Other conformational studies have been performed using ^{13}C n.m.r. on LH-RH,[95]

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and a comparison of possible conformations for the hyperactive [D-Leu^6]-LH-RH(1—9)NHEt and the hypoactive [L-Leu^6]-LH-RH(1—9)NHEt [96] has been published. Low-energy conformations of peptide analogues of LH-RH have been calculated, and a correlation with biological activity has been attempted.[97] Circular dichroism, n.m.r., and charge-transfer investigations have been reported for LH-RH and analogues mainly substituted in positions 6 and 8.[98]

Bacitracin has been found to be an effective inhibitor of the *in vitro* degradation of LH-RH by guinea-pig hypothalamic and whole brain homogenates and rat hypothalamic homogenates and subcellular fractions.[42]

Growth Hormone Release Inhibiting Hormone (GH-RIH, Somatostatin).—A number of informative reviews have appeared,[1-3, 7, 9, 99-101] and several additional syntheses have been published.[102-104] The structure of the recently isolated porcine hypothalamic somatostatin has been elucidated and found to be identical with the ovine somatostatin.[105] Evidence pointed to the presence of a second somatostatin, active both biologically and immunologically, but differing in physicochemical properties, *e.g.* being more basic and having a higher molecular weight.[105] A similar observation leading to the postulation of a big form of somatostatin was made earlier in rat pancreatic extracts. Recently, however, this form was converted into somatostatin by agents which dissociate non-covalent bonds, which indicates that the proposed big form is probably not a classical prohormone.[106] Another radioimmunoassay for the hormone has been described.[107] The wide spectrum of activities going even beyond the endocrine system and the wide distribution throughout the body have been further confirmed. In animal experiments (rat) it could be shown that the formation of stress ulcers is drastically reduced by somatostatin infusion before and during stress.[108]

The *in vivo* inhibition of platelet aggregation caused by somatostatin in rabbits and baboons [109, 110] and the same phenomenon observed in man [111] was

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considered as a serious drawback for further clinical studies in man. Quite recently it has been demonstrated, however, that somatostatin administration in man at a reasonable dosage programme does not lead to clinically relevant changes in coagulation and platelet function.[112] Haemorrhagic complications therefore may not be expected. By contrast, peptic ulcer bleeding in one patient stopped 60 min after starting GH-RIH infusion.[112] The effect of somatostatin on gastric acid secretion and gastrin release in man [113, 114] has been investigated. It has been shown that somatostatin infusion in healthy men induced hyperglycaemia in all subjects.[115] In another study with six diabetic patients, insulin doses could be reduced during a three-day somatostatin infusion, and as a consequence diabetic control appeared to improve without any adverse metabolic effects.[116] In eight insulin-dependent juvenile diabetic patients somatostatin failed to alter the response to intravenously administered glucose while it suppressed the response to orally administered glucose, indicating that it improves postprandial hyperglycaemia in diabetes by decreasing glucose absorption rather than enhancing glucose disposal.[117]

The wide range of activities and the short duration of action have further stimulated the synthesis of analogues in the hope of improving some of these drawbacks for therapeutic application. Original expectations to use somatostatin for the treatment of acromegalic patients have been reduced due to reports on successful application of bromo-ergocryptin, which is orally active.[118, 119] The analogues of somatostatin whose activities have been reported are listed in Table 3 and 4.

That the ring size is not crucial for somatostatin activity has further been exemplified by deleting the amino-acids Asn^5 (151) and Ser^{13} (183) leading to analogues which retain substantial activity. Simultaneous omission of the *N*-terminal dipeptide does not reduce the activity any further. There were some indications that (121) did not lower the arginine-elevated glucagon level in rats, whereas it did inhibit the release of growth hormone and insulin suggesting a possible separation of activities.[123] The [D-Trp^8]-analogue (162), which is more resistant to enzymatic degradation,[128] is eight to ten times as potent as somatostatin in inhibiting the secretion of growth hormone, insulin, and glucagon. It is interesting to note that the separation of activities resulting from the deletion of asparagine in position 5 can be potentiated by introducing D-tryptophan in position 8. The des-Asn^5-[D-Trp^8]-analogue (152) is six to eight times as potent as des-Asn^5-somatostatin (151) in inhibiting insulin secretion while it does not affect glucagon secretion. An activity pattern similar to des-Asn^5- was found for [D-Ser^{13}]-somatostatin (182). A higher potency for inhibition of glucagon than

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Table 3 *Analogues of somatostatin*

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Thr-Phe-Ser-Cys
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Compound number	*Structure*	*Biological activity [a] against: Growth hormone release in vitro*	*Growth hormone release in vivo*	*Insulin release in vivo*	*Glucagon release in vivo*	*Gastric acid release*	*Ref.*
A. Oxidized form							
(108)	Gly_2-somatostatin	++++	++++				120
(109)	Gly_3-somatostatin	++++	++++				120
(110)	Gly_3-somatostatin	+++		+++++	++++		48
(111)	Leu-Gly_2-somatostatin	++++	++++				120
(112)	Descarboxy-Leu-Gly_2-somatostatin	++++	++++				120
(113)	[D-Trp^8]-Gly_3-somatostatin	++++		+++++	+++++		48
(114)	Desamino-					++++	121
		++++	++++				120
(115)	Desamino, descarboxy-		+++				122
						+++	121
		++++	++++				120
(116)	(3—14)	+++		+++	−		48
(117)	Ac-(3—14)	+++		++++	++++		48
(118)	Bz-(3—14)		++++				122
(119)	Desamino-(3—14)	+++		++++	++++		48
(120)	Des-Lys^4-(3—14)		+++				123
(121)	Des-Asn^5-(3—14)			+++	++++		125
			+++				123

[120] H. Immer, N. Abraham, V. Nelson, W. Robinson, and K. Sestanj, in 'Peptides', ed. A. Loffet, Editions de l'Université de Bruxelles, Bruxelles, 1976, p. 471.
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Table 3 (*cont.*)

Compound number	*Structure*	*Biological activity [a] against: Growth hormone release in vitro*	*Growth hormone release in vivo*	*Insulin release in vivo*	*Glucagon release in vivo*	*Gastric acid release*	*Ref.*
(122)	[Ala^5, D-Trp^8]-(3—14)			++++			48
(123)	Desamino, descarboxy-(3—14)	++++	++++				120
(124)	[D-Trp^8]-(3—14)-dicarba		+++	+++	++		124
(125)	Desamino-(3—14)-dicarba		+++			++++	126
(126)	Desamino, descarboxy-(3—14)-dicarba		+++			+++	126
(127)	*Cyclo*(Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Glu)		+++	++	−		127
(128)	*Cyclo*(Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Glu)		+++	++	−		124
(129)	*Cyclo*(γ-Abu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Asp)		+++	++	++		127
(130)	Somatostatin-amide	+++		+++	+++		48
(131)	[D-Ala^1]		++++				122
		++++		+++	+++		48
(132)	[Tyr^1]	++++		+++	+++		48
(133)	[Tyr^1, D-Trp^8]	++++		++++	++++		48
(134)	[Ala^2]	++++	++++				78
(135)	[D-Ala^2]	+++	++++	++++	++++		128
(136)	[D-Ala^2, Ala^5]	−		++++	−		48
(137)	[Ala^{2,5}, D-Trp^8]	−		++++	−		48
(138)	[D-Cys^3]	+++		++	++		48
(139)	[Ala^4]	+++		+++	+++		48
(140)	[Arg^4]	++++		+++	++++		48
(141)	[D-Lys^4]	+++		++	++		48
(142)	Des-Lys^4	++		++	−		48
(143)	[D-Lys^{4,9}]	−		+	+		48
(144)	[D-Asn^5]	+		+	+		48
(145)	[Asp^5]			+++	+++		48
(146)	[Leu^5]	+++		+++	+++		48
(147)	[Lys^5]	−		+++	+++		48
(148)	[D-Lys^5]	−		+	+		48

(149)	[Phe^5]	−		++++	++++	48
(150)	[Ala^5, D-Trp^8]			++++		48
(151)	Des-Asn^5			+++	+	129
		+++		+++	++	48
(152)	Des-Asn^5-[D-Trp^8]			+++	++	129
		+++		+++	++	48
(153)	[D-Phe^6]	++		+	+	48
(154)	[Tyr^6]	+++		+++	+++	48
(155)	Des-Phe^6	+		+	+	48
(156)	[Ala^7]	++	*b*	*b*	−	128
(157)	[D-Phe^7]	+		++	++	48
(158)	[Tyr^7]	++++		+++	++++	48
(159)	[MePhe^7]	++		+	+	48
(160)	[MePhe^{7,11}, D-Trp^8]	−		++	++	48
(161)	[D-Ala^8]	+		++	++	48
(162)	[D-Trp^8]	++++		++++	++++	128
(163)	[D-Tyr^8]	+++		+++	++	48
(164)	Des-Trp^8	+		++	++	48
(165)	[Ala^9]	+		+	+	48
(166)	[Arg^9]	++		++	++	48
(167)	[D-Lys^9]	+		+	+	48
(168)	[Orn^9]	−		+++	+++	48
(169)	Des-Lys^9	+		+	+	48
(170)	[Nle^9]		+			123
(171)	[D-Thr^{10}]	+		+	+	48
(172)	Des-Thr^{10}	+		+	+	48
(173)	[Ala^{11}]	++		*b*	−	128
(174)	[D-Phe^{11}]	+++		++	++	48
(175)	[MePhe^{11}]	+	+++	++		48
(176)	[Tyr^{11}]	+++		++++	+++	48
(177)	Des-Phe^{11}	++		+	+	48

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Table 3 (*cont.*)

Compound number	*Structure*	*Biological activity [a] against: Growth hormone release in vitro*	*in vivo*	*Insulin release in vivo*	*Glucagon release in vivo*	*Gastric acid release*	*Ref.*
(178)	[Ala^{12}]	++	+++	+++	++		128
(179)	[D-Thr^{12}]	+++		+++	+++		48
(180)	Des-Thr^{12}	−		+	+		48
(181)	[Ala^{13}]	++	*b*	+++	−		128
(182)	[D-Ser^{13}]			+++	++		129
		+++		+++	+		48
(183)	Des-Ser^{13}	−		+	+		48
			+++				123
(184)	[D-Cys^{14}]	++++		+++	++++		48
				+++	++++		129
(185)	Descarboxy	++++	++++				120
B. Reduced form							
(186)	H_2 (somatostatein)	++++		++++			48
(187)	[Ala^2]-H_2			+++	+++		128
(188)	[D-Ala^2]-H_2	++++					128
(189)	[Ala^{3,11}]	+		*b*	*b*		48
(190)	[Ala^{3,14}]	+		*b*	*b*		128
(191)	[Cys(Acm)^{3,14}]	+		−	−		48
(192)	[Cys(Me)^{3,14}]	++		−	−		48
(193)	[Ala^5]-H_2		−	++++	++++		128
(194)	[Ala^6]-H_2	−		*b*	*b*		128
(195)	[Ala^7]-H_2	−		*b*	*b*		128
(196)	[Ala^8]-H_2	−		*b*	*b*		128
(197)	[Ala^{10}]-H_2	−		*b*	*b*		128
(198)	[Ala^{11}]-H_2	−		+++	*b*		128
(199)	[Ala^{12}]-H_2	−		+++	*b*		128
(200)	[Ala^{13}]-H_2	−		*b*	*b*		128
(201)	[Ala^{14}]-H_2	+		*b*	*b*		128
(202)	[Ala^{3,14}]-H_2	+		*b*	*b*		128
(203)	[D-Trp^8]-H_2	++		++++	−		128
(204)	(3—14)-H_2			++++	++++		125

[a] The symbols for potency are used as in Table 1. [b] < 10%.

Table 4 *Alanine analogues of somatostatin and somatostatein* (H_2)

Compound number	*Structure*	*Biological activity against:* *Growth hormone release in vitro*	*Insulin release in vivo*	*Glucagon release in vivo*	*Ref.*
(205)	Somatostatin	100	100	100	128
(134)	[Ala^2]	190	135	279	128
(187)	[Ala^2]-H_2		46	35	128
(135)	[D-Ala^2]	55	227	240	128
(206)	[D-Ala^2]-H_2	103	—	—	128
(207)	[Ala^3]-H_2	<0.1	<10	<10	128
(139)	[Ala^4]	20	20	20	48
(208)	[Ala^5]	130	132	—	128
(193)	[Ala^5]-H_2	—	176	112	128
(209)	[Ala^6]	1	<10	—	128
(194)	[Ala^6]-H_2	—	<10	<10	128
(156)	[Ala^7]	3	<10	—	128
(195)	[Ala^7]-H_2	—	<10	<10	128
(210)	[Ala^8]	<0.5	<10	—	128
(196)	[Ala^8]-H_2	—	<10	<10	128
(165)	[Ala^9]	<1	<0.5	<1	48
(211)	[Ala^{10}]	25	14	—	128
(197)	[Ala^{10}]-H_2	—	<10	<10	128
(173)	[Ala^{11}]	2	<10	—	128
(198)	[Ala^{11}]-H_2	—	29	<10	128
(178)	[Ala^{12}]	4	26	—	128
(199)	[Ala^{12}]-H_2	—	22	<10	128
(181)	[Ala^{13}]	6	27	—	128
(200)	[Ala^{13}]-H_2	—	<10	<10	128
(201)	[Ala^{14}]-H_2	0.5	<10	<10	128
(202)	[Ala^{3,14}]-H_2	0.6	<10	<10	128

for insulin secretion has been determined for [D-Cys[14]]-somatostatin (184). High doses, however, lead also to inhibition of insulin secretion.[129]

The question concerning the biological activities of the cyclic and the linear forms as well as the question concerning the importance of the thiol groups for biological activities has been further investigated. From the high biological activities and the qualitatively similar pattern of the analogues (125) and (126) it was concluded that the cyclic form of somatostatin can be active, that the disulphide bridge does not play a functional role, and that even the presence of both terminal groups is not required.[126] A similar conclusion was reached from analogues in which the sulphur bridge has been replaced by coupling the γ-carboxy-group of glutamic acid in position 14 with the α-amino-group of lysine in position 4 with tryptophan either in the L- or D-configuration (127) and (128). Especially the latter shows a prolonged growth hormone release inhibiting activity without effect on glucagon and insulin after s.c. injections into rats.[124]

From the biological activities of a number of Tyr analogues of somatostatin it seems that the three Phe residues play different roles. The Phe[6] residue might be functionally involved in the receptor activation process whereas the Phe[7] could be of importance for the internal stabilization of somatostatin tertiary structure through the stacking of aromatic rings, while the phenyl side-chain of Phe[11] is responsible for the creation of a hydrophobic environment. Furthermore, the indole ring at the 8 position seems to be a prerequisite for biological potency.[130] From a highly interesting series of alanine-substituted analogues of somatostatin and H_2-somatostatin (Table 4) it can be seen that, with the exception of the [Ala[2]] (134) and [Ala[5]] (208) analogues of somatostatin, all the analogues have lower specific activities than somatostatin itself.

A study of gastric acid secretion shows that somatostatin and some of its analogues inhibit basal and pentagastrin-induced gastric acid secretion in the rat. Also for this activity the terminal amino-group is not important, while elimination of the terminal carboxy-group causes some reduction in activity.[121]

The two previously known methods of growth hormone stimulation in the rat by pentobarbital and morphine have been used in combination, resulting in a new model with better precision and reproducibility where plasma growth hormone levels are rapidly increased and maintained constant for up to 180 min.[122]

The conformation of somatostatin has been investigated.[131, 132]

Other Releasing Hormones.—The search continues for the proposed additional Releasing Hormones. Further evidence for their existence was reported, but none have been identified to date. Since administration of antiserum to somatostatin into rats raised the lowest serum levels of growth hormone without abolishing its fluctuation the probable involvement of a GH-RH has been discussed.[133] Comparison of the release of TSH and prolactin of extracts from porcine hypothalamic fragments with TRH has given further evidence of a

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[131] L. A. Holladay and D. Puett, in 'Peptides', ed. R. Walter and J. Meienhofer, Ann Arbor Science Publishers, Ann Arbor, 1975, p. 175.
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[133] A. Arimura, W. D. Smith, and A. V. Schally, *Endocrinology*, 1976, **98**, 540.

prolactin releasing factor distinct from TRH.[134, 135] A partially purified material from porcine hypothalami showing prolactin release inhibiting activity was found to contain catecholamines.[136] Prolactin release and prolactin release inhibiting activity has also been reported to be present in extracts of the pineal gland.[137] Thus the question whether a specific inhibitor for prolactin release exists or whether catecholamines are physiologically responsible for this activity still remains unanswered.

The existence of one or several corticotropin releasing factors has been further substantiated. CRH activity was found in two peaks with molecular weight of 1500 and 30 000, respectively, by chromatography on Sephadex G-50. The latter was proved to be associated with aggregated forms.[138] Also two peaks, albeit both with feeble CRH activity, were separated by chromatography on Sephadex G-25. Since full activity was regained by recombining both peaks the existence of a co-factor was postulated.[139] With some reservation, CRH activity has been reported in rat plasma [140] and in rat and human peripheral blood.[141, 142] A dose-related secretion of ACTH was found, when pitressin (posterior pituitary extract) but not when arginine or lysine vasopressin was tested in an *in vitro* system of rat adenohypophyseal cells.[143] CRH-like activity was detected in some ectopic ACTH-producing tumours.[144, 145] *In vitro* incubation of anterior pituitary cells with CRH led to the secretion of predominantly low molecular weight (5000) ACTH, whereas in the absence of CRH more high molecular weight (10 000) ACTH was observed.[146] An attempt to map regions in the rat hypothalamus which possess ACTH-releasing activity has been published.[147] The *in vitro* secretion of CRH has been reviewed.[148]

The situation in the case of MSH release inhibiting factor (MIF) is still controversial. In spite of numerous attempts, no unambiguous proof for any MIF-activity of the postulated MIF, prolyl-leucyl-glycinamide, has yet been furnished.

3 Anterior Pituitary Hormones

Contributed by R. Schwyzer

In this section, the main emphasis is on structure–activity relationships evidenced by studies of hormone analogues or derivatives produced by chemical or enzymatic

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[136] A. V. Schally, A. Dupont, A. Arimura, J. Takahara, T. W. Redding, J. Clemens, and C. Shaar, *Acta Endocrinol.*, 1976, **82**, 1.
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modification or by chemical synthesis. However, information on closely related subjects like hormone precursors, isolation, chemical structure, biological activity, and hormone–receptor interactions is included.

The literature for 1976 and early 1977 is covered. For comparison, some reference to previously published articles is made.

The nomenclature is according to the suggestions of the IUPAC–IUB Commission on Biochemical Nomenclature,[149] although it appears to be in conflict with the system of International Nonproprietary Names, INN.[150] The source designation is: h = human, b = bovine, c = canine, e = equine, o = ovine, and p = porcine.

The following compounds are treated (with the exception of the endorphins which are covered in Section 6).

A. The Corticotropin–Melanotropin–Lipotropin–Endorphin Family
   - Subfamily 1: Corticotropin, α-melanotropin
   - Subfamily 2: Lipotropin, β-melanotropin, endorphins (including enkephalins)

B. The Glycoprotein Hormone Family (α = 'common', β = 'specific' subunit)
   - Subfamily 1: Thyrotropin, follitropin
   - Subfamily 2: Choriogonadotropin, lutropin

C. The Single-chain Protein Hormone Family
   - Subfamily 1: Prolactin
   - Subfamily 2: Choriomammotropin, somatotropin

General Introduction.—It is becoming increasingly clear that particularly relevant information on structure–activity relationships can be gained by studying complete log dose/response curves (for reviews, see ref. 151). This allows the assessment of two important pharmacological parameters: potency and intrinsic activity.[152] A measure of the (reciprocal) potency is the hormone concentration necessary to evoke 50% of the maximal effect (ED_{50}, effective dose at the 50% response level). A measure of the intrinsic activity is the ratio (α) of the maximal effect attainable by a given hormone analogue divided by the maximal effect of the native, parent hormone ($0 \leqslant \alpha \leqslant 1$).

These parameters have recently been supplemented by 'binding' data to receptors, such as the number of binding sites (binding capacity), the apparent association or dissociation constants (K_{ass}, K_{diss}), or the hormone concentration necessary to achieve a 50% occupation of the binding sites. The receptors themselves are chemically still rather ill-defined and the experiments are usually carried out with target tissue, target cells, target cell membranes, or homogenates. It is furthermore very difficult to decide whether or not the observed binding occurs at functional or at non-functional sites. Circumstantial evidence for functionality includes: (i) the close coincidence of K_{diss} with ED_{50} for a particular effect, (ii) the interaction specificity (competitive inhibition of the binding by close analogues, but not by distantly related peptides), and infrequently

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[152] A. J. Ariëns and A. M. Simonis, *J. Pharm. Pharmacol.*, 1964, **16**, 137.

(iii) the parallel influence of certain compounds on the intrinsic activity and the number of binding sites displaying a given affinity.[153]

By comparing the data obtained by these methods, it is possible to gain insight into the specific organization of biological information within hormone molecules, into the specific requirements of receptors for hormone recognition and stimulation, into the chemical processes of receptor stimulation and stimulus–response coupling, and into the nature of functionally important components of the receptors.

Precursors and Special Discharge Phenomena.—Many polypeptide hormones, like the classical example of insulin, are most probably derived from larger precursors, or prohormones, by enzymatic modification. At least four major corticotropin species with different molecular size were found in foetal sheep pituitaries.[154] High molecular weight forms (6500—9000 and 20 000—30 000) were also isolated from the mouse pituitary and a corresponding tumour cell line, and identified as glycoproteins [155] (see also ref. 156). Corticotropin itself is the precursor of α-melanotropin.[157] β-Lipotropin, previously a 'hormone without a function', is obviously the precursor of β-melanotropin and the endorphins.[158]

'Big growth hormones', possible educts for somatotropin, may simply be disulphide bond polymers (h pituitary) [159] or larger polypeptide chains, like those that were obtained by cell-free translation of rat pituitary tumour cell RNA.[160] Whether or not somatotropin is further cleaved to produce smaller, biologically active peptides is not known. However, under natural conditions, somatotropin activity and immunological properties are connected with a family of closely related polypeptides.[161]

The discharge, or release, of anterior pituitary hormones is regulated not only by hypothalamic 'liberins' or 'statins', but also by other factors. Somatotropin and the related prolactin are reported to be released by Substance P in rats,[162] prolactin even by enkephalin.[163] The intensity of lutropin discharge in ovariectomized rats was greater under the influence of thyroxin, but occurred with unchanged frequency.[164]

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Isolation and Structure.—The primary structures of polypeptide hormones have been listed,[165] and compiled with respect to molecular evolution and three-dimensional structures.[166] Local primary-structure similarities have been pointed out for the groups: prolactin and somatotropin; lutropin and thyrotropin β-subunits; β-lipotropin, proinsulin, and parathormone.[167]

Two strongly lipolytic peptides without other activities (Fettstoffwechselhormon) were purified from p-pituitaries[168] (are they possibly related to the synthetic lipolytic fragments of somatotropin, Table 11?). An h-pituitary 'lipolytic hormone' similar to β-lipotropin was partially characterized,[169] and a [1-pyroglutamic acid]-β-lipotropin isolated from o-glands.[170] A primary structure of h-β-lipotropin was proposed.[171]

The three-dimensional structures of the corticotropin–melanotropin–lipotropin–endorphin family have received much attention (enkephalin and endorphins, see Section 6). The situation was briefly reviewed in the 'New York Academy of Sciences' Conference on ACTH and Related Peptides'.[172] The random conformation of corticotropin in aqueous solution was confirmed by a careful c.d. study.[173] However, a change of dichroitic properties was observed with increasing amounts of solvent trifluoroethanol and interpreted as the appearance of local α-helical structures,[174] such as had been suggested earlier by Squire for aqueous solutions (see ref. 172). The confusion is enhanced by reports based on c.d. and i.r. spectroscopy claiming M-helical, poly-L-proline II- (and twisted β-) -type conformations for corticotropin, β-melanotropin, and β-lipotropin in water.[175] An increase in the NaCl and dioxan concentrations causes an increase in o-β-lipotropin helix content as seen by c.d.[176] Much as earlier in the simpler case of gramicidin S, c.d. studies appear to overestimate helix content and not to be definitively conclusive.

In the field of glycoprotein hormones, an h-placental factor with uterine-growth and DNA-promoting effects has been studied.[177] A mouse placental gonadotropin, immunologically related to choriogonadotropin, has also been

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described.[178] Evidence for a gonadotropin from non-pregnant subjects that has physical, immunological, and biological similarities to h-choriogonadotropin was provided.[179] Pituitary gonadotropins were isolated from salmon,[180] sturgeon,[181] amphibians,[182] and turtle[183] (with lutropin- and follitropin-like fractions from the latter two). Studies on the purification and the biological properties of b- and p-follitropin[184] and a comparison of various lutropins with different assay procedures[185] have appeared. The carbohydrate components of the glycopeptides from o-lutropin and from the α-subunits of p-lutropin, follitropin, and thyrotropin were studied.[186] The primary structures of the h-follitropin[187] and h-lutropin[188] β-subunits and of the p-thyrotropin α- and β-subunits[189] were elucidated, and studies on the disulphide bridges of o-lutropin[190] carried out. The secondary structures of h-choriogonadotropin and h- and o-lutropin were investigated by c.d. spectroscopy.[191]

In the field of single-chain protein hormones, rabbit and b-chorioprolactins occupy a position more intermediate between pituitary prolactin and somatotropin in the evolutionary tree than the primate placental lactogens.[192] Furthermore, o-chorioprolactin was purified and characterized.[193] h-Choriomammotropin appears to exist as several molecular species (iso-hormones).[194] Pituitary prolactins were isolated from humans[195] and monkeys,[196] from rats,[197] dogs,[198]

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birds,[199] and fish.[200] The hydrophobic core in the primary structure of b-prolactin was studied,[201] and the primary structures of p-prolactin,[202] e-somatotropin,[203] and of choriomammotropins[204] were studied. Somatomedin, a mediator of somatotropic activity, was isolated from b-material and purified 120 000-fold.[205]

Biological Effects.—*Corticotropin.* For the construction of log dose/response curves in the study of steroidogenesis and cyclic AMP accumulation, a counter-streaming centrifugation technique for the superfusion of adrenocortical cell suspensions appears to be a valuable asset.[206] The purification of b-adrenocortical cell membrane vesicles containing a highly corticotropin-sensitive adenylate cyclase system (and angiotensin II-binding sites)[207] and their stabilization by concanavalin A[208] have been described.

Glycoprotein Hormones. A theoretically interesting differential effect of lutropin on cyclic AMP and progesterone in immature female rats has been observed.[209] While 0.01 μg did not affect cyclic AMP levels, an increase in ovary progesterone was found. In order to increase cyclic AMP levels, 0.1 μg was necessary. This corresponds to observations on the effects of corticotropin in isolated adrenocortical cells, where the phenomenon has been discussed in terms of the straightforward 'cyclic AMP Second Messenger Concept' as an indication of an extremely large receptor reserve, and in terms of a 'Dual Receptor Concept'.[210] The effects of lutropin on cyclic AMP and protein kinase activity in interstitial cell tissue have been reported.[211]

The effects of thyrotropin on the refractoriness of the thyroid slice cyclic AMP system,[212] on the inhibition of its adenylate cyclase stimulation by propranolol,[213] on iodide transport,[214] on ornithine decarboxylase in connection with growth promotion in the thyroid,[215] and on the circulating thyroxine concentration in ducks[216] were investigated. Biological properties and cell binding of h-choriofollitropin were studied in rat ovaries.[217] An interesting 'universality' of the effect of mammalian follitropin was demonstrated: stimulation of

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spermatogenesis in the ovotestis of snails, but no effect on oogenesis.[218] Unlike commercially available preparations of h-choriogonadotropin, a highly purified specimen showed no inhibitory effect on the phytohaemagglutinin-induced stimulation of lymphocytes.[219]

Single-chain Protein Hormones. The present state of knowledge about h-prolactin and its role in human reproduction has been reviewed.[220] Various effects of prolactin in animals and in perfused livers have been studied,[221] as have actions of somatotropin on enzyme systems (mixed-function oxidase and RNA polymerase,[222] lecithin-cholesterol acyl transferase [223]), drug metabolism,[222,224] cyclic AMP/cyclic GMP ratios and translation in heart muscle,[225] on serum lipids (no effect),[226] and as a suppressor of basal insulin secretion in man.[227]

Receptor Binding.—*Corticotropin.* A very pure, biologically active preparation of ^{125}I-labelled corticotropin was used to study binding to isolated rat adrenocortical cells. About 3000 sites per cell with $K_{diss} = 2.5 \times 10^{-10}$ mol l^{-1} and 30 000 sites per cell with $K_{diss} = 10^{-8}$ mol l^{-1} were found. Only *ca.* 12% of the total number of 33 000 sites had to be occupied in order to produce the maximum rate of steroidogenesis ($ED_{50} \simeq 4 \times 10^{-10}$ mol l^{-1}). The dissociation constant of the low-affinity sites corresponds more closely to ED_{50} of cyclic AMP accumulation. The data were discussed in terms of the 'cyclic AMP Second Messenger Concept' and of 'spare receptors'.[228] According to the usual interpretation of the 'cyclic AMP Second Messenger Concept', cyclic AMP is both a necessary and a sufficient condition for evoking the hormone-specific response of a target cell. The Reporter feels that the data are better compatible with either the 'Dual Receptor Concept' (cyclic AMP is sufficient for producing steroidogenesis, but unnecessary, because the hormone can trigger a 'by-pass'),[210] or the 'Compartment Guidance Concept', in which cyclic AMP needs complementation by a corticotropin-stimulated process in order to produce steroidogenesis (cyclic AMP is a necessary but insufficient condition).[229]

Thyrotropin Receptor Binding. It has been claimed that thyrotropin binding to thyroid slices is partly persistent and, at least to thyroid plasma membranes, partly reversible with $K_{diss} \simeq 7 \times 10^{-8}$ and 9×10^{-6} mol l^{-1}.[230] A very

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thorough and most illuminating series of studies has been carried out by an NIH group.[231] The binding of [^{125}I]thyrotropin to bovine thyroid plasma membranes, guinea-pig retro-orbital tissue plasma membranes, and human adipocyte membranes was investigated. It was found that all these tissues have thyrotropin-specific binding sites. In addition, a thyrotropin-specific binding inhibitor that may help to regulate the thyrotropin effects is released extracellularly. This inhibitor is inactivated by γ-globulins from patients with exophthalmos. In this disease, binding to retro-orbital tissue becomes excessive. Furthermore, gangliosides with a critical disposition of sialic acid residues appear to be constituents of the receptors; they are missing in the membranes of a tumour with strongly reduced thyrotropin-binding capacity. Added to the assay, such gangliosides inhibit thyrotropin-receptor interaction by competitive binding to thyrotropin (albumin, glucagon, insulin, prolactin, follitropin, somatotropin, and corticotropin do not interfere). Effective gangliosides change the conformation of the hormones. The interaction is reminiscent of ganglioside–choleratoxin B-chain binding. The B-chain has, in fact, certain structural features in common with the thyrotropin β-subunit. This work provides the first clear indication that certain gangliosides are structural or functional components of hormone receptors. It was shown by others that *N*-acetylneuraminic acid may become incorporated into deficient thyroid membranes, restoring their thyrotropin-binding capacity.[232]

Follitropin. Follitropin binding to rat granulosa cells[233] and rat testes tubule membranes[234] has been studied. In the latter case, treatment with phospholipase (A or C) abolished the binding capacity (D had no effect), and supplementation with phosphatidylserine restored it. Chemical treatment of the membranes suggested that disulphide bonds, tyrosine, histidine, and tryptophan residues in or about the membrane receptor site may be involved in the binding interaction.

h-Choriogonadotropin Receptors. In plasma membranes of bovine corpora lutea, phospholipase D again had no effect, A produced binding-inhibitory products, whereas C inhibited by directly altering the receptor sites.[235] A very interesting correlation of h-choriogonadotropin binding, cyclic AMP accumulation, and testosterone production in isolated Leydig (interstitial) cells from adult rat testes again indicated a marked dissociation of effects: a 1% occupation of receptor sites ($\simeq 60$ out of a total of 6000 with a 'uniform' $K_{diss} \simeq 10^{-10}$ mol l^{-1}) produces maximal steroidogenesis, but no measurable cyclic AMP accumulation. Increasing receptor occupancy at higher hormone concentrations produced cyclic AMP accumulation at progressively earlier time intervals, but did not alter the 20—30 min time lag for testosterone synthesis[236] (see also the similar situation with corticotropin).

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High-affinity choriogonadotropin binding sites were 'solubilized' with detergent[237] or with 30% ethanol in water.[238] These studies should prove valuable for attempts at isolation of receptor molecules or complexes. Finally, changes of binding capacity were observed during follicle development,[239] after pretreatment with choriogonadotropin,[240] and under the influence of follitropin.[241]

The lutropin-binding capacity of testicular interstitial cells first increases, then declines during storage. The decline phase can be retarded by protease inhibitors and by hormone addition.[242] The number of binding sites is reduced by choriogonadotropin pretreatment.[243] However, early prolactin treatment increases the lutropin-binding capacity of luteal cells and their responsiveness with respect to progesterone synthesis.[244]

Prolactin. Prolactin binding has been studied in various rabbit tissues,[245] in rat, b-, and h-ovaries,[246] in p-corpora lutea,[247] in rat mammary glands during pregnancy and lactation,[248] and in crop sacs of juvenile, mature, parent, and prolactin-injected pigeons.[249] Prolactin and somatotropin binding in mouse liver and its physiological, pharmacological, and surgical modulation were also examined.[250] Similar studies with somatotropin were described by other authors.[251]

Somatotropin. Somatotropin receptor concentrations in IM-9 lymphocytes are subject to active regulation by the hormone: preincubation reduces the number of cell surface receptors which is restored by *de novo* protein synthesis.[252] These receptors (and those for insulin) are also under the control of microfilaments: cytochalasins reduce the binding capacity, whereas microtubule modifiers (vincristine, vinblastine, colchicine) have no effect.[253] This is especially interesting because a rapidly increasing number of observations on the transmembrane control of receptors in general is being made (for a review, see ref. 254).

In summary, hormone receptors are emerging as integral membrane lipoproteins (with carbohydrate either on the protein or lipid, or both) that are probably connected with the microfilament system on the cytoplasmatic side of

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the membrane. Their number can be regulated by unknown mechanisms. After enzymatic destruction, they are rather rapidly resynthesized within the cell and put into position in the membrane. Receptor deficiencies can lead to serious diseases: this is an especially interesting practical aspect of their study (for a review of their physiological and pathogenic significance see ref. 255).

Hormone Modification.—The following types of modification that are potentially useful for structure–activity studies have been described during the review period: (i) the chemical synthesis of hormones and analogues using new methods; (ii) the introduction of radioactive labels; (iii) the synthesis of photo-affinity labels; (iv) the preparation of compounds containing the hormone or its fragments covalently attached to an insoluble carrier for affinity chromatography or to a protein for enhancing its antigenicity; and (v) the specific chemical modification of certain groups within the hormone molecule in order to determine either structure or conformation, or the specific contributions of such groups to biological activity.

(i) Corticotropin-(1—24)-tetracosapeptide [256] and human corticotropin [257] were synthesized by solution methods using the pentachlorophenyl trichloroacetate ester exchange and the *N*-hydroxy-5-norbornene-2,3-dicarboximide active ester methods, respectively.

(ii) A method utilizing a thin-layer electrochemical cell for the constant-potential radio-iodination of microgram protein quantities has been described, with emphasis on electrode effects.[258] Several papers deal with the preparation, purity, and stability of radio-iodinated hormones (Table 5); one utilizes iodination

Table 5 *Radio-labelled derivatives obtained by modification of the native hormones*

Hormone	*Label*	*Method*	Ci mmol^{-1}	*Ref.*
Corticotropin	^{125}I	Lactoperoxidase	⩽3200	*a*
α-Melanotropin	^{125}I	Lactoperoxidase	1000	*b*
		Electrolytic	200	*b*
h-Prolactins [g]	^{125}I	Lactoperoxidase, chloramine T	—	*c*
h-Choriogonadotropin	^{125}I	Lactoperoxidase, chloramine T	—	*d*
h-Thyrotropin	^{125}I	Lactoperoxidase, chloramine T	—	*e*
h-Somatotropin	^{3}H	Iodination, then sodium boro[^{3}H]hydride	1.7	*f*

(*a*) R. A. J. McIlhinney and D. Schulster, *Endocrinology*, 1974, **94**, 1259. (*b*) A. Eberle, Thesis, ETH Zürich, No. 5735, 1976. (*c*) A. D. Rogol and A. Chrambach, *Endocrinology*, 1975, **97**, 406. (*d*) B. W. Griffiths and A. Godard, *Clin. Biochem.*, 1975, **8**, 400. (*e*) J. M. Burrin, *Clin. Chim. Acta*, 1976, **70**, 153. (*f*) H. Kawauchi, T. A. Bewley, J. Ramachandran, and C. H. Li, *Biochim. Biophys. Acta*, 1976, **420**, 214. (*g*) From the pituitary and the amniotic fluid.

and replacement of iodine by tritium to produce h-[^{3}H]somatotropin. More radiolabelled derivatives of corticotropin and melanotropin are being reported (Table 6). These compounds have been prepared synthetically and contain the label to 95% and more in one, well-defined, rather stable position. Therefore, they are especially suited for biological and biochemical studies.

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Table 6 *Radio-labelled derivatives of corticotropin and α-melanotropin obtained by synthetic procedures*

Compound number	*Amino-acid sequence*	Ci mmol^{-1}	*Ref.*
(1) [j]	SYSMEHFRWGKPVGKKRRPVKVYP	—	*a*
(2) [k]	S----------^{t}YP	46	*b*
(3) [k]	^{t}SY---------- P	29	*c*
(4) [l]	S-----^{t}F----------P	27	*d*
(5) [m]	^{t}SF-V′----------P	7.4	*e*
(6) [k]	E----------^{t}YP	8.8	*f*
(7) [k, n]	^{Dt}SY----------KK	17	*c*
(8) [o]	Ac·SYSMEHFRWGKPV·NH$_2$	—	*g*
(9) [k]	Ac·^{i}Y----------V·NH$_2$	10—20	*h*
(10) [p]	Ac·^{Dt}AF′----------V·NH$_2$	11	*i*

(*a*) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, 1961, **44**, 1136; R. Schwyzer and H. Kappeler, *ibid.*, 1963, **46**, 1550. (*b*) D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1973, 2875. (*c*) D. E. Brundish, J. R. Martin, and R. Wade, *J.C.S. Perkin I*, 1976, 2182. (*d*) D. E. Brundish and R. Wade, *ibid.*, 1976, 2186. (*e*) R. Schwyzer and G. Karlaganis, *Annalen*, 1973, 1298. (*f*) G. Karlaganis and R. Schwyzer, *Helv. Chim. Acta*, 1977, **60**, in press. (*g*) A. Eberle, J.-L. Fauchère, G. I. Tesser, and R. Schwyzer, *ibid.*, 1975, **58**, 2106. (*i*) A. Eberle and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2421. (*h*) Ref. *b*, Table 5. (*j*) The sequence of corticotropin–(1—24)–tetracosapeptide is shown as reference. (*k*) ^{t}Y stands for [3,5-^{3}H] tyrosine. (*l*) $^{t}_{D}$F stands for [4-^{3}H] phenylalanine. (*m*) ^{t}V′, stands for 4,5-dehydro-[4,5-^{3}H] norvaline. (*n*) S stands for D-serine. (*o*) The sequence of α-melanotropin, synthesized by the same methods as (9) and (10), is shown. (*p*) ^{t}F′ stands for 4-azido-[3,5-^{3}H] phenylalanine.

(iii) The synthesis of a radioactive photoaffinity-labelled hormone, [1-D-alanine-2-(4′-azido-3′,5′-ditritio)-phenylalanine]-α-melanotropin [(10), Table 6], has been reported. The use and limitations of *p*-azidophenylalanine as a photolabel in model systems with chymotrypsin have been studied.[259]

(iv) The covalent attachment of α-melanotropin to human serum albumin exclusively through either the side-chain carboxy-group of glutamic acid or the modified *N*-terminal acetyl group leads to a complex antigen with excellent antigenic properties, producing a very selective, high-titre antiserum in rabbits.[260] (For another radioimmunoassay of α-melanotropin, see ref. 261.) This finding supports the idea that chemically well-defined linkages can lead to improved complex antigens and affinity-chromatographic materials.[262]

(v) Systematic modification of the tryptophan side-chain in corticotropin with a variety of substituted *o*-nitrophenylsulphenyl chlorides has been reported.[263] Furthermore, γ-irradiation of thyrotropin for sterilization appears to produce rather slight modifications in the β-subunit or in its connection with α; however, the activity is substantially decreased 3—5 months after irradiation.[264] Sialic acid cleavage by neuraminidase destroys follitropin *in vivo* activity, but leaves

[259] W. Fischli, O. Leukart, and R. Schwyzer, *Helv. Chim. Acta*, 1977, **60**, in press.
[260] H. G. Kopp, A. Eberle, P. Vitins, W. Lichtensteiger, and R. Schwyzer, *European J. Biochem.*, 1977, **75**, 417.
[261] A. J. Thody, R. J. Penny, D. Clark, and C. Taylor, *J. Endocrinol.*, 1975, **67**, 385.
[262] J.-L. Fauchère and G.-M. Pelican, *Helv. Chim. Acta*, 1975, **58**, 1984.
[263] E. Canova-Davis and J. Ramachandran, *Biochemistry*, 1976, **15**, 921.
[264] V. Štrbák, L. Macho, J. Sedlák, and M. Hromadová, *Endocrinol. Exp.*, 1976, **10**, 3.

that of lutropin unchanged.[265] Table 7 lists a number of interesting modifications carried out with intact protein hormones or with their isolated subunits.

Disulphide bonds of protein hormones have been reduced and reoxidized, or reduced and alkylated. In b-lutropin, the reduced α-chain contains the complete information for refolding to the native subunit, as evidenced by electrophoresis and recombination with lutropin β and thyrotropin β. The first disulphide bridge to be cleaved in b-lutropin β is Cys-93—Cys-100, in b-thyrotropin β, Cys-88—Cys-95, indicating a structural relationship between the two activity-specific hormone subunits. Conditions leading to the cleavage of both disulphide bridges in h-somatotropin caused the reduction of only one bridge in b-somatotropin. It is the *C*-terminal bridge. Its cleavage and alkylation result neither in a dissociation of the dimeric form of the hormone nor in a significant loss of biological activity.

Carboxy-group modification of b-lutropin leads to cross-linking and dissociation into subunits. Studies with the isolated subunits showed that α incorporates eight glycine residues. This causes a complete loss of its ability to recombine with β. The β subunit incorporates six or seven residues and retains its property of recombining with α to a partially active hormone derivative.

Arginine residues of o-lutropin were modified with cyclohexane-1,2-dione. No disruption of the quaternary structure was observed, but biological activity was lost in parallel to the number of substituted arginine residues. Hydroxylamine treatment partially restored activity. Modified α- and β-subunits retained their ability to recombine to more or less active hormone derivatives.

Histidine residues of b- and p-lutropin were acylated with ethoxyformyl anhydride. The reactivity is pH-dependent: at pH 5, seven histidines react, and at pH 7 only five. Positions are tentatively assigned to the unreactive His residues. The modification of four histidines in b-lutropin does not dissociate the molecule, but almost completely inactivates it.

Methionine residues of b-lutropin and its α- and β-subunits were modified at pH 3 with iodo[^{14}C]acetic acid and the positions of the modified residues in the primary sequence determined. A comparison with modified h-lutropin and a study of receptor-binding properties revealed that Met-8α of b-lutropin is not essential, but that Met-33α and Met-52β are involved in optimal binding.

Tyrosine residues were acetylated and nitrated in h-choriogonadotropin and nitrated in o-lutropin. In h-choriogonadotropin, acetylation of Tyr-2 reduces the biological activity to *ca.* 50% (partly restored by treatment with hydroxylamine). Tyr-3 in isolated α- and Tyr-2 in isolated β-subunits are reactive. Acetylated α loses its ability to recombine with β, but acetylated β still recombines with unmodified α (see the parallel behaviour in the case of carboxy-group modification).

In o-lutropin, Tyr-37β and Tyr-59β are nitrated in the isolated β-subunit, but not in the intact hormone. In the isolated α-subunit or the intact hormone, residues 21α, 92α, and 93α reacted equally fast. Tyr-41α reacts more slowly and only in the isolated subunit; 30α is buried and reacts only after exposure to urea. Polymerization was observed as a side-reaction. Only Tyr-37β of isolated

[265] E. Finné, *Endokrinologie*, 1975, **66**, 279; H. J. Grimek, L. C. Nuti, K. M. Nuti, and W. H. McShan, *Endocrinology*, 1976, **98**, 105.

Table 7 *Chemical modification of specific groups within protein hormones*

Hormone	*Disulphide bonds*	*Carboxy-groups*	*Arginine*	*Histidine*	*Methionine*	*Tyrosine*
h-Choriogonadotropin	red./alkyl [a]					*N*-acetyl-imidazole [k] tetranitro-methane [l]
b-Lutropin	red./oxid. [b]	DCCI + HGlyOMe [f]		EtO_2COCO_2Et [h]	ICH_2CO_2H [i]	
o-Lutropin			cyclohexane-1,2-dione [g]			tetranitro-methane [m]
p-Lutropin				EtO_2COCO_2Et [h]		dansyl chloride, dinitrofluoro-benzene [n]
b-Thyrotropin	red./oxid. [c]					
h-Somatotropin	red./alkyl [d]					
b-Somatotropin	red./alkyl [e]					
o-Prolactin					H_2O_2 [j]	

(*a*) O. P. Bahl, M. R. Pandian, and R. D. Ghai, *Biochem. Biophys. Res. Comm.*, 1976, **70**, 525. (*b*) J. R. Reeve, K.-W. Cheng, and J. G. Pierce, *Biochem. Biophys. Res. Comm.*, 1975, **67**, 149; L. C. Giudice and J. G. Pierce, *J. Biol. Chem.*, 1976, **251**, 6392. (*c*) J. G. Pierce, L. C. Giudice, and J. R. Reeve, *J. Biol. Chem.*, 1976, **251**, 6388. (*d*) L. Gráf, J. Borvendég, E. Barát, I. Hermann, and A. Patthy, *F.E.B.S. Letters*, 1976, **66**, 233. (*e*) L. Gráf, C. H. Li, and T. A. Bewley, *Internat. J. Peptide Protein Res.*, 1975, **7**, 467. (*f*) M. R. Faith and J. G. Pierce, *J. Biol. Chem.*, 1975, **250**, 6923. (*g*) M. R. Sairam, *Arch. Biochem. Biophys.*, 1976, **176**, 197. (*h*) P. de la Llosa, *Biochimie*, 1976, **58**, 611. (*i*) K.-W. Cheng, *Biochem. J.*, 1976, **159**, 71, 79. (*j*) R. A. Houghten and C. H. Li, *Biochim. Biophys. Acta*, 1976, **439**, 240. (*k*) V. G. Hum, H. Botting, and K. F. Mori, *Endocrine Res. Comm.*, 1976, **3**, 145. (*l*) R. B. Carlsen and O. P. Bahl, *Arch. Biochem. Biophys.*, 1976, **175**, 209. (*m*) B. D. Burleigh, W.-K. Liu, and D. N. Ward, *J. Biol. Chem.*, 1976, **251**, 308; W.-K. Liu and D. N. Ward, *ibid.*, p. 316. (*n*) Y. Combarnous and G. Hennen, *Internat. J. Peptide Protein Res.*, 1976, **8**, 491.

p-lutropin β-subunit reacts with dansyl chloride. This modification prevents reassociation with the native α-subunit. Dinitrofluorobenzene reacts preferentially with Tyr-59β. This substitution does not affect reassociation. Mono-dansylated α-subunit reacts with one Tyr, and on recombination with β the fluoroescence is markedly quenched. Both substituted dimers are biologically inactive.

Relationships between Primary (and Secondary) Structure and Activity.—For corticotropin and α-melanotropin, these aspects have been reviewed in the 'New York Academy of Sciences' Conference, 1976'.[172] The following conclusions were drawn and discussed in detail. Corticotropin, α-melanotropin, β-melanotropin, the lipotropins, and the endorphins are members of a chemically and biogenetically related polypeptide family. Corticotropin is a linear nonatriacontapeptide with species differences in the *C*-terminal two-thirds of the molecule. Corticotropin stimulates steroidogensis and cyclic AMP accumulation in adrenocortical cells, and adenylate cyclase activation in adrenocortical cell membranes. It also stimulates lipolysis and a transient cyclic AMP accumulation in fat cells, as well as Mg^{2+} transport into fat cells and fat cell membranes.[266] Fat cell membrane adenylate cyclase is also activated. Corticotropin mimics α-melanotropin with its ability to disperse melanin in amphibian melanophores (melanocytes). On the other hand, α-melanotropin is able to mimic the effects of corticotropin on adrenocortical cells (at very high doses) and on rabbit lipocytes (lipolysis), but not on rat lipocytes.[267] Both α-melanotropin and corticotropin show effects on the central nervous system, enhancing the acquisition of learned behaviour.[271a] The *N*-terminal part (amino-acid residues 1—24) contains the essential molecular information for these effects, and the *C*-terminus facilitates transport and stabilizes the *N*-terminus in the body. Corticotropin is a pleiotropic effector based on the principles of flexibility and sychnologic organization.

Further details of the information storage in corticotropin-(1—24)-tetracosapeptide [267a] and in α-melanotropin have recently been discovered.[268–270] The situation is summarized in Tables 8 and 9. Both hormones are sychnologically organized. This means that discrete sequences of adjacent amino-acids are responsible for different components of the biological activity (in contrast to rhegnylogically organized hormones, like insulin).[172] A definite dissociation of adenylate cyclase stimulation from cell-specific hormonal effects (steroidogenesis, cyclic AMP accumulation,[210, 269] and lipolysis [268]) is indicated for corticotropin. In α-melanotropin, the message for the CNS receptors is centred around Met-Glu. In contrast, frog melanophores are triggered independently by Phe-Arg and

[266] D. A. Elliott and M. A. Rizack, *J. Biol. Chem.*, 1974, **249**, 3985; D. A. Elliott, M. W. Draper, and M. A. Rizack, *J. Med. Chem.*, 1977, **20**, 584.

[267] J. Ramachandran and V. Lee, *Biochim. Biophys. Acta*, 1976, **428**, 339; J. Ramachandran, S. W. Farmer, S. Liles, and C. H. Li, *ibid.*, p. 347.

[267a] H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, 1961, **44**, 1136; R. Schwyzer and H. Kappeler, *ibid.*, 1963, **46**, 1550.

[268] U. Lang, J.-L. Fauchère, G.-M. Pelican, G. Karlaganis, and R. Schwyzer, *F.E.B.S. Letters*, 1976, **66**, 246.

[269] J.-C. Bonnafous, J. L. Fauchère, W. Schlegel, and R. Schwyzer, *F.E.B.S. Letters*, 1977, **78**, 247.

[270] A. Eberle and R. Schwyzer, *Helv. Chim. Acta*, 1975, **58**, 1528; *Clin. Endocrinol.*, 1976, **5** (Suppl.), 41s; in 'Surface Membrane Receptors', ed. R. A. Bradshaw, W. A. Frazier, R. C. Merrell, D. I. Gottlieb, and R. A. Hogue-Angeletti, Plenum, New York, 1976, p. 291.

Lys-Pro-Val-NH_2. (It was claimed also that Ser-Tyr-Ser-Met tetrapeptides could stimulate frog melanophores.[271] However, the statement could not be confirmed: even Ser-Tyr-Ser-Met-Glu-His was found to be inactive.[271a])

Puzzling observations[272] that corticotropin analogues that inhibit the action of corticotropin-(1—24)-tetracosapeptide on b-adrenocortical cell membrane adenylate cyclase fail to inhibit its effect on steroidogenesis and cyclic AMP accumulation may find their simple explanation in the fact that most corticotropin peptides, even though they may be full or partial agonists at low

Table 8 *Organization of biological information for adrenocortical and fat cells in corticotropin-(1—24)-tetracosapeptide*

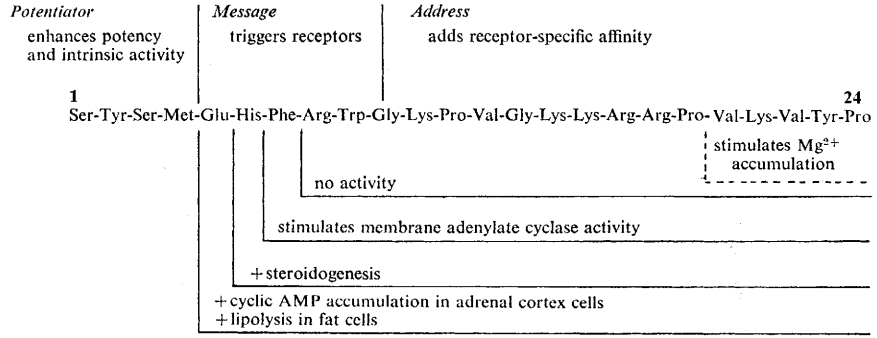
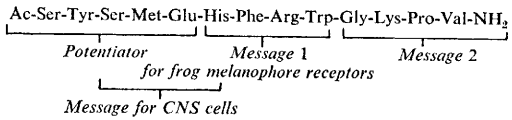


Table 9 *Organization of biological information for melanophores and central nervous cells in α-melanotropin*

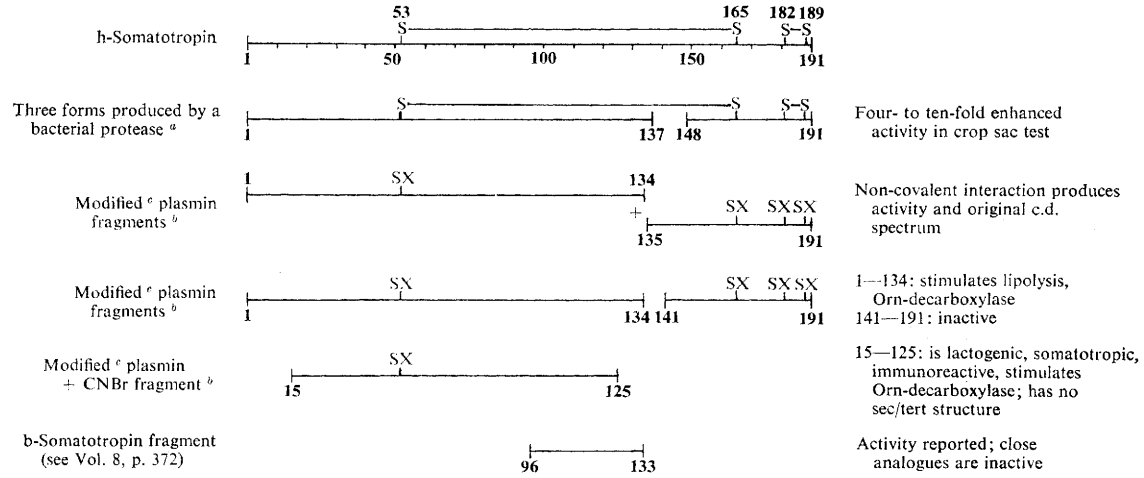


concentrations, inhibit adenylate cyclase activity in the reported concentration range (10—200 μmol l^{-1}).[269] This auto-inhibition persists in the presence of guanylyl imidodiphosphate[269, 273] and was also observed with lipocyte membranes.[274] The statement,[272] 'these findings support the postulate that cyclic AMP accumulation and steroidogenesis in adrenocortical cells are coupled processes', is not supported by the results[210, 269] summarized in Table 8. The peptide Phe-Arg-Trp--- stimulates adrenocortical membrane adenylate cyclase activity. The peptide His-Phe-Arg-Trp--- adds steroidogenesis to that property. But only if glutamic acid is added, as in Glu-His-Phe---, does cyclic AMP become accumulated in adrenocortical cells.

Interesting but unexplained observations have been reported: (i) injection of the tripeptide NH_2CO-Arg-Pro-Val-NH_2 in extremely large doses increases the

[271] K. Medzihradszky and H. Medzihradszky-Schweiger, *F.E.B.S. Letters*, 1976, **67**, 45.
[271a] A. Eberle, thesis, ETH Zürich, No. 5735, 1976.
[272] F. M. Finn, P. A. Johns, N. Nishi, and K. Hofmann, *J. Biol. Chem.*, 1976, **251**, 3576.
[273] H. Glossmann and C. J. Struck, *Naunyn-Schmiedeberg's Archiv Pharmacol.*, 1976, **294**, 199.
[274] U. Lang and R. Schwyzer, in 'Peptide Hormones', ed. J. A. Parsons, Macmillan, London, 1976, p. 337.

Table 10 *Somatotropin fragments produced enzymatically and by CNBr cleavage*

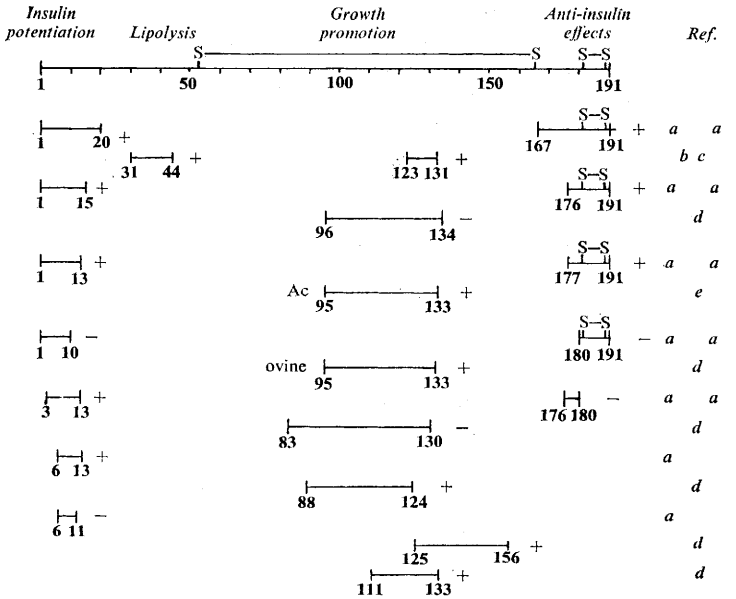


(*a*) U. J. Lewis, S. J. Pence, R. N. P. Singh, and W. P. VanderLaan, *Biochem. Biophys. Res. Comm.*, 1975, **67**, 617. (*b*) C. H. Li and T. A. Bewley, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1476; C. H. Li, T. Hayashida, B. A. Doneen, and A. J. Rao, *ibid.*, 3463; A. J. Rao, J. Ramachandran, and C. H. Li, *Proc. Soc. Exp. Biol. Med.*, 1976, **151**, 285; C. H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 3878; C. H. Li and T. A. Bewley, in 'Growth Hormone and Related Peptides', ed. A. Pecile and E. E. Müller, Excerpta Medica, Amsterdam and Oxford, 1976, p. 14. (*c*) X = CH_2CONH_2.

ascorbic acid depletion caused by subsequent administration of corticotropin; it also potentiates the action of corticotropin on the rat ascending colon;[275] (ii) the *in vitro* lipolytic effect of corticotropin is potentiated by the tetrapeptide amide Lys-Pro-Val-Gly-NH_2.[276]

Somatotropin information was investigated with the aid of enzymatically produced fragments (Table 10) and synthetic partial sequences (Table 11). Fragments generated with cyanogen bromide were used to determine immunologically active zones.[277] Cleavage of somatotropin between residues 137 and 148 yields products with enhanced activity. A mixture of fragments 1—134 and 135—191 is claimed to be equipotent with the native hormone, although the disulphide bonds have been reduced and alkylated. Shorter peptides between residues 1 and 134 are reported to be active. Many sequences have been prepared

Table 11 *Synthetic somatotropin fragments and a tentative interpretation in terms of sychnological organization of information (+ indicates biological activity, − lack of activity)*



(*a*) J. Bornstein, in 'Growth Hormone and Related Peptides', ed. A. Pecile and E. E. Müller, Excerpta Medica, Amsterdam and Oxford, 1976, p. 41. (*b*) N. A. Yudayev, Yu. P. Shvachkin, M. N. Ryabtsev, S. G. Chukashev, Yu. A. Pankov, and Yu. M. Keda *Bioorg. Khim.*, 1975, **1**, 1531; N. A. Yudayev, Yu. A. Pankov, Yu. M. Keda, Yu. P. Shvachkin, M. N. Ryabtsev, and S. G. Chukashev, *Biokhimiya*, 1976, **41**, 843; N. A. Yudayev, Yu. A. Pankov, Yu. M. Keda, Yu. N. Shvachkin, and M. N. Ryabtsev, *ibid.*, p. 1484. (*c*) S. S. Wang, C. C. Yang, I. D. Kulesha, M. Sonenberg, and R. B. Merrifield, *Internat. J. Peptide Protein Res.*, 1974, **6**, 103. (*d*) F. Chillemi, A. Aiello, A. Pecile, and V. R. Olgiati, in 'Growth Hormone and Related Peptides', ed. A. Pecile and E. E. Müller, Excerpta Medica, Amsterdam and Oxford, 1976, p. 50. (*e*) J. Blake and C. H. Li, *Internat. J. Peptide Protein Res.*, 1973, **5**, 123.

[275] E. A. Protopopov, V. E. Klusha, and G. I. Chipens, *Biokhimiya*, 1976, **41**, 389.
[276] J. A. Porunkevich, A. A. Skujinsh, and G. I. Chipens, *Biokhimiya*, 1976, **41**, 1448.
[277] E. Poskus, M. M. Zanin, H. N. Fernández, and A. C. Paladini, *European J. Immunol.*, 1976, **6**, 409.

by solid-phase peptide synthesis and investigated for activity. If the results are taken at face value, the picture of a sychnologically organized hormone with pleiotropic action (definitions and concepts [212]) emerges. Insulin potentiation and lipolysis would be located towards the *N*-terminus, anti-insulin effects at the *C*-terminus, and the effects on growth promotion, ornithine decarboxylase activity, *etc.* around the centre. However, here the confusion is great as indicated by the conflicting reports on biological activity (+ and − signs). A very critical team of authors [277a] points out that they find 96—134 inactive, 95—133 active, 83—130 inactive, 125—156 active, and 111—133 active. In view of the fallacies of both the synthetic and the test procedures one cannot be sure how to judge the work or even the heading of Table 11.

A prolactin *C*-terminal, heterodetic cyclic undecapeptide was synthesized and found to be devoid of pigeon crop sac stimulating or immunological activity.[278]

Quaternary Structure and Activity.—It is well known that the α- and β-subunits of different glycoprotein hormones can be recombined to hybrids with biological activity. It was found that h-follitropin dissociation and recombination can lead to a purification (90% reduction of the lutropin contamination).[279] A recombinant from the b-lutropin α-subunit and one of the subunits of a fish gonadotropin produced a gonadotropic activity in frogs that had not been present in either one of the starting materials.[280]

The mechanism and kinetics of the recombination process have been studied with respect to changes of the surroundings of tyrosine in h-choriogonadotropin,[281] the influence of the α-subunit *C*-terminus in lutropin,[282] and co-operativity phenomena in h-choriogonadotropin using fluorescence techniques.[283]

4 Pancreatic Hormones

Contributed by D. Brandenburg

Reviews have appeared on pancreatic hormones,[284, 285] on the biosynthesis of insulin and glucagon,[286–288] the mode of action of insulin,[289–291] and diabetes mellitus.[292] Molecular perspectives in diabetes research,[293] the impact of pro-

[277a] Table 11, ref. *d*.
[278] D. Yamashiro, M. Rigbi, T. A. Bewley, and C. H. Li, *Internat. J. Peptide Protein Res.*, 1975, **7**, 389.
[279] R. C. Shownkeen, A. S. Hartree, F. Stewart, K. Mashiter, and V. C. Stevens, *J. Endocrinol.*, 1976, **69**, 263.
[280] E. Burzawa-Gérard and Y.-A. C. R. Fontaine, *Compt. rend.*, 1976, **282**, *D*, 97.
[281] J.-C. Pernollet, J. Garnier, J. G. Pierce, and R. Salesse, *Biochim. Biophys. Acta*, 1976, **446**,262.
[282] A. G. Kiseleva and Yu. A. Pankov, *Biokhimiya*, 1976, **41**, 941.
[283] K. C. Ingham, C. Tylenda, and H. Edelhoch, *Arch. Biochem. Biophys.*, 1976, **173**, 680; K. C. Ingham, B. D. Weintraub, and Edelhoch, *Biochemistry*, 1976, **15**, 1720; K. C. Ingham, H. A. Saroff, and H. Edelhoch, *ibid.*, 1975, **14**, 4745, 4751.
[284] J. Meienhofer, *Ann. Reports Medicin. Chem.*, 1976, **11**, 158.
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[287] D. F. Steiner, in 'Peptide Hormones', Ed. J. A. Parsons, Macmillan, London, 1976, p. 49.
[288] C. L. Hew and C. C. Yip, *Canad. J. Biochem.*, 1976, **54**, 591.
[289] I. D. Goldfine, *Diabetes*, 1977, **26**, 148.
[290] A. Jakob and J. Zapf, *Ther. Umschau*, 1976, **33**, 586.
[291] N. P. Mertvetsov and O. E. Gordienko, *Voprosy med. Khim.*, 1976, **22**, 3.
[292] A. Y. Chang, *Ann. Reports Medicin. Chem.*, 1976, **11**, 170.
[293] D. F. Steiner, *IDF Bull.*, 1976, **21**, 7.

insulin on insulin research,[294] the interrelationships of insulin, glucagon, and somatostatin [295] as well as the diagnostic implications of serum C-peptide [296] have been discussed. Of particular importance in the context of this chapter is the review on the chemistry of insulin [297] and also its chemical synthesis.[298]

The literature on insulin-receptor studies continues to be extensive and is discussed, partially in relation to hormone action, in several reviews on membrane receptors.[299–304] The role of the insulin receptor in insulin resistance and diabetes is described.[305] The 1975 Minkowski award lecture, on interactions of insulin and glucagon with their receptors, has been published.[306] The physiology and pathophysiology of glucagon [307] and somatostatin [308–311] have been reviewed.

The proceedings of symposia on cell surface receptors,[312, 313] immunological aspects of diabetes mellitus,[314] insulin,[315a] and glucagon,[315b] and the abstracts of the International Diabetes Congress [316] have been published.

Insulin.—It has now been found that biosynthesis of proinsulin proceeds *via* a precursor, preproinsulin, with a molecular weight *ca.* 11 500 daltons, which contains an additional polypeptide sequence of 23 amino-acids at the *N*-terminus.[317] This precursor was synthesized by a cell-free ribosomal system derived from wheatgerm and primed with a mRNA containing fraction from rat pancreatic islets. The partial amino-acid sequence was found to be

Phe
H-X-Leu-Lys-Met-X-Phe-Leu-Phe-Leu-Leu-Lys-Leu-Leu-X-Leu-
X-X-X-X-X-X-X-X-proinsulin

[294] N. R. Lazarus, in 'Plasma Protein Turnover, 1974. Proceedings of the Sixth Meeting of the Plasma Protein Group, 1974', ed. R. Bianchi, G. Marinani, and A. S. McFairlane, University Park Press, Baltimore, 1976, p. 331.
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[296] D. L. Horwitz, H. Kuzuya, and A. H. Rubenstein, *New England J. Med.*, 1976, **295**, 207.
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[311] R. Assan, *Diabète Metabol.*, 1976, **2**, 135.
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[313] 'Cell Membrane Receptors for Viruses, Antigens, and Antibodies, Polypeptide Hormones and Small Molecules', Miles International Symposium Series No. 9, ed. R. F. Beers and E. G. Basset, Raven Press, New York, 1976.
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[315a] 'Insulin: Islet Pathology, Islet Function, Insulin Treatment', ed. R. Luft, *Acta Med. Scand.*, 1976, Suppl. 601.
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The extension is believed, as in the case of other presecretory proteins, to play a role in the sequestration of the nascent polypeptide chain within the microsomal cisternae. It is very rapidly cleaved by enzymes. Evidence for the synthesis of carp preproinsulin has also been found.[318]

The evolutionary origin of proinsulin has been analysed by new statistical methods, and the observed sequence similarities of proinsulin and serine proteases are interpreted to indicate a common genetic ancestor of the two protein families.[319] A first example of defective proinsulin–insulin conversion, manifested by a hyperproinsulinemia, has been detected.[320] C-peptide may have a physiological role in the down-regulation of insulin.[321]

The amino-acid sequence of rattlesnake (*Crotalus atrox*) insulin has been determined.[322] As compared to porcine insulin, replacements in the A-chain are [Glu^{8}], [Asn^{9}], and [Thr^{10}], and in the B-chain [Ala^{1}], [Pro^{2}], [Arg^{5}], [Phe^{16}], [Ile^{18}], [Tyr^{25}], and [Arg^{29}]. These largely conservative substitutions have no effect on the biological activity *in vivo* (*ca.* 90%). The biological activity of insulins from vertebrates has been discussed [323] in relation to the chemical structure.

Work on structure–function relationships of insulin has been extended in two directions: (i) the preparation of new insulins with altered structure, by synthesis or, in most cases, by chemical and/or enzymatic modification of the native molecule, and their primary characterization by the standard *in vivo* or *in vitro* bioassays; (ii) more detailed biological and conformational studies with known analogues and derivatives. The modified insulins are listed in Table 12, and information on biological activity as well as conformational studies is included.

Table 12 *Analogues and derivatives of insulin* [a]

Compound number	*Structure*[b]	*Species*[c]	*Biological activity*[d]	*Test*[e]	*Ref.*
1	Insulin[f]	h	++++	B	324
2	[Ala^{A1}]	p	++	E	325
3	[D-Ala^{A1}]	p	+++	E	325
*4	[β-Ala^{A1}]	p	+++	B	326
			+++	E	327
*5	[Guanido-acetyl^{A1}]	b	+++	C	327
*6	[Lys^{A0}]	b	+++	C	327
7	[Arg^{A0}]	b	+++	C	327
*8	[Met^{A0}]	b	+++	C	327
*9	[Phe^{A0}]	b	+++	C	327
*10	[Glu^{A0}]	b	+++	C	327

[318] T. A. Rapoport, W. E. Höhne, D. Klatt, S. Prehn, and V. Hahn, *F.E.B.S. Letters*, 1976, **69**, 32.
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[325] H.-D. Summ, H. Schiemann, and H.-D. Volkmann, *Z. physiol. Chem.*, 1976, **357**, 683.
[326] R. Geiger, K. Geisen, G. Regitz, and H.-D. Summ, *Z. physiol. Chem.*, 1976, **357**, 1267.
[327] H. Zahn, *Schriftenreihe der Bundesapothekerkammer zur wissenschaftlichen Fortbildung*, 1976, Band IV, Gelbe Reihe, 279.

Table 12 (*cont.*)

Compound number	*Structure*[b]		*Species*[c]	*Biological activity*[d]	*Test*[e]		*Ref.*
*11	Me_3N^+Np	A1	b	+++	A		328
12	Tfa	A1	b			*g*	329
13	$(Tfa)_2$	A1, B1	b			*g*	329
14	$(Tfa)_2$	A1, B29	b			*g*	329
15	$(Ac)_2$	A1, B29	b	+++	B	*i*	330
				+++	C		
16	Oxalyl [h]	A1, B29	b	+++	B	*i*	330
				++	C		
17	Suberoyl [h]	A1, B29	b	+++	B	*i*	330
				++	C		
18	Dodecoyl [h]	A1, B29	b	+++	B	*i*	330
				++	C		
19	$CO(Met)_2$ [h]	A1, B29	b	++	D	*g*	331
*20	Dsu [h]	A1, B1	b	++	C	*g*	332
*21	$Ad(Lys)_2$ [h]	A1, B1	b	++	C	*g*	332
*22	$(Z\text{-}Lys)_2(Ad)$ [h]	A1, B1	b	+	C	*g*	332
*23	$(^3H\text{-}Ac)_3$	A1, B1, B29	b	+++	D, E	*j*	333
24	$[Tyr(NO_2)^{A14}]$ [k]		b		F		334
*25	$[Iso\text{-}Asn^{A21}]$		o	+++	A		335
*26	$[Sar^{A1}$, iso-$Asn^{A21}]$		o	+++	A		335
*27	$[Asn\text{-}imide^{A21}]$		b	+++	A, C		336
28	Des-Asn^{A21}, des-Ala^{B30}		b		F		334
	Des-Asn^{A21}, des-Ala^{B30} [l]		b			*m*	337
*29	Des-Asn^{A21}, des-Asp^{B30}		gp	+	C, E		338
30	$(Msc)_2$des-Gly^{A1}	B1, B29	p	++	E		325
31	$[^3H\text{-}Phe^{B1}]$		p			*m*	339
*32	$[I_2\text{-}Tyr^{B1}]$		p	++++	B		340
*33	$(N\text{-}MePy)_3^+$	B1, Tyr	b	−	A	*n*	341
*34	$[Leu^{B9}]$		h	+++	A		342
35	Des(B23—30)	desoctapeptide	p	++	E		325
35	(= R)					*o*	343
					F		334

328 D. A. Sutton, B. R. D. Easter, and S. E. Drewes, *Z. physiol. Chem.*, 1976, **357**, 971.
329 R. A. Paselk and D. Levy, *Biochim. Biophys. Acta*, 1976, **439**, 479.
330 R. H. Jones, D. I. Dron, M. J. Ellis, P. H. Sönksen, and D. Brandenburg, *Diabetologia*, 1976, **12**, 601.
331 W.-D. Busse and F. Carpenter, *Biochemistry*, 1976, **15**, 1657.
332 D. Brandenburg, W. Schermutzki, A. Wollmer, H. P. Vogt, and J. Gliemann, in 'Peptides: Chemistry, Structure and Biology. Proceedings of the Fourth American Peptide Symposium, 1975', ed. R. Walter and J. Meienhofer, Ann Arbor Science Publishers, Ann Arbor, 1975, p. 497.
333 S. A. Kaplan, J. W. Morris, M. B. Davidson, L. E. Gerschenson, and M. Scott, *Metabolism*, 1976, **25**, 1209.
334 E. R. Arquilla, R. J. Dorio, and T. M. Brugman, *Diabetes*, 1976, **25**, 397.
335 A. Cosmatos, Y. Okada, and P. G. Katsoyannis, *Biochemistry*, 1976, **15**, 4076.
336 H.-G. Gattner and E. W. Schmitt, *Z. physiol. Chem.*, 1977, **358**, 105.
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341 S. E. Drewes and D. A. Sutton, *Z. physiol. Chem.*, 1976, **357**, 1261.
342 G. Schwartz and P. G. Katsoyannis, *Biochemistry*, 1976, **15**, 4071.
343 M. D. Hollenberg, *Life Sci.*, 1976, **18**, 521.

Table 12 (*cont.*)

Compound number	*Structure*[b]		*Species*[c]	*Biological activity*[b]	*Test*[e]		*Ref.*
*36	R-Gly	desheptapeptide	b	++	A		344
			b, p				345
37	R-Gly-Phe	deshexapeptide	b	+++	A		344
*38	R-Gly-Ala		b	++	A		344
39	R-Gly-Phe-Phe	despentapeptide	p				346
	(= R′)		b	+++	E	*p*, *g*, *n*	347—351
			b	+++	A		344
*40	R-Gly-Phe-Ala		b	+++	A		344
41	R′-Tyr	destetrapeptide	b	+++	A		344
*42	R′-Ala		b	+++	A		344
43	R′-Tyr-Thr		b	+++	A		344
44	Des-Ala^{B30}					*o*	343
*45	Insulin dimer	$N^{\alpha A1}$-$C^{\beta A21'}$	b	−	C		327
*46	Insulin dimer	$N^{\alpha B1}$-$C^{\beta A21'}$	b	+++	C		327
*47	Des-Arg^{31}, des-Arg^{32}-proinsulin		b	+++	B	*g*	352
				++	C		
*48	Des-Lys^{59}, des-Arg$^{60'}$-proinsulin		b	+++	B		352
				+++	C	*g*	

(*a*) New compounds are marked by an asterisk. (*b*) Me_3N^+-Np = 2-nitro-4-trimethylammoniophenyl; *N*-MePy$^+$ = *N*-methylpyridinium. (*c*) Human = h; bovine = b; porcine = p; ovine = o; guinea-pig = gp. (*d*) Symbols for potency are used as in Table 1. (*e*) The following tests are indicated: A, mouse convulsion assay; B, *in vivo* blood glucose assay; C, isolated fat cell assay; D, *in vitro* glucose oxidation; E, receptor binding; F, immunological studies. (*f*) Semisynthetic. (*g*) Conformational studies. (*h*) Cross-linked. (*i*) Half-life and effect on glucose studied. (*j*) Induction of enzymes in liver cells. (*k*) 76% A14. (*l*) Iodinated. (*m*) Degradation studies. (*n*) *X*-ray studies. (*o*) AIB incorporation into fibroblasts. (*p*) Glucose incorporation into rat diaphragma.

Immunoreactivity values are not given, for the same reasons as in Vol. 8, and because these depend on the particular antiserum used and are thus not comparable.

The analogues (25), (26), and (27) have been obtained by total synthesis of the corresponding A-chains and combination with natural B-chain, (34) from the synthetic [Leu^{13}] B-chain and natural A-chain. Compound (4) was obtained semisynthetically as described for the [Ala^{A1}]-analogue,[297] and (32) by removal of [Phe^{B1}] and its replacement by I_2-Tyr following the strategy described by

344 G. Weitzel, F.-U. Bauer, and K. Eisele, *Z. physiol. Chem.*, 1976, **357**, 187.
345 Y. P. Shvachkin, V. F. Krivtsov, and G. A. Shmeleva, *Zhur. obshchei Khim.*, 1976, **46**, 1911.
346 G. A. Shmeleva, V. F. Krivtsov, F. V. Abramova, A. I. Ivanova, V. P. Fedotov, and Y. P. Shvachkin, *Zhur. obshchei Khim.*, 1976, **46**, 1628.
347 Insulin Research Group, Shanghai, Peking Insulin Structure Research Group and Biochemical Preparation Group, *Sci. Sinica*, 1976, **19**, 351.
348 Peking Insulin Structure Research Group, *Sci. Sinica*, 1976, **19**, 358.
349 Insulin Research Group, Shanghai, *Sci. Sinica*, 1976, **19**, 475.
350 Conformational Study Group and Peking Insulin Structure Research Group, *Sci. Sinica*, 1976, **19**, 486.
351 Conformational Study Group, *Sci. Sinica*, 1976, **19**, 497.
352 J. Markussen and L. G. Heding, *Internat. J. Peptide Protein Res.*, 1976, **8**, 597.

Geiger [297] under temporary protection of A1- and B29-amino-groups with the methylsulphoethyloxycarbonyl residue. The monofunctional derivatives (11) and (33), as well as the A1—B1-cross-linked ones (20)—(22) were isolated by fractionating the arylation/acylation products of unprotected insulin. For the preparation of the A1-modified insulins (5)—(10), A1-Tfa-insulin was first protected at the amino-groups in positions B1 and B29 with the t-butyloxycarbonyl residue, then deblocked at A1. This was followed by acylation at this site with 1-amidino-3,5-dimethylpyrazole (5) or Boc-protected activated esters, and then complete deblocking.

Carboxypeptidase-A treatment of guinea-pig insulin gave the analogue (29). Ruttenberg's procedure [353] did not lead to reproducible results in several laboratories, it could be modified to give the series of analogues (36)—(44) by coupling the corresponding peptides to A1,B1-$(Boc)_2$-desoctapeptide(B23—30)-insulin$(OMe)_5$. Attempts to saponify insulin$(OMe)_6$ led, under certain conditions, to cyclization at [Asn^{A21}] and formation of (27). The difficulties inherent in esterification and saponification were circumvented in the semisynthesis without any protection of carboxy-groups, by which fully active, crystalline human insulin (1) could be obtained in a yield of *ca.* 10%, based on porcine insulin.

The split proinsulins (47) and (48), as well as the insulin dimers (45) and (46), in which two monomers are linked *via* isopeptide bonds involving [Asn^{A21}] and the α-amino-group of [Gly^{A1}] and [Phe^{B1}], respectively, have been isolated from the b-component which is separated from crude insulin by gel filtration. Of the other, previously described insulins, compounds (2), (3), (15), (16), (28), (31), (35), (36), (39) and (44) as well as (19), for which full experimental details are now given, were listed in Table 7 of Vol. 8.

The joint study on the receptor binding area of insulin,[354] in which A1-acylated derivatives were analysed by *X*-ray, c.d., and biological methods, was discussed in Vol. 8. Further modifications have been carried out at this site. The replacement of [Gly^{A1}] by L-Ala results in a decrease of half-maximal binding to partially purified rat liver receptors to 6%, while the affinity of the D-Ala analogue is 43%.[325] Shifting the amino-group of glycine by one CH_2-group (~1.5 Å), as in compound (3), reduces binding affinity to 46—54% and, likewise, potency *in vivo* to 45%.[326] Almost full activity (87%) is retained *in vitro* when glycine is exchanged for the strongly positive guanido-acetyl residue.[327] The side-chain of amino-acids added to the *N*-terminus modulates the activity of the analogues: compounds (6) and (7) have potencies of 40—45%, whereas (10) with a negative charge is only 22% active.[327]

Addition of the positively charged, large 2-nitro-4-trimethylammoniophenyl group (11) reduces the potency to 60% *in vivo*.[328] As all the compounds (5)—(11) give well-formed two-zinc rhombohedral crystals, structural changes are presumably not extensive (*cf.* Pullen *et al.*).[354] Linking the *N*-terminals of insulin causes almost complete loss of biological activity *in vitro*.[332] This effect is independent of the length of the cross-link (8, 12, and 20 atoms). C.d. spectra indicate substantial changes of conformation.

[353] M. A. Ruttenberg, *Science*, 1972, **177**, 623.
[354] R. A. Pullen, D. G. Lindsay, S. P. Wood, I. J. Tickle, T. L. Blundell, A. Wollmer, G. Krail, D. Brandenburg, H. Zahn, J. Gliemann, and S. Gammeltoft, *Nature*, 1976, **259**, 369.

The proinsulin intermediate (47), in which a chain of 28 amino-acids is attached to the A-chain, has a potency of 9% *in vitro*.[352] *In vivo*, a protracted effect is observed, and the activity is markedly higher (*ca.* 50%). The c.d. spectrum indicates that this molecule contains less α-helix than insulin or proinsulin, probably due to an unwinding of the α-helix in the region A2—8.[352] The dimer (45) is inactive.[327] This shows that attachment of a whole insulin molecule to either terminus of the A-chain is detrimental for the receptor binding region of both monomer moieties.

At the *C*-terminus of the A-chain, [Asn^{21}] can be replaced by iso-Asn with virtually no activity loss *in vivo*, as demonstrated with the analogues (25) and (26).[335] It was concluded that the α- and β-carboxy-groups of Asn are nearly equivalent in terms of their contribution to the expression of biological activity. Incorporation of the carboxy-group into the imide ring with loss of negative charge gives the compound (27), which is still quite active *in vitro* (40%) and *in vivo* (55%).[336] On the other hand, removal of [Asn^{21}] from guinea-pig insulin results in almost complete loss of activity.[338] The potency measured with rat fat cells drops from 4% to *ca.* 0.2%, and the affinity for rat liver membrane receptors is similarly reduced. Although in guinea-pig insulin [Arg^{B22}] is exchanged for Asp, the relative effect of inactivation upon removal of [Asn^{A21}] is comparable to that observed with the 'classical' analogue des-Asp-des-Ala-insulin (28). These joint findings are evidence against the predominant role of a salt-linkage between the α-carboxy-group of [Asn^{A21}] and [Arg^{B22}] in maintaining insulin in an active conformation, and point towards a more direct involvement in receptor binding.

In the B-chain, replacement of [Phe^{B1}] by di-iodo-Tyr is possible without change of activity, an important finding with respect to tracer studies.[340] The ^{125}I-labelled analogue has been obtained in preliminary experiments. The inactivation caused by replacement of [Ser^{B9}] by Leu is moderate (56%).[342]

Compound (33) is a very interesting derivative, since it crystallizes readily in the two-zinc form, but is devoid of any biological activity.[341] Unfortunately, it is as yet unclear to which tyrosines the two *N*-methylpyridinium residues are attached. Interpretations have therefore to await further investigations.

Des-heptapeptide-insulin (36) is, like the well-known des-octapeptide-insulin (35), almost inactive.[344] Addition of [Phe^{B24}], but not of Ala, results in a small increase of activity (14%). A marked rise to 36% is observed when the sequence is further elongated to give des-pentapeptide-insulin (39) or the [Ala^{B25}]-analogue. Addition of [Tyr^{26}] brings the potency up to 61%. The [Ala^{B26}]-analogue is equipotent. Finally, coupling of a further Ala-residue gives an [Ala^{B27}]-des-tripeptide-insulin with 91% activity. Weitzel *et al.*[344] concluded that the activity of insulin proceeds from the [Arg^{B22}]-residue and is only intensified by the aromatic amino-acids (B24—26), and drew parallels to the activity of their synthetic oligopeptides described earlier.

The potency of des-pentapeptide-insulin corresponds well to the values found previously *in vivo* [355, 356] and *in vitro* (17—27%).[356] In contrast, the Chinese groups find essentially full activity, in agreement with an earlier report,[357] and

[355] P. G. Katsoyannis, J. Ginos, G. P. Schwartz, and A. Cosmatos, *J.C.S. Perkin I*, 1974, 1311.
[356] H.-G. Gattner, *Z. physiol. Chem.*, 1975, **356**, 1397.
[357] S. Terris and D. F. Steiner, *J. Biol. Chem.*, 1975, **250**, 8389.

high receptor binding.[347] The values are 80% in the mouse convulsion assay, 77% with respect to glucose incorporation into rat diaphragm, and 88% in the binding assay. Monoclinic Cd^{2+} and orthorhombic Zn^{2+} crystals have now been obtained.[348] The analogue was subjected to a number of physicochemical studies.[349–351] Gel filtration at pH 7 confirmed the findings[356] that self-aggregation does no longer occur, and difference u.v. spectroscopy showed all three tyrosines to be unmasked.[349] The far c.d. spectrum is similar to the spectrum of insulin, but there are significant differences in the near u.v.[349] (*cf.* ref. 356). High-resolution n.m.r. studies (250 MHz) also suggest local conformational changes in solution.[351] According to fluorescence spectroscopy using 1-*p*-toluidinylnaphthalene-8-sulphonate, the hydrophobic region of (39) at physiological pH is more exposed than in insulin, and the conformation of the analogue less stable.[350]

These findings of partially altered conformation would lend themselves to explain the *decreased* activity of des-pentapeptide-insulin[344, 355, 356] along the lines of reasoning by Pullen *et al.*[354] However, on the basis of *high* activity[347] in spite of the absence of the β-pleated sheet structure, it is assumed[351] that this structure is important only in dimerization, not for the exhibition of the hormonal activity.

The intermediate of the proinsulin–insulin conversion (48) is more potent *in vivo* (*ca.* 50%) than *in vitro* (19%). The c.d. spectrum indicates some smaller conformational changes.[352] The activity of the A21—B1′-linked insulin dimer is 25% *in vitro i.e.* one dimer molecule is about half as active as one insulin molecule.[327]

The ability to stimulate α-aminoisobutyrate uptake by cultured human fibroblasts decreases in the order insulin = des-Ala^{B30}-insulin (44) > proinsulin > des-octapeptide-insulin (35). It parallels exactly the relative potency of the compounds with respect to glucose metabolism in fat and muscle preparations, but the concentrations for half-maximal stimulation are higher.[343] Labelled triacetyl-insulin (23) exhibits decreased activity in the induction of tyrosine-α-ketoglutarate transaminase in cultured liver cells, as well as in fat-pad glucose oxidation. Receptor binding to liver cell membranes was concentration dependent, indicating an impairment of negative co-operativity.[333]

The apparent discrepancy between low *in vitro* and much higher *in vivo* biopotency of modified insulins like compounds (15), and particularly (16)—(18), as well as proinsulin, can be largely explained by a lower rate of metabolism.[330] This was demonstrated by monitoring the plasma concentration of glucose *and* insulin derivative during *in vivo* experiments using a priming-dose constant infusion technique. For example, the dose-related *in vivo* activity of suberoyl-insulin (17) is 63%, while the potency calculated in relation to the actual plasma concentration is only 6.9%, in good agreement with the (concentration-related) value of 5.6% in the usual fat-cell assay. It can, therefore, be rather misleading to base conclusions about the effect of structural modifications on the biological activity of insulin *only* on the results of *in vivo* experiments, like the standard blood glucose assay or mouse convulsion assay, as it is still frequently done. Within the field of structure–function studies, structure–metabolism relationships require adequate attention. These certainly will be aided by new labelled insulins such as compounds (31), (32), and (23).

The mechanism of insulin degradation by the liver, the major site of inactivation, becomes clearer. Degradation of ^{125}I-labelled insulin by isolated hepatocytes [357] and perfused rat liver [337] is preceded by binding to recognition sites. In contrast to insulin, retention and degradation by the liver of two iodinated analogues of low potency, (28) and proinsulin, are low.[337] Likewise, a series of modified insulins inhibited the degradation of labelled insulin roughly in parallel to their biopotency *in vitro*.[357] ^{3}H-Labelled triacetyl-insulin (23) is also degraded more slowly than insulin by cultured liver cells and in other systems.[333] Furthermore, the acetyl groups were shown to be stable. The results indicate that the three-dimensional structure of insulin not only determines the hormone's ability to exert its action, but also directs its degradation *via* interaction with recognition sites. The mode of degradation in isolated liver cells has been shown to be sequential,[358] *i.e.* a cleavage of disulphide bonds by glutathione-insulin transhydrogenase followed by proteolysis of the A- and B-chains. The ^{3}H-labelled insulin (31) is degraded by perfused mouse liver at the same rate as native insulin, and thus is a valid tracer.[339] While the specific activity (15 Ci mmol^{-1}) is of the order of 1—2% compared with the preparations of iodinated insulins usually employed, the site of label in the B-chain is favourable. With respect to insulin degradation in man, further support for the concept of an autoregulatory system, in which the rate of insulin metabolism changes in response to its blood levels, has been produced.[359]

Chemically modified insulins have been used to study the antibody response to bovine insulin in the guinea-pig.[360, 361] Immunological studies with antiserum raised against (28) in guinea-pigs show that insulin and derivatives as (24), in which the tyrosines A14 and, in part, A19 are modified, exhibit high cross-reactivity, whereas that of bovine proinsulin and (35) is markedly lower.[334] The connecting peptide of proinsulins seems to be in close proximity to the solvent surface of the A-chain, including the region A14/A19.

Different methods for the preparation of ^{125}I-insulin by means of iodide/chloramine T have been compared and the distribution of iodine atoms found to be random and to depend only on the average iodination number.[362]

The pentamethyl esters of bovine des-Lys^{B29}-des-Ala^{B30}- and des-nonapeptide-(B22—30)-insulin, as well as of the analogues (44), (39), and (35) with one free carboxy-group at the *C*-terminus of the B-chain, have been prepared as intermediates for semisyntheses.[363] Unprotected insulin has been *N*-acylated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and then coupled to β-D-galactosidase to give a conjugate with sufficient immunoreactivity and enzymatic activity for an enzyme-linked immunoassay of insulin.[364] Oxidation

[358] P. T. Varandani and M. A. Nafz, *Diabetes*, 1976, **25**, 173.
[359] T. Uete, N. Shimano, S. Shimizu, and M. Morikawa, *Metabolism*, 1976, **25**, 375.
[360] P. J. Hollins, G. Howard-Smith, and R. L. Himsworth, *Clin. Exp. Immunol.*, 1976, **24**, 507.
[361] P. J. Hollins and R. L. Himsworth, *Clin. Exp. Immunol.*, 1976, **23**, 507.
[362] B. Schneider, E. Straus, and R. S. Yalow, *Diabetes*, 1976, **25**, 260.
[363] H.-G. Gattner, E. W. Schmitt, and H. Zahn, in 'Peptides, 1976. Proceedings of the 14th European Peptide Symposium,' Wépion, Belgium, 1976, ed. A. Loffet, Editions de l'Université de Bruxelles, p. 279.
[364] T. Kitagawa and T. Aikawa, *J. Biochem.* (*Japan*), 1976, **79**, 233.

of insulin with peroxidase and hydrogen peroxide leads to cross-linking under formation of 3,3′-dityrosine.[365]

Preparation and properties of insulin derivatives with intra- and intermolecularly cross-links, formed by a variety of dicarboxylic acids and *m*-phenylene di-isothiocyanate, are discussed.[366] Reoxidation of reduced $CO(Met)_2$-insulin (19) and removal of the cross-link with cyanogen bromide give a fully active insulin in a yield of 60%, based on the reduced derivative.[331] C.d. spectroscopy and partial enzymatic degradations have been used to study the reaction and products. Reoxidation of reduced A1—B1-linked insulins is less favoured, but active insulin crystals have been obtained from the derivative (20).[332] Improved yields in the combination of separated A- and B-chains have been reported when *S*-sulphonated B-chain was bound to anti-insulin antibodies coupled to Sepharose and then treated with reduced A-chain.[367]

The crystal structure of porcine four-zinc insulin has been elucidated and compared with that of two-zinc insulin.[368] A surprisingly large change in the conformation of molecule I was found. There is a major rearrangement of the first eight amino-acid residues of the B-chain into a helical conformation, in contrast to the original extended form. The regions B9—30 of both monomers are very similar and the dimerizing site is preserved. The A-chains of the monomers I and II are found to be quite similar but not in position relative to the B-chains. This results in the appearance of a cleft within monomer I, exposing [Tyr^{A19}]. Encouraging results have been obtained in calculating the c.d. of the thyosines on the basis of the atomic co-ordinates of two-zinc insulin.[369] The conformational potential of C-peptides has been analysed on the basis of c.d. data.[370] ^{19}F N.m.r. spectroscopy, in conjunction with c.d. spectroscopy and sedimentation velocity studies, have been used to analyse the effects of zinc ions, salts, and denaturants on the structure and aggregation properties of trifluoroacetylated insulins (12)—(14).[329] In the absence of perturbants, the Tfa-group on A1 showed considerably more motional freedom than that on B1. Sulphonylureas affect the c.d. spectrum of insulin at pH 7.4, but the meaning of this observation with respect to the pharmacological action of sulphonylureas is not yet clear.[371]

Evidence has been found for the occurrence of extra-pancreatic immunoreactive insulin in the gastric mucosa of pancretectomized pigs.[372] The primary structure of NSILA I and II (non-suppressible insulin-like activity) has been partially resolved.[373] There is a striking similarity between the *N*-terminal sequence (1)—(31) and the B-chain of human insulin. Of the amino-acid residues 47%, including the half-cystines, are identical. A common evolutionary origin

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[372] C. Kühl, S. L. Jensen, and O. V. Nielsen, *Endocrinology*, 1976, **99**, 1667.
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of insulin and NSILA is assumed, and a new name, insulin-like growth factor (IGF), proposed.

An attempt to discuss the abundant new literature on insulin receptors (see also refs. 299—306, 312, 313) is beyond the scope of this review. The solubilized receptor (mol. wt. *ca.* 300 000 daltons) appears to be an oligomeric structure comprised of four or more binding sites.[374] Under the influence of insulin, a dissociation into subunits has been observed, and related to negative co-operativity. This phenomenon has been further characterized in a detailed study,[375] but it is disputed by others.[376] Present concepts about relations between insulin and glucagon receptors and the cause of diabetes have been discussed.[377] It appears that one of the major causes of maturity-onset diabetes is a defect in insulin binding to receptors on the target cell surface. Binding of insulin and glucagon to human lymphocytes derived from diabetic patients is substantially reduced,[378] an observation which might find diagnostic application. Insulin binding and its relation to action on glucose metabolism has been studied in the rat.[379] Specific binding sites for ^{125}I-labelled insulin were detected in purified nuclei from rat liver cells.[380] Des-octapeptide-insulin (35) and pro-insulin were effective in inhibiting binding, although with low affinity. It is suggested that the nucleus might be an intracellular site of insulin action.

Circulating antibodies to the insulin receptor have been found to be the cause of severe insulin resistance in several patients. Such antibodies are now being used as new tools to study the insulin receptor. They act as competitive inhibitors of insulin binding and are also able to stimulate glucose oxidation *in vitro*.[381]

Glucagon.—The proceedings of the glucagon symposium,[315b] containing the papers and discussions in condensed form, present an up-to-date survey of the entire field of glucagon research, including valuable information on the chemistry and structure–function relationships. Unfortunately they were received too late to be integrated into this text.

The biosynthesis of glucagon is less clear than that of insulin. When it was studied in isolated pigeon islets,[382, 383] the formation of three species, a 20 000 dalton protein, 'big glucagon' (8200 daltons), and 'little glucagon' (3500 daltons) was observed. The relation of the high molecular weight form, which was also found in turkey, dog, and ox pancreas and in the dog circulation, to the other two forms is as yet unclear. No precursor–product relationship was evident between big and little glucagon. A 9000 dalton protein has been isolated [382] which contains 76 amino-acids, including all those of turkey glucagon,

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and is similar with respect to amino-acid composition to angler fish 'proglucagon'. Purified big glucagon from bovine pancreas [384] (mol. wt. 8200, according to gel filtration) is equipotent, on a molecular basis, to glucagon in stimulating lipolysis in isolated chicken fat cells, and mouse islet adenylate cyclase, but, in contrast to glucagon, it inhibits basal liver adenylate cyclase. It is *ca.* one-sixth as effective in displacing labelled glucagon from its liver membrane receptor. The half-life in the rat is prolonged (6.9 *vs.* 32 min). Big glucagon is without effect on blood glucose concentration (up to 5 $\mu g\,kg^{-1}$) in the rat. No similarity, except for immunoreactivity in some systems, was found between pancreatic porcine glucagon and a highly purified protein from porcine gut with a molecular weight of 11 625.[385] Neither the *N*- nor the *C*-terminal amino-acid sequences were identical.

A new synthesis of glucagon has been described in which the four fragments 22—26, 16—21, 7—15, and 1—6 were prepared in solution and then coupled successively to the resin-bound *C*-terminal tripeptide 27—29.[386] The efficiency in each of the coupling steps was better than 95%, giving an overall yield of *ca.* 17%. The hyperglycaemic activity of the crystallizable hormone in rabbits was close to that of native glucagon. The interaction of glucagon and related peptides with chicken adipose tissue has been studied.[387] The lipolytic activities of glucagon and the synthetic fragment 1—23 in human adipocytes are equal, but very low.[388] However, in rat fat cells, in which glucagon is much more active, the full effect can only be achieved with the complete molecule.

From the finding that des-His-glucagon produces similar Arrhenius plots to glucagon upon incubation with rat liver membranes, it was concluded that the analogue, without activating adenylate cyclase, causes a structural coupling between the receptor and the catalytic unit.[389]

A derivative, in which the C-2 position of [Trp^{25}] is substituted with the *o*-nitrophenylthio (Nps) group, and a dimer, in which two molecules are cross-linked between the same positions with the 2,4-dinitrophenyl-1,5-dithio (N_2pS_2) residue, have been prepared according to previously published methods, and were subjected to physicochemical and biological studies.[390] C.d. spectra suggest an increased content of secondary structure, especially at low temperatures and low pH. Stimulation of glucose release from liver slices by the monomeric derivative was not impaired, while the dimer was significantly less active on an equal weight basis. When compared to glucagon in their ability to stimulate liver adenylate cyclase on a molar basis, the monomer exhibited a slightly enhanced activity; and the dimer was about as active as the native hormone. Thus, an intact Trp residue is not essential for biological activity.

Further studies on the iodination of glucagon and the effect of iodination on receptor binding and biological action have been carried out.[391] Radioiodination

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with the lactoperoxidase method is pH-dependent.[392] At high pH, both the substitution and the monoiodo/di-iodotyrosine ratio increase. Thus, labelled glucagon with a very low content of di-iodotyrosine could be prepared at pH 10.

Other chemical work on glucagon includes the coupling to serum albumin by means of difluorodinitrobenzene, leading to a preparation in which an average of about nine glucagon molecules were bound as haptens to the amino-groups of one carrier molecule.[393] A selective cleavage of glucagon at the [Trp^{25}] residue has been achieved, with simultaneous transformation of Met into the sulphoxide, by oxidative chlorination with *N*-chlorosuccinimide (32% yield).[394] Glucagon and glucagon peptides have been subjected to conformational studies by n.m.r. spectroscopy.[395]

Somatostatin.—In addition to somatostatin, the D-cell of human pancreatic islets contains also gastrin.[396] A previously described 'big' form of pancreatic somatostatin can be dissociated with 8M-urea and thus represents a non-covalent complex and not a 'classical' prohormone.[397]

Several studies of the mechanism of somatostatin action on the secretion of insulin and glucagon [398, 399] and on Arg-induced secretion [400, 401] have been carried out; only a few references are given here. A concept has been put forward according to which a local 'paracrine' restraint is exerted by D-cells upon the secretory activity of neighbouring cells, particularly the glucagon-producing A-cells.[401]

Several analogues were assayed with respect to their ability to inhibit spontaneous [402] and Arg-induced [403] insulin and glucagon release in rats. [D-Trp^{8}]-somatostatin was 6—8 times more active.[403] This analogue possesses enhanced stability against degradation in serum.[404] Two cyclic analogues, [Glu^{14}]-somatostatin-(4—14)-undecapeptide, in which the side-chains of [Lys^{4}] and [Glu^{14}] are linked, and [Abu^{3}, Asp^{14}]-somatostatin-(9—14)-dodecapeptide, in which a similar ring is present between the side-chains of residues 3 and 14, have been prepared.[405] They had no effect on insulin and glucagon release, but were active in inhibiting the release of growth hormone. Other analogues could be obtained which inhibit the release of glucagon and growth hormone more than the release of insulin.[406]

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Pancreatic Polypeptide.—The polypeptide, which contains 36 amino-acids, is thought to be a hormone, but its physiological role is still poorly understood. Recent work has been mainly concerned with the localization of PpP-roducing cells and their structural and ultrastructural features. Rat pancreas contains two populations of islets located at different parts, one with numerous PP-cells in the periphery, the other with glucagon cells, besides the predominant B-cells in both types.[407] By means of light and electron microscopic immunocytochemistry it could be demonstrated that in a large number of mammals, PP is present in endocrine-like cells distinct from the A,- B,- and D-cells.[408]

5 Gastrointestinal Hormones

Contributed by M. Bodanszky and A. Bodanszky

Gastrointestinal hormones have been reviewed.[409] A review of peptide hormones[410] presents considerable material on gastrointestinal hormones. These can be arranged in two major groups. The 'gastrin family' consists of gastrin, cholecystokinin-pancreozymin (CCK-PZ), and caerulein. The last-named is not found in the gut, but in the skin of the frog, yet is structurally related to gastrin and CCK-PZ (Figure 1). Secretin, glucagon (a pancreatic

CCK-PZ Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-

G-34 Glp-Leu-Gly-Pro-Gln-Gly-His-Pro-Ser-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-

CCK-PZ -Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr*-Met-Gly-Trp-Met-Asp-Phe-NH_2

G-34 -Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr*-Gly-Trp-Met-Asp-Phe-NH_2

Caerulein Pyr-Gln-Asp-Tyr*-Thr-Gly-Trp-Met-Asp-Phe-NH_2

Figure 1 *The gastrin family (regions of homology underlined) Tyr* denotes a sulphate ester of tyrosine; CCK-PZ is a porcine peptide; G-34 isthe sequence in humans*

hormone, but found also in the intestines), the vasoactive intestinal peptide (VIP), and the gastric inhibitory peptide (GIP) are the members of the 'secretin family' (Figure 2). Motilin (Figure 3), so far, stands alone.

Gastrin.—A valuable comprehensive review has been published.[411] It deals, in addition to the best studied 'little gastrin' with 17 residues (G-17), also with human 'big gastrin' (G-34) and 'mini-gastrin' (G-14) (Figure 4). Syntheses of human G-34 and its 32-leucine analogue were accomplished.[412] The radio-immunoassay[413] of gastrin necessitated a study of the biological activity of the

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	1							10						
Secretin	His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg-
VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg-
Glucagon	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu-
GIP	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met-

	15							25			
Secretin	Asp	Ser	Ala	Arg	Leu	Gln	Arg	Leu	Leu	Gln	Gly	Leu	Val	NH_2
VIP	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn-NH_2
Glucagon	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn-Thr-
GIP	Asp	Lys	Ile	Arg	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala-Gln-

	30									40				
GIP	Gln	Lys	Gly	Lys	Lys	Ser	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln-

Figure 2 *The secretin family (porcine sequences)*

iodinated hormone.[414] Its immuno-reactivity led to the discovery of a gastrin in the mollusc *Apylysia californica* (sea hare).[415] The determination of the exact physiological role of gastrins has received increasing attention.[416] Such studies are

1				5				10				15			
Phe	Val	Pro	Ile	Phe	Thr	Tyr	Gly	Glu	Leu	Gln	Arg	Met	Gln	Glu	Lys	Glu	Arg-
	20														
Asn	Lys	Gly	Gln												

Figure 3 *The sequence of porcine motiline*

now facilitated by the availability of tritiated hormones.[417] Numerous analogues of the *C*-terminal portion of gastrin were synthesized and studied [418] to determine the structural requirements for biological activity. The conformational freedom of the *C*-terminal tetrapeptide was examined by proton spin–lattice relaxation.[419]

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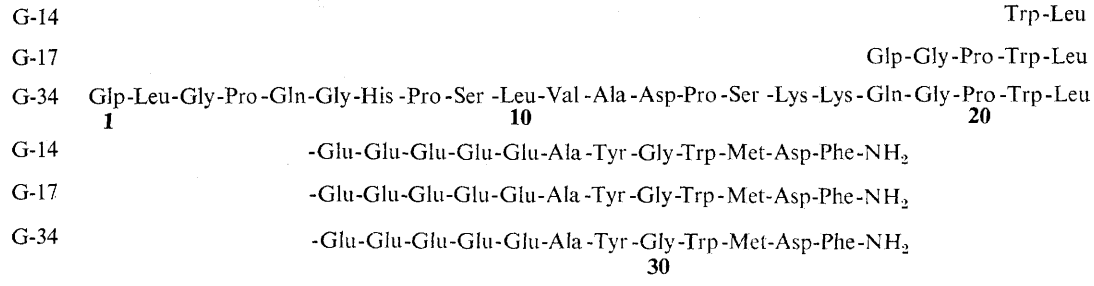

Figure 4 *Human gastrins. Glp, pyroglutamyl. Numbering of residues corresponds to G-34. Tyr-28 is present as sulphate ester in gastrin II-s*

Cholecystokinin-Pancreozymin (CCK-PZ).—The entire sequence of the 33-residue chain (Figure 1) was synthesized, except that the tyrosine sulphate residue in position 27 was replaced by tyrosine.[420] The synthetic material is about 250 times less potent than the natural hormone. A heptapeptide sequence (17—23) was prepared in protected form.[421] The sequence of a 39-residue variant of CCK-PZ, with Tyr-Ile-Gln-Gln-Ala-Arg at the *N*-terminal, was determined.[422] The action of CCK-PZ on calcium transport in pancreatic acinar cells,[423] and the interactions between CCK-PZ and other gastrointestinal hormones, were studied.[424] The related (*cf.* Figure 1) caerulein and some of its analogues inhibit gastric secretion in humans.[425] Structure–activity relationships in ceruletide (=caerulein) like peptides were examined.[426]

Secretin.—Porcine secretin was synthesized in solution by the repetitive application of the mixed anhydride method[427] and also on a solid, benzhydrylamine-type support.[428] Segments of an ornithine analogue of the hormone were built on a soluble polymer.[429] The *C*-terminal tricosapeptide portion of secretin, together with the [Gln^9], [Asn^{15}], and [Gln^9-Asn^{15}] analogues of this peptide, were synthesized simultaneously by the *in situ* technique.[430] The activities of these tricosapeptides on pancreatic acinar cells were also studied.[431] The chiroptical properties of the corresponding Asp^9-Lys^{21} analogue suggest that the short helical stretch in secretin is located near the *C*-terminus of the chain.[432]

Methods for the radioimmunoassay of secretin and VIP have been developed and applied in several laboratories.[433] On pancreatic membranes of the cat, separate receptors could be recognized for secretin and VIP,[434] although VIP is a competitive inhibitor of secretin. The secretin-stimulated bile-flow involves a receptor different from the one stimulated by glucagon.[435] There are two

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independent message sequences in secretin.[436] The results of treatment of duodenal ulcer in patients with secretin are not encouraging: notwithstanding a definite alkalinization by the hormone, it was not more effective than a placebo.[437]

Vasoactive Intestinal Peptide (VIP).—The sequence of the *C*-terminal hendecapeptide of chicken VIP, a cyanogen bromide fragment, has been confirmed by synthesis.[438] Immunoassays of lung tissues revealed the presence of peptides closely related to VIP.[439] The radioimmunoassay of VIP allowed its detection in specific endocrine cells of pancreatic islets [440] and, perhaps more surprisingly, in neural cell-lines.[441] This latter finding raises questions about the physiological role of VIP and its potential significance as a neurotransmitter.

A general theory of gastrointestinal hormones [442] includes the proposal of two separate actions, one local (paracrine) and one distant (endocrine), and also the modulation of integrated neural control. The expression 'eupeptide systems' was coined for this complex activity.

The mode of action of VIP on pancreatic acinar cells of guinea-pigs was studied,[443] and its presence in tumours and its causative role in watery diarrhoea examined,[444] both with the help of radioimmunoassays.

The tetradecapeptide bombesin, isolated from the European frogs *Bombina bombina* and *Bombina variegata variegata*, has CCK-PZ-like activity, while its sequence is clearly homologous [445] with that of VIP. The physiological effects of bombesin have received growing attention.[446]

Gastric Inhibitory Peptide (GIP).—A 43-membered chain with the sequence proposed for the porcine peptide has been synthesized and details of the synthesis were reported.[447] Since no direct comparisons between synthetic and natural GIP were published, the synthesis cannot be regarded at this time as proof of

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structure. The role of GIP in the release of insulin [448] and its effect under pathological conditions [449] were studied.

Motilin.—The sequence of this biologically active intestinal peptide (Figure 3) shows no obvious homology with other gastrointestinal hormones. Two syntheses,[450, 451] both by segment-condensation, have been completed and syntheses of the [Leu^{13}] and [Nle^{13}] analogues reported.[452] The effect of motilin on gastrointestinal smooth muscle preparations was studied.[453] The peptide could be localized in the gastrointestinal tract by immunofluorescence.[454] The proceedings of a symposium on motilin, held in Erlangen, Germany, in June 1976, have been published.[455]

Other Gastrointestinal Hormones.—The history of the discovery, isolation, structure elucidation, and synthesis of the principal gastrointestinal hormones, given in the lectures [456] at the presentation of the first Beaumont prize, provides

Bombesin	Glp-Gln-Arg-Leu-Gly-Asn-
Porcine VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-
Bombesin	Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Porcine VIP	Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2

Figure 5 *Homology between bombesin and (porcine) VIP*

a clear indication of the presence of additional hormones in the gut. For example, a fraction from the extract of porcine upper small intestines [457] stimulates pepsin secretion in the cat. The peptide with glucagon-like immunoreactivity, enteroglucagon, was implicated [458] in pathological conditions.

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6 Enkephalins and Endorphins

Contributed by B. A. Morgan and G. Metcalf

Introduction.—The activity and possible physiological function of peptides which act on the central nervous system (CNS) have attracted increased interest during recent years. In particular there has been a dramatic explosion of research relating to the so-called opioid peptides since December 1975. This explosion was set off by the identification of two pentapeptides, methionine enkephalin and leucine enkephalin, as possible endogenous ligands for opiate-receptors. The search for such an endogenous ligand had been initiated earlier when it was discovered that 'opiate-receptors' isolated from brain tissue did not interact with known neurotransmitter substances. Most of the work published during 1976 has sought to examine the possible physiological significance of the enkephalins and other opioid peptides, or to probe their therapeutic possibilities.

This review covers work published or disclosed at scientific meetings during 1976. Information disclosed in patent literature has not been included. The initial sources of information have been 'Current Contents – Life Sciences' (Institute for Scientific Information, U.S.A.) and 'Ringdoc (Derwent Publications Ltd., England). A large section of the work reviewed was presented at the meeting of the International Narcotics Research Club, held in Aberdeen during July 1976. The proceedings of this meeting have now been published.[459]

Discovery and Distribution.—There can be little doubt that if neuroscientists were to name 1976 according to the Chinese custom it would be appropriate to entitle it 'The year of the opioid peptides'.[459] The first opioid peptides to be reported were methionine enkephalin and leucine enkephalin (see Table 13).[460] These two pentapeptides were isolated from pig brain, characterized, and finally synthesized. It was confirmed that, in isolated tissues, their pharmacological properties were similar to morphine and it was noted that both could be considered to be derived from the pituitary peptide β-lipotropin (β-LPH; see Figure 6).[460] This initial discovery was rapidly followed by other reports of opioid peptides with related structures. These compounds, now known generically as endorphins, are shown in Table 1. As noted in the original report, most of these peptides are related to β-LPH. Thus β-endorphin[462] (or C-fragment[463]) is composed of an amino-acid sequence identical with β-LPH 61—91. Similarly α-endorphin[461] corresponds to β-LPH 61—76 and γ-endorphin[464] to β-LPH 61—77. The only known exception to this β-LPH relationship is leucine enkephalin (Table 13) which has a Leu residue at position 65. A β-LPH fragment with Leu at this position has yet to be isolated from animal tissue. Also when porcine β-LPH was subjected to tryptic digestion,[469] no trace of Leu was detected in the 61—69 fragment produced. Other endorphin-like materials of unknown structure have been reported to occur in brain,[466] pituitary,[467] cerebrospinal fluid,[468] and blood.[465]

Various workers have investigated the distribution of enkephalins in animal tissue either the mouse vas deferens,[470] the receptor binding assay,[471] or radioimmunoassay[472] to detect activity. In the brain high levels of activity were

[459] 'Opiates and Endogenous Opioid Peptides', ed. H. W. Kosterlitz, North Holland, Amsterdam, 1976.

Table 13 *Endorphins reported during* 1976

Name	*Structure*	*Source of material*	*Ref.*
Methionine enkephalin	Tyr-Gly-Gly-Phe-Met (*i.e.* β-LPH 61—65)	Pig brain	460
Leucine enkephalin	Tyr-Gly-Gly-Phe-Leu	Pig brain	460
α-Endorphin	β-LPH 61—76	Pig hypothalamus	461
β-Endorphin	β-LPH 61—91	Camel hypothalamus	462
C-fragment (*i.e.* β-endorphin)	β-LPH 61—91	Pig pituitary	463
γ-Endorphin	β-LPH 61—77	Pig hypothalamus	464
Anodynin	not known	Rat blood, human blood	465
Un-named	not known	Calf pituitary, pig pituitary	466
Un-named	not known	Rat brain, calf brain	467
Un-named	not known	Human CSF	468

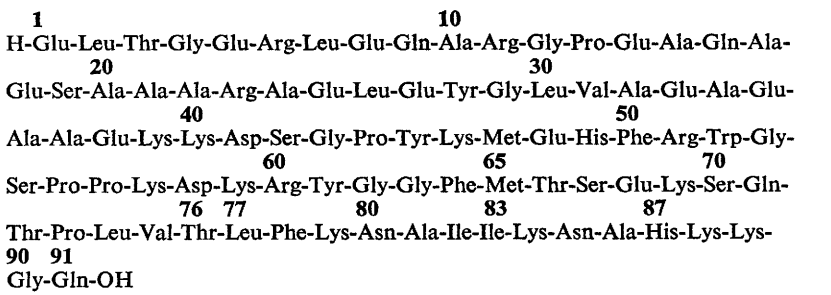

Figure 6 *Amino-acid sequence for ovine β-lipotropin (β-LPH). For porcine β-LPH,* 83 = *Val; human β-LPH,* 87 = *Tyr and* 91 = *Glu*

detected in the thalamus, hypothalamus, and striatum; the midbrain, brainstem, and hippocampus contained intermediate levels, whilst lower levels of activity were detected in the cerebral cortex. The cerebellum contained barely detectable amounts. Interestingly the proportion of [Met^5] to [Leu^5] varied both

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[467] M. Ross, T. P. Su, B. M. Cox, and A. Goldstein, ref. 459, p. 35.
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[470] T. W. Smith, J. Hughes, H. W. Kosterlitz, and R. P. Sosa, ref. 459, p. 57.
[471] S. J. Bird, S. F. Atweh, and M. J. Kuhar ref. 459, p. 199.
[472] R. Simantou and S. H. Snyder, ref. 459, p. 41.

with the species and the brain regions investigated. When the pituitary gland was assayed for endorphin content, most was detected in the intermediate lobe.[473] Hypophysectomy did not decrease levels of endorphin in brain, suggesting that it is synthesized *in situ*.[474] Enkephalins have also been detected in peripheral tissues. Considerable quantities have been detected in the small intestine of several species with the highest concentration in the myenteric plexus.[470] Preliminary reports concerning the subcellular distribution of enkephalins indicate that they are associated with the synaptosome or nerve ending fraction.[475]

Biological Activity.—A large number of enkephalin and endorphin analogues have been investigated for their biological activity. Opiate-like activity may be detected using isolated tissues, receptor binding, biochemical reactions, or behavioural effects in animals. Given this range of procedures and the variety of test conditions which can be applied, it is not surprising that ideas relating to structure–activity relationships are still liable to abrupt change. For the purpose of this review information relating to different test methods will be considered separately. In the following tables when similar results have been reported more than once by the same authors, only the first account is included.

Isolated Tissue Assays. Table 14 lists endorphins and enkephalins whose activity has been assessed using either the electrically stimulated mouse vas deferens

Table 14 *The activity of enkephalins and endorphins in isolated tissue assays*

Compound number	*Structure* [a]	*Biological activity* [b] *MVD* [c]	*GPI* [d]	*Ref.*
1	Met[5]	100	100	460
			(0.9 ± 0.2 × NM) [e]	476, 469
2	Leu[5]	50	20	460
		170	37	477
			17	478
3	β-LPH 61—76		36	479
4	β-LPH 61—91		137	476
			450	479
5	β-LPH 61—77		23	479
6	β-LPH 61—79		42	476
7	β-LPH 61—69		(0.4 ± 0.05 × NM) [e]	476, 469
			60	479
			12	478
8	β-LPH 1—69		inactive	469
9	β-LPH 1—91		inactive	469
10	Gly[5]	3	2	477
11	β-LPH 61—64	1	1	477
			0.1	479
			< 5	478

[473] G. Queen, C. Pinsky, and F. Labella, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 1021.
[474] A. L. Cheung and A. Goldstein, *Life Sci.*, 1976, **19**, 1005.
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[479] N. Ling and R. Guillemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3308.

Table 14 (*cont.*)

Compound number	*Structure* [a]	*Biological activity* [b] *MVD* [c]	*GPI* [d]	*Ref.*
12	Desamino-Tyr^1	inactive	inactive	477
		< 0.15		480
13	Phe^1	0.03	0.2	477
		< 0.15	0.2	479
		< 0.15	< 0.15	480
14	Tyr^4	0.1	0.3	477
15	β-LPH 62—65	0.1		481
16	Gly-Gly-Phe-Leu	0.1		481
17	D-Ala^2	500		481
		1000		482
18	Tyr-Gly		inactive	479
19	Tyr-Gly-Gly		inactive	479
20	Met^5-NH_2		302	479
		53	130	480
21	Met^5-NHEt		246	479
22	Met(O)^5		20	479
23	Tyr(O-Me)^1		17	479
24	N-Ac-Tyr^1		'low' (non-parallel assay)	479
25	D-Ala^3		4	479
		< 0.1		482
26	β-LPH 61—76 NH_2		72	479
27	Sar^2	0.6		483
28	Sar^3	< 0.1		483
29	Tyr-γ-amino-valeryl-Phe-Met	0.03		483
30	Gly^4	< 0.01		483
31	D-Phe^4	0.06		483
		< 0.1		482
32	Phe^5	11		483
33	Tyr(OBu^t)^1, Leu^5	0.1		484
34	Z-Tyr^1, Leu^5	2		484
35	*N*-MeTyr^1	20		484
36	β-LPH 61—67	52		484
37	D-Tyr^1	< 0.1		482
38	D-Met^5	0.2		482
39	D-Ala^2-NH_2	1000		482
40	Met^5-NHMe	24		480
41	Descarboxy Met^5	7		480
42	β-LPH 61—64 NH_2	0.5		480
43	Nle^5		45	478
44	Arg-Tyr-Gly-Gly-Phe-Met		25	478

[a] The numbers denoting the position of individual amino-acids refer to the Met^5-enkephalin sequence, *i.e.* Tyr^1-Gly^2-Gly^3-Phe-^{4}-Met^5, unless a β-LPH sequence is specified. [b] Activity is quoted as a percentage relative to Met^5-enkephalin unless otherwise stated. [c] MVD assay; see text. [d] GPI assay; see text. [e] NM refers to normorphine.

480 R. C. A. Frederickson, R. Nickander, E. L. Smithwick, R. Shuman, and F. H. Norris, ref. 459, p. 239.

481 J. M. Hambrook, B. A. Morgan, M. J. Rance, and C. F. C. Smith, *Nature*, 1976, **262**, 782.

482 D. H. Coy, A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, and C. A. Sandman, *Biochem. Biophys. Res. Comm.*, 1976, **73**, 632.

483 B. A. Morgan, Fifth International Symposium on Medicinal Chemistry, Paris, July, 1976.

484 J. D. Bower, K. P. Guest, B. A. Morgan, L. Fothergall, J. Hughes, H. W. Kosterlitz, T. Smith, and H. R. Morris, Chemical Society Peptide and Protein Group Meeting, Exeter, April, 1976.

(MVD) or the electrically stimulated guinea-pig ileum (GPI). It had been established previously [485] that for narcotic analgesics both of these tests correlate well with *in vivo* antinociceptive test results.

Examination of the results listed shows that the complete pentapeptide sequence of [Met^5]-enkephalin (1) or [Leu^5]-enkephalin (2) is important for potency in these tests. The dipeptide Tyr-Gly (18) and the tripeptide Tyr-Gly-Gly (19) are inactive,[479] as is the *C*-terminal tetrapeptide (15).[481] The *N*-terminal tetrapeptide (11) has low potency although it exerts full activity.[479] When the *C*-terminus of [Met^5]-enkephalin is extended along the β-LPH sequence there is a reduction in GPI activity, *e.g.* (36), (7), (3), (5), (6). In contrast β-LPH 61—91 (4) (β-endorphin) is at least as potent as [Met^5]-enkephalin in the GPI test.[47, 479]

Structure–activity relationships within the pentapeptide sequence have also been revealed using these test procedures. Blockade of the nitrogen function on [Tyr^1] as in (24) and (34) reduces potency, whilst removal of this function as in (12) leads to complete inactivity. Similarly, blockade or removal of the hydroxy-group on [Tyr^1] also reduced potency, *e.g.* (13),[477, 479] (23),[479] and (33).[484] Interestingly, arginyl-[Met^5]-enkephalin (44) and the *N*-methylated [Tyr^1] analogue (35) both retain some activity.

Variations at position 2 have produced the most noteworthy improvements so far. Whilst *N*-methylation at this position (27) reduces activity, the introduction of the unnatural amino-acid D-Ala at position 2 (17) produced a compound with 5—10 times the activity of the parent molecule in the MVD assay [481, 482] and also produced a dramatic increase in the biological half-life of the compound (see later). In contrast, introduction of D-Ala at position 3, *i.e.* (25), decreased activity [479] as did the introduction of a Sar grouping (28) at this position.[483] Similarly introduction of various substituents into position 4, *e.g.* (31),[482, 483] (30),[483] and (14),[477] decreased activity so that the conformation of the Phe grouping appears important for good activity in these tests. Alteration at position 5 can produce a variety of changes. Removal of the side-chain (10) or removal of the carboxy-group (41) both decrease activity.[477, 481] Amidation of the Met^5 moiety (20), (21), and (40) increases potency in the GPI but decreases activity in the MVD.[480, 479] Replacement of the [Met^5] grouping with another substituent almost always decreases activity, *e.g.* (22), (38), (32), and (43), but in the case of leucine enkephalin (2) the result is worthy of further comment. [Leu^5]-enkephalin has almost twice the activity of [Met^5]-enkephalin in the MVD but only about 0.3 of the activity of [Met^5]-enkephalin in the GPI, thus strongly suggesting that the structural requirements for receptor interaction are different in each tissue. Further light may be shed on such questions by the use of other isolated tissue preparations.[486]

Opiate Receptor Binding.—Results obtained with this technique were critical both to the concept of an endogenous morphine-like substance and to the characterization of the enkephalins and endorphins. Latterly the technique has been used to study the structure–activity relationships for opioid peptides. Assays of this type can be performed in a wide variety of ways. Variables include tissue source, preparation of tiss,sue fraction temperature and duration of

[485] H. W. Kosterlitz and A. A. Waterfield, *Rev. Pharmacol.*, 1975, **15**, 29.
[486] J. Knoll, *European J. Pharmacol.*, 1976, **39**, 403.

incubation, composition of incubation medium, and choice of radio-labelled ligand. Despite all these points of potential disagreement, there has been a large measure of agreement between the results obtained by various workers. In addition, structure–activity relationships derived using this technique correspond well with the relationships evolved using isolated tissues.

Table 15 sets out the data reported using this technique. The importance of the intact [Tyr^1] residue is again evident, although certain analogues such as *N*-tyrosyl (52) and arginyl (44) enkephalins [459] appear to have a relatively high affinity for the receptor.[478, 488] The enhanced activity of [D-Ala^2] (17) and its amide analogue is also evident.[482, 489] Longer peptides retaining the enkephalin

Table 15 *The activity of enkephalins and endorphins in receptor binding assays*

Compound number	*Structure*	*Assay procedure*	*Activity* [i] *Naloxone*	*DHM* [l]	*Enkephalin*	*Ref.*
(1)	Met^5	*a*		3 × morphine		460
		b, c	90	8 [j]		487
		e		300 [k]		488
		f		0.009		478
		h	200			489
(2)	Leu^5	*h*	700			489
(4)	β-LPH 61—91	*b, c*	2.6	2.2		487
		d		3.4 × normorphine		462
		h	200			489
(45)	β-LPH 61—89	*b, c*	5	2.2		487
(46)	β-LPH 61—87	*b, c*	100	42		487
(7)	β-LPH 61—69	*b, c*	60	21		487
(47)	β-LPH 61—68	*b, c*	160	45		487
(11)	β-LPH 61—64	*b, c*	1000	300		487
(9)	β-LPH 1—91	*b, c*	inactive	inactive		487
(48)	β-LPH 1—58 (γ-LPH)	*b, c*	inactive	inactive		487
(49)	β-LPH 1—38	*b, c*	inactive	inactive		487
(50)	β-LPH 41—58 (β-MSH)	*b, c*	inactive	inactive		487
(51)	β-LPH 70—79	*b, c*	inactive	inactive		487
(11)	β-LPH 61—64	*e*		7 [j]		488
		f		100		478
(42)	β-LPH 61—64 amide	*b*	600			490
(3)	β-LPH 61—76	*h*	3000			489
(9)	β-LPH 1—91	*h*	> 100 000			489
(52)	Tyr-Tyr-Gly-Gly-Phe-Leu	*e*	100 [k]			488
(53)	Leu^5, Leu^6	*e*	60 [k]			488
(54)	Tyr-Gly-Gly-Gly-Phe-Leu	*e*	15 [k]			488
(55)	Tyr-Gly-Phe-Leu	*e*	< 1 [j]			488

[487] A. F. Bradbury, D. G. Smyth, C. R. Snell, N. J. M. Birdsall, and E. C. Hume, *Nature*, 1976, **260**, 793.

[488] L. Terenius, A. Wahlstrom, G. Lindeberg, S. Karlsson, and U. Ragnarsson, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 175.

[489] C. B. Pert, D. L. Bowie, B. T. W. Fong and J. K. Chang, ref. 1, p. 79.

[490] A. F. Bradbury, W. F. Feldberg, D. G. Smyth, and C. R. Snell, ref. 1, p. 9.

Table 15 (*cont.*)

Compound number	*Structure*	*Assay procedure*	*Activity* [i] *Naloxone*	*DHM* [l]	*Enkephalin*	*Ref.*
(16)	Gly-Gly-Phe-Leu	*e*	< 1 [j]			488
(56)	(3,5-di-I)Tyr[1], Leu[5]	*e*	< 1 [j]			488
(57)	Tyr[4], Leu[5]	*e*	3 [j]			488
(58)	Phe[1], Leu[5]	*e*	< 3 [j]			488
(59)	Ala[2], Leu[5]	*e*	3 [j]			488
(60)	Ala[3], Leu[5]	*e*	6 [j]			488
(61)	Ala[2], Ala[3], Leu[5]	*e*	< 1 [j]			488
(43)	Nle[5]	*f*	0.05			478
(44)	Arg-Tyr-Gly-Gly-Phe-Met	*f*	2			478
(17)	D-Ala[2]	*g*			120 [j]	482
(37)	D-Tyr[1]	*g*			< 1 [j]	482
(62)	D-Leu[2]	*g*			< 1 [j]	482
(63)	D-Phe[2]	*g*			< 1 [j]	482
(25)	D-Ala[3]	*g*			10	482
(31)	D-Phe[4]	*g*			< 1 [j]	482
(38)	D-Met[5]	*g*			10 [j]	482
(39)	D-Ala[2]-NH_2	*g*			150 [j]	482
(64)	L-DOPA[1]	*h*	100 000			489
(65)	Sar[1]	*h*	40 000			489
(13)	Phe[1]	*h*	200 000			489
(66)	Trp[1]	*h*	500 000			489
(67)	His[1]	*h*	> 500 000			489
(39)	D-Ala[2]-NH_2	*h*	400			489
(68)	Ala[2]-NH_2	*h*	4000			489
(69)	Pro[2]-NH_2	*h*	90 000			489
(70)	Sar[2]-NH_2	*h*	8000			489
(71)	D-Val[2]-NH_2	*h*	4000			489
(72)	D-Phe[2]-NH_2	*h*	2000			489
(74)	NH_2CO-Tyr[1]	*b*	> 100 000			490
(35)	*N*-MeTyr[1]	*b*	20			490
(70)	Sar[2]	*b*	300			490
(75)	Ala[2]	*b*	> 10 000			490
(76)	Pro[2]	*b*	> 10 000			490
(20)	Met[5]-NH_2	*b*	30			490
(77)	*N*-MeTyr[1] NH_2	*b*	30			490
(78)	*N*-MeTyr(OBz)[1]	*b*	> 1000			490
(79)	*N*-MeTyr(OBz)[1] NH_2	*b*	> 1000			490
	Morphine	*h*	100			489

[a] Inhibition of [^{3}H]-naloxone binding in Na^+-free guinea-pig brain homogenate. [b] Inhibition of [^{3}H]-naloxone binding to lysed synaptosomes from rat brain. Incubation conditions: 15 min; 30 °C; 100 mM-Na^+. [c] Inhibition of [^{3}H]-dihydromorphine binding. Conditions as in (b). [d] No details available. [e] Inhibition of [^{3}H]-dihydromorphine binding to synaptic membranes from rat brain. Incubation conditions: 40 min; 25 °C. [f] Inhibition of [^{3}H]-dihydromorphine binding to mouse brain homogenate at 35 °C. [g] Inhibition of [^{3}H]-Met[5]-enkephalin binding to P2 fraction from rat brain. Incubation conditions: 70 min; 0 °C. [h] Inhibition of [^{3}H]-naloxone binding to rat brain membrane preparation. Incubation conditions: 60 min; 0 °C. [i] Activity quoted as *n*-molar concentration to produce 50% inhibition of binding. [j] Percentage receptor affinity relative to Met[5]-enkephalin. [k] Percentage receptor affinity relative to Leu[5]-enkephalin. DHM = dihydromorphine.

N-terminal sequence are also potent in these assays. It has been reported that β-endorphin (4) and β-LPH 61—89 (45) have a substantially greater affinity than

[Met^5]-enkephalin, and it has been suggested that this reflects the importance of the *C*-terminus in binding.[487, 463] Other workers report that the affinities of the enkephalins and β-endorphin are similar.[489]

Snyder and his co-workers have, in the past, used a variation of this technique to comment on the agonist/antagonist character of narcotic drugs (see ref. 491 for previous work). Briefly these workers have reported that narcotic agonists have a lower affinity for opiate receptors in the presence of an excess of sodium ions, whilst narcotic antagonists bind equally well under both conditions. Similarly, Terenius has suggested that narcotic agonists will displace other agonists rather than antagonists.[492] Using these arguments [Met^5]-enkephalin has been classified as a pure agonist [463, 474, 491] whilst β-endorphin may be regarded as a partial-agonist. Terenius has also reported the unrelated peptides somatostatin and ACTH 1—24 to be partial-agonists in character.[493]

Behavioural Effects.—The results obtained from both *in vitro* and binding tests reviewed in the previous sections suggested that [Met^5]- and [Leu^5]-enkephalins would induce antinociception in animals. However, only weak, transient effects have been obtained to date even when the peptide was administered directly into the CNS.[476, 494–496, 505] In contrast, β-endorphin has proved to be a potent and long-lasting analgesic after both CNS and intravenous administration.[497,–501] It has been estimated that β-endorphin is 20—50 timesmore potent than morphine on a molar basis after intraventricular administration and 3—4 times more potent than morphine after intravenous injection.[498,502] As would be predicted for an opiate effect, the analgesic effects are reported to be naloxone reversible. Interestingly, β-LPH 1—91 has been reported active.[503] It may be that this represents exposure of a β-LPH 61—65 like sequence after enzymatic degradation. Hambrooke and her colleagues have reported that the enephalinks are rapidly degraded *in vivo*, and they suggest that β-endorphin is able to resist [D-Ala^2] cleavage of the [Tyr^1-Gly^2] bond. Such reasoning is supported by the antinociceptive effects reported[500] for (39) with the unnatural However, (39) was not active after intravenous administration.[504]

Conventional opiate drugs produce a wide spectrum of pharmacological effects in animal tests, and several authors have investigated such activities for opioid peptides. Intraventricular (1) has been reported to increase locomotor activity in mice, decrease gastrointestinal propulsion in the same species, and

[491] R. Simantov and S. H. Snyder, *Mol. Pharmacol.*, 1976, **12**, 987.
[492] L. Terenius, *Acta Physiol. Scand.*, 1976, Suppl. 440, 5.
[493] L. Terenius, *Europ. J. Pharmacol.*, 1976, **38**, 211.
[494] J. D. Belluzzi, N. Grant, V. Garsky, D. Sarantakis, D. C. Wise, and L. Stein, *Nature*, 1976, **260**, 625.
[495] H. H. Büscher R. C. Hill, D. Römer, F. Cardinaux, A. Close, D. Hauser, and J. Pless, *Nature*, 1976, **261**, 423.
[496] J. B. Malik and J. M. Goldstein, *Pharmacologist*, 1976, **18**, 120.
[497] C. H. Li, S. Lemare, D. Yamashiro, and B. A. Doneen, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 19.
[498] H. H. Loh, L. F. Tseng, E. Wei, and C. H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 2895.
[499] W. F. Feldberg and D. G. Smyth, *J. Physiol.*, 1976, **260**, 30P.
[500] A. Pert, ref. 459, p. 87.
[501] L. F. Tseng, H. H. Loh, and C. H. Li, *Nature*, 1976, **263**, 239.
[502] J. Goldfarb and J. W. Hu, *Neuropharmacology*, 1976, **15**, 785.
[503] A. Z. Ronai, J. I. Szekely, L. Graf, Z. D. Kovacs and S. Bajusz, *Life Sci.*, 1976, **19**, 733.
[504] C. B. Pert, A. Pert, J. K. Chang, and B. T. W. Fong, *Science*, 1976, **194**, 330.

cause hyperthermia in cats.[505] After intravenous administration to dogs it caused bradycardia, hypotension, and depression of respiration.[505] In another study whilst (1) and (2) caused some sedation and immobility, (4) caused profound catalepsy lasting several hours. This catalepsy induced by β-endorphin was reversed by naloxone.[506] The cataleptic effect of β-endorphin has been confirmed by the same workers, and they also reported analgesia when the peptide was injected directly into the midbrain.[507] Further confirmation for naloxone-reversible catalepsy, analgesia, and hypothermia after intracisternal or intraventricular administration of β-endorphin to rats was provided by Bloom *et al.*[508] In contrast, these authors reported that (5) produced hyperthermia in rats and that (3), (4), and (5) all induced 'wet-dog shakes' – but only when injected into the lateral ventricles.[508] It was suggested in this report that the behavioural effects produced by β-endorphin may imply a role in the aetiology of mental disease. Preliminary studies on the levels of endorphins in the cerebrospinal fluid of schizophrenic patients appear to support such a suggestion.[492]

Motomatsu *et al.* claim to differentiate between the catalepsy and antinociception produced by β-endorphin. These authors report that L-dopa will selectively antagonize the catalepsy whilst naloxone selectively antagonizes the antinociceptive effects.[509] Such findings are in conflict with those of Jacquet and Marks[506, 507] and Bloom *et al.*[508] The findings need confirmation for they imply separation of dopaminergic and opiate effects. Some dopaminergic effects may also be inferred for (1) and (39) by the potentiation of the behavioural effects induced by dopa in mice.[510] Again confirmation is required, because the latter authors use the intraperitoneal route of administration and it is hard to reconcile the activity reported with the route of administration and the established lability of (1). Finally, Gispen *et al.* concluded that β-LPH 61—69

Table 16 *The activity of enkephalins and endorphins in antinociceptive tests*

Compound number	*Structure*	*Test*	*Dose*	*Effect*	*Ref.*
(1)	Met[5]	*a*	100 μg, icv	Brief analgesia	494
		b	170 mg kg^{-1}, icv	Brief analgesia	495
		a	30 μg, icv	Inactive	469
		c	—	Equipotent to morphine	496
		g	400 μg, icv	Inactive	499
		a	675 nmol, icv	Brief analgesia	476
		j	1000 μg, icv	Inactive	505
		i	—	Inactive	506
		k	125—500 μg, icv	Active	505
(2)	Leu[5]	*a*	100 μg, icv	Brief analgesia	494
		b	320 mg kg^{-1}, icv	Weak	495
		i	—	Inactive	506
		j, k	1000 μg, icv	Inactive	505

[505] A. Cowan, J. C. Doxey, and G. Metcalf, ref. 459, p. 95.
[506] Y. F. Jacquet, N. Marks, and C. H. Li, ref. 459, p. 411.
[507] Y. F. Jacquet and N. Marks, *Science*, 1976, **194**, 632.
[508] F. Bloom, D. Segal, N. Ling, and R. Guilleman, *Science*, 1976, **194**, 630.
[509] T. Motomatsu, M. Lis, N. Seidah and M. Chretien, *Clin. Res.*, 1976, **24**, 660A.
[510] N. P. Plotnikoff, A. J. Kastin, D. H. Coy, C. W. Christensen, A. V. Schally, and M. A. Spirtes, *Life Sci.*, 1976, **19**, 1283.

Table 16 (*cont.*)

Compound number	*Structure*	*Test*	*Dose*	*Effect*	*Ref.*
(4)	β-LPH 61—91	*a*	—	Active	497
		d	—	20 times morphine	498
		e	—	17 times morphine	498
		f	—	33 times morphine	498
		g	—	100—200 times morphine	499
		c	10 μg, ic	Active	500
		a	—	50 times morphine	476
		i	—	Active	506
(17)	D-Ala^2	*c*	10 μg, ic	Active	500
(39)	D-Ala^2, amide	*c*	5 μg, ic	Active	500
		h	50 mg kg^{-1}, iv	Inactive	504
(68)	Ala^2, amide	*c*	10 μg, ic	Inactive	500
(71)	D-Val^2 amide	*c*	20 μg, ic	Active	500
(19)	Tyr-Gly-Gly	*b*	—	Inactive	495
(12)	DesaminoTyr^1	*b*	—	Inactive	495
(9)	β-LPH 1—91	*a*	—	ED_{50} 17 nmol	503
		h	130 mg kg^{-1}, iv	Inactive	503
(3)	β-LPH 61—76	*d*	—	Inactive	498
		e	—	Inactive	498
		f	—	Inactive	498
		c	15 μg, ic	Weak activity	500
		i	—	Inactive	506
(6)	β-LPH 61—79	*a*	40 nmol, icv	Active	476

[a] Rat Tail Flick, radiant heat stimulation, icv administration. [b] Mouse Tail Flick, icv administration. [c] Rat Tail Flick, radiant heat stimulation, injection into periaqueductal grey matter (PAG), of midbrain. [d] As (*a*); unspecified intracerebral injection. [e] Rat Hot Plate, intracerebral injection. [f] Mouse acetic acid writhing test, intracerebral injection. [g] Cat tail pinch, injection into third ventricle. [h] As (*a*); intravenous administration. [i] Battery of tests, injection into PAG [j] Rat Tail Flick, hot water (55 °C) stimulation, icv administration. [k] Rat hypertonic-saline writhing test, icv administration.

is the essential sequence for the excessive grooming behaviour produced in rats by several enkephalin analogues and lipotropin fragments.[511]

Single Unit Recording Experiments. Much work has been carried out on the effects of opioid peptides on the firing rate of single neurones. In either rats or cats the enkephalins have been found to depress the firing rate of neurones in the cerebral cortex,[480, 512, 513] striatum,[480, 512, 513] brainstem,[514, 552] thalamus,[515] reticular formation,[513] midbrain,[480, 513] and spinal cord,[516] but there does not seem to be any agreement between the various authors as to whether such effects are antagonized by naloxone. Perhaps more pertinently, (1) depressed the response of cat substantia gelatinosa cells to painful stimuli, and the effect was antagonized by naloxone.[517] Outside the CNS both (1) and (2) inhibited the

[511] W. H. Gispen, V. M. Wiegant, A. F. Bradbury, E. C. Hulme, D. G. Smyth, C. R. Snell, and D. de Wied, *Nature*, 1976, **264**, 794.
[512] W. Zeiglgansberger and J. P. Fry, ref. 459, p. 231.
[513] R. C. A. Frederickson and F. H. Norris, *Science*, 1976, **194**, 440.
[514] P. B. Bradley, I. Briggs, R. J. Gayton and L. A. Lambert, *Nature*, 1976, **261**, 425.
[515] R. E. Hill, C. M. Pepper, and J. F. Mitchell, *Nature*, 1976, **262**, 604.
[516] J. Davies and A. Dray, *Nature*, 1976, **262**, 603.
[517] A. W. Duggan, J. G. Hall and P. M. Headley, *Nature*, 1976, **264**, 456.

firing of neurones in the myenteric plexus of guinea-pigs,[518] but (1) did not affect spontaneous excitatory junction potentials in a similar preparation.[519]

Biochemical Effects.—There is increasing evidence that cyclic nucleotides may be 'secondary messengers' for synaptic events and recently opiates have been shown to influence these 'messengers'.[520] The effect of enkephalins on the adenylate cyclase activity of neuroblastoma × glioma hybrid cells has been investigated. Like morphine, the enkephalins have been found to inhibit both basal and prostaglandin-stimulated adenylate cyclase activity.[520] The authors estimated the enkephalins to be 25—100 times as potent as morphine in this test and they found that the effect was antagonized by naloxone.[520, 521] Using bacitracin to prevent their metabolism, the enkephalins have also been found to increase the accumulation of cyclic GMP in slices of rat neostriatum.[522] Again similar effects were produced by morphine and both the enkephalins and morphine were antagonized by naloxone. Other authors have reported that in the rat, enkephalins provoke prolactin and growth hormone release in a similar manner to opiates.[523, 524] The enkephalins have also been reported to stimulate MSH release *in vivo*.[525]

Several workers have investigated whether opioid peptides inhibit transmitter release in a manner similar to opiate drugs. [Met^5]-enkephalin has been found to inhibit the overflow of tritiated 'noradrenaline' induced by either electrical stimulation or potassium depolarization of rat cortical slices. The authors suggest that the enkephalins are presynaptic inhibitors of central noradrenergic transmission.[553] Similarly, β-endorphin has been found to inhibit the evoked release of tritiated 'dopamine' from slices of rat striatum.[526] Both effects were antagonized by naloxone.

Addictive Liability.—When the endogenous opioid peptides were first isolated it was hoped that they would prove free of the addictive properties that are associated with alkaloid-derived opiate drugs. Addiction is characterized by two phenomena: tolerance and physical dependence. The former implies that progressively larger doses must be administered to achieve a constant effect, whilst during the latter a withdrawal syndrome is produced if administration of the dependence-provoking drug is stopped (or an antagonist drug is administered).

Soon after their characterization the enkephalins were shown to exhibit cross tolerance to morphine.[527] In these experiments ID_{50} values for both morphine and (1) on the MVD and GPI increased in animals implanted with morphine pellets. Similarly, using isolated tissues, it was concluded that the enkephalins would maintain dependence produced by morphine.[528]

These *in vitro* investigations have been extended into whole animals by several workers. In one of the earlier reports a novel osmotic pump was used to infuse

[518] R. A. North and J. T. Williams, *Nature*, 1976, **264**, 460.
[519] G. Henderson, *European J. Pharmacol.*, 1976, **39**, 409.
[520] W. A. Klee, A. Lampert, and M. Nirenberg, ref 459, p. 153.
[521] W. A. Klee and M. Nirenberg, *Nature*, 1976, **263**, 609.
[522] K. P. Minneman and L. L. Iversen, ref. 459, p. 137.
[523] E. L. Lien, R. L. Fenichel, V. Garsky, D. Sarantakis, and N. H. Grant, *Life Sci.*, 1976, **19**, 837.
[524] L. Cusan, A. Duport, M. Garon, and D. H. Coy, *Clin. Res.*, 1976, **24**, 656A.
[525] K. Medzihradszky and H. Medzihradszky-Schweiger, *F.E.B.S. Letters*, 1976, **67**, 45.
[526] H. H. Loh, D. A. Brase, S. K. Sumathy, J. B. Mar, E. L. Way, and C. H. Li, *Nature*, 1976, **264**, 567.
[527] A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, *Nature*, 1976, **260**, 624.
[528] R. Schulz and A. Herz, *European J. Pharmacol.*, 1976, **39**, 429.

the peptides directly into the appropriate part of the brain thus minimizing the problem of metabolism. It was found that both [Met^5]-enkephalin and β-endorphin could cause physical dependence, *i.e.* the animals displayed signs of abstinence when challenged with naloxone.[529, 530] In a similar experiment the stable enkephalin analogue (39) also induced physical dependence after repeated intraventricular injections.[500] This latter paper also reported cross-tolerance between the analgesic effects of morphine and (39) administered intraventricularly.[500] Probably because of its stability and potency, β-endorphin has been most thoroughly evaluated. Cross-tolerance with morphine has been confirmed for this peptide in mice [531] and rats.[532, 533]

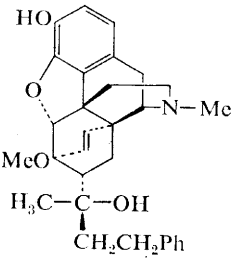
Electrophysiological techniques have also been used to evaluate the cross-tolerance to opiates produced by peptides. The inhibitory effects of (1) and (2) on cortical [512, 534] and striatal [512] neurones were found to be reduced in morphine dependent as compared to control animals. When an adenylate cyclase assay is used in conjunction with neuroblastma × glioma hybrid cells [Met^5]-enkephalin, like morphine, produces a biphasic effect on enzyme activity. The authors have interpreted the results as a possible biochemical mechanism to explain tolerance and dependence.[520, 535] Finally, enkephalin brain levels have been compared in normal and morphine-dependent rats, and were found to be greater elevated in the dependent animals.[536] This result may be interpreted as support for the earlier suggestion that there may be diminished enkephalin release in tolerant or dependent animals.[537]

The apparent low addictive potential of partial agonist opiate drugs [538, 539] suggests that partial-agonist peptides may also have desirable properties. Arginyl-[Met^5]-enkephalin has been suggested to possess antagonist properties *in vivo*,[540] but the report has not been confirmed. If endogenous opiate-like peptides are involved in the processing of sensory information, it would follow that the opiate-antagonist drug naloxone should increase awareness of pain in untreated animals. Experiments designed to examine this possibility have given conflicting results to date.[502, 541, 542]

Structural Conformation.—The structural conformation of the enkephalins has been studied and compared with opiate analgesics to ascertain whether there are any topographical similarities. Obviously the tyramine moiety is common to both enkephalins and classical opiates but by itself tyrosine does not react with opiate receptors. It is not until the extended sequence Tyr-Gly-Gly-Phe is attained that even minimal opiate-like activity emerges.[477, 479] Addition of a

[529] E. Wei and H. H. Loh, *Science*, 1976, **193**, 1262.
[530] E. Wei and H. H. Loh, ref. 459, p. 303.
[531] L. F. Tseng, H. H. Loh, and C. H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 4187.
[532] J. M. Van Ree, D. deWied, A. F. Bradbury, E. C. Hulme, D. G. Smyth, and C. R. Snell, *Nature*, 1976, **264**, 792.
[533] J. Bläsig and A. Herz, *Arch. Pharmacol.*, 1976, **294**, 297.
[534] W. Zeiglgärisberger, J. P. Fry, A. Herz, L. Maroder, and E. Wünsch, *Brain Res.*, 1976, **115**, 160.
[535] A. Lampert, M. Nirenberg and W. A. Klee, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3165.
[536] R. Simantov and S. H. Snyder, *Nature*, 1976, **267**, 505.
[537] H. W. Kosterlitz and J. Hughes, *Life Sci.*, 1975, **17**, 91.
[538] D. L. Francis, N. J. Cuthbert, L. C. Dinneen, C. Schneider, and H. O. J. Collier, ref. 1, p. 177.
[539] J. J. Jacob, E. C. Tremblay, and G. M. Michaud, ref. 459, p. 377.
[540] G. Ungar, A. L. Ungar, and D. H. Malin, ref. 459, p. 121.
[541] A. Goldstein, G. Pryor, L. S. Otis, and F. Larsen, *Life Sci.*, 1976, **18**, 599.
[542] B. Pomeranz and D. Chiu, *Life Sci.*, 1976, **19**, 1757.

fifth residue, which possesses a lipophilic side-chain, *e.g.* (1), increases such activity by 100 times.[477, 479] Thus the nature of the residues at the *C*-terminal is critically important in determining the degree of opiate character conferred on the molecule. It has been reported that if the pentapeptides are assumed to adopt a β-turn structure, with a hydrogen bond between the CO grouping of the tyrosine and the NH grouping of the phenylalanine, then the relative positions of the tyrosine and phenylalanine side-chains correspond to the position of similar moieties in the phenethyloripavine analgesics such as (80).[484, 543]



(80)

However, from n.m.r. results in dimethyl sulphoxide, other workers have proposed that the Gly-Gly-Phe-Met sequence of [Met^5]-enkephalin adopts a β_1-turn involving a hydrogen bond between the CO grouping of [Gly^2] and the NH grouping of methionine.[544, 545, 546, 547] Another approach has been to compare the enkephalins with opiates using molecular parameters obtained from published *X*-ray data.[548] Finally, a study of the interaction of sodium and potassium ions with [Met^5]-enkephalins using c.d. techniques has been reported.[549]

Conclusion.—The year 1976 has seen an avalanche of papers relating to short-chain and long-chain opioid peptides. The pace of the scientific progress has been extremely rapid, so much so that some manipulation of data has been admitted.[550] Leaving aside such personal misfortunes, it is fair to say that a whole area of medicinal chemistry and pharmacology has been opened up. Whether or not the peptides themselves will provide a new opiate-like drug is still to be determined (a recent report suggests that the short-chain peptides are capable of attaining the potency associated with β-endorphin in this review [551]). Undoubtedly the endorphins will give fresh insight into opiate/receptor interactions and the role of peptides in brain neurochemistry.

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6

Metal Complexes of Amino-acids, Peptides, and Proteins

BY R. W. HAY AND D. R. WILLIAMS

1 Introduction

This chapter describes work published during the years 1975 and 1976 in the general field indicated by the title. Attention has been concentrated on those aspects which relate to the modification of the reactivity and other properties of the organic ligand when attached to a metal; however, it is clear that many inorganic chemists find the chapter of value and so we have included appropriate material. There has been little in the way of unusual developments but some very elegant applications of known chemistry to more complicated systems have occurred.

The area defined in the title can be regarded as a section of the wider field of bio-inorganic chemistry. A considerable number of general review articles and books have appeared: 'Metal Chelates and Physiological Reactions',[1] 'Recent Progress in the Study of Metalloenzymes',[2] 'Electron-transfer Proteins',[3] 'Calcium in Biological Systems',[4] 'Mechanisms for the Reactions of Molybdenum in Enzymes',[5] 'Lanthanide Ions as Structural Probes in Biological and Model Systems',[6] 'Trace Metals in Biological Systems',[7] 'Copper Chelates as Possible Active Forms of the Antiarthritic Agents',[8] 'Selenium and Cancer',[9] 'Inorganic Oxygen Carriers as Models for Biological Systems',[10] 'Inorganic Medicinal Chemistry',[11] 'Transition Metal Complexes Containing Tridentate Amino-acids',[12] 'Metallothionein',[13] 'Binding of Metal Ions to Membranes and its Consequences',[14] and 'Structural Aspects of Molybdenum(IV), Molybdenum(V), and Molybdenum(VI) Complexes'.[15]

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Volume 5 of Sigel's 'Metal Ions in Biological Systems' has been published,[16] and contains the following articles: formation of Schiff bases in the co-ordination sphere of metal ions; metal-ion promoted hydrolysis of amino-acid esters and peptides; the catalytic activity of poly-L,α-amino-acid metal complexes and their use as enzyme models, molybdenum complexes as models for biological systems and the interaction of copper(I) complexes with dioxygen. A new edition of Falk's 'Porphyrins and Metalloporphyrins' has been published,[17] as has an introductory text on the general area of bio-inorganic chemistry.[18]

There has been a growing awareness of the possible uses of metal complexes in chemotherapy.[11] The use of platinum complexes in cancer chemotherapy has done much to stimulate interest in this area. Sorenson[8] has observed that copper(II) acetate monohydrate has anti-inflammatory activity in the carrageenan foot edema model of inflammation. (It is noteworthy that all clinically useful antiarthritic compounds are chelating agents.) Various copper(II) amino-acid complexes have been screened[8] for anti-inflammatory and antiulcer activity and many of these have high activity. The superoxide ion ($O_2^{\cdot-}$) has been implicated in the initiation of inflammatory types of arthritis,[19] in ageing, and in carcinogenesis. The enzyme superoxide dismutase[20] is a blue copper(II) protein (mol. wt. 34 000, 0.35% copper, with a broad *d–d* band at 675 nm) which catalyses the dismutation of superoxide, $2H^+ + 2O_2^{\cdot-} \rightarrow 2H_2O_2 + O_2$. Copper(II) complexes have been shown[21] to act as superoxide scavengers, and it is suggested that this accounts for their activity as anti-inflammatory agents.

2 Amino-acids

Binding.—There seems to have been a resurgence of interest in the determination of formation constants of complexes in solution. Formation constants are being reported with greater precision as a result of improved equipment and computational aids. Groups of authors are collaborating to collect critical surveys of published constants.[22] Ternary complex formation is attracting much attention.

A number of unsaturated amino-acids have been synthesized and their complexing with Cu^{II}, Ni^{II}, Zn^{II}, Co^{II}, Cd^{II}, and Ag^{I} has been studied.[23] Silver(I) is the only ion where significant bonding to the olefin occurs, in spite of the fact that the bonds are non-linear about the metal ion.

Previously little work has been reported on uranium and vanadium complexes; however, this lack is now being rectified. The U^{IV}–alanine system has been

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studied,[24] and a spectrophotometric investigation [25] carried out on the interaction of dioxovanadium(v) with *N*-methyliminodiacetic acid, nitrolotriacetic acid, and ethylenediamine-*NN'*-diacetic acid.

Binary amino-acid–transition-metal ion systems for which stability constants have been reported are: DL-4-amino-3-hydroxybutanoic acid–Cu^{II};[26] penicillamine– and cysteine–Co^{II};[27] DL-3,4-dihydroxyphenylalanine–Cu^{II} [28] (a system investigated because of its relationship to L-dopa and adrenaline *in vivo*); histidylhistidine–Cu^{II} and –Zn^{II} and mixed ligand complexes with histidine;[29] 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, ornithine, lysine, and arginine with Co^{II}, Ni^{II}, and Cu^{II};[30] cysteine with Cd^{II};[31] a range of potentially terdentate amino-acids with Ni^{II} and Zn^{II};[32] DL-3-amino-2-hydroxypropanoic acid with Co^{II}, Ni^{II}, Cu^{II}, and Zn^{II};[33] adrenaline, L-dopa, and a catechol derivative with Fe^{III};[34] *NN*-aminoethylglycine and diethylenetriamine-*N*-acetic acid with Co^{II};[35] L- and DL-serine and DL-threonine with Ni^{II} and Cu^{II};[36] alanine with Ni^{II} and Cu^{II} in dioxan–water and methanol–water [37] (in these latter two papers values of ΔH^0 and ΔS^0 were determined and the bonding discussed in terms of solvation effects); a variety of amino-acids with a range of first transition series cations;[38] mixed amino-acid systems with Cu^{II};[39] amino-acids with UO_2^{2+}.[40]

The introduction of improved glass electrode potentiometric techniques and the use of more sophisticated least-squares computational approaches for data analysis over recent years have allowed the study of ternary complex formation in solution to begin. In addition, there has been an increasing awareness of the pivotal role played by amino-acid complexes *in vivo*. It is becoming apparent that the most important complexes in biological systems are ternary and quaternary.

Formation constants have been reported for the *N*(3)-benzyl-L-histidine and *N*(α)*N*(3)-dibenzyl-L-histidine with Cu^{II}, Ni^{II}, and Zn^{II} and D- and L-histidine. Stereoselective effects were observed.[41] Enthalpies of formation and formation constants are now available for the ternary systems, asparagine–glycine–Cu^{II}, asparagine–serine–Cu^{II}, and glutamine–serine–Cu^{II}.[42] The enthalpy data suggest that above pH 9.5, the amide group of asparagine is deprotonated and bound to

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copper(II). A pH–titration study has been reported for the ligands (considered as pairs or triplets) cysteine, orthophosphoric acid, and either citric or nitrilotriacetic acid with Al^{III}, Ca^{II}, or Cd^{II}. The means by which such mixed complexes are introduced into the roots of plants is also discussed.[43] The mixed complexes of 5-sulphosalicylic acid and an α-amino-acid with Cu^{II} have been studied and values of $\Delta H°$ and $\Delta S°$ determined.[44] Two papers have appeared concerning mixed amino-acid complexes of Ni^{II} and Cu^{II}.[45, 46]

The concentrations of the individual complexes formed by trace elements and amino-acids *in vivo* are too low to be detectable by current analytical techniques. If one accepts the caveat that biological solutions approximate to equilibrium conditions, formation constants determined *in vitro* can be used to calculate the relative concentrations of species present *in vivo*. An approach of this type was used to assess agents for oral iron and zinc administration (a neutral complex which is membrane transmittable at pH 6.5 is required).[47, 48] Similar investigations have revealed the salient complexes formed between asparagine, threonine, and histidine and Cu^{II}.[49] Possible structures for these ternary complexes were suggested on the basis of the $\Delta H°$ and $\Delta S°$ values obtained.

Some essential elements and polluting metal ions can be removed from blood plasma by carefully choosing the ligand to sequester selectively the metal ion in question. Thus, treatment with calcium diethylenetriaminepenta-acetate encourages the excretion of zinc(II).[50] As a result of a study of the binding of amino-acids to Cd^{II} and Pb^{II} it was concluded that glutathione deserves investigation as a ligand for mobilizing Pb^{II} and Cd^{II} in plasma.[51] Thermodynamic studies of the binding of glycine, glycylglycine, triglycine, cysteine, and glutathione to Pb^{II} have been reported and possible structures suggested.[52]

Ternary complexes of glutathione containing two dissimilar metal ions, *e.g.* Ca^{II} and Zn^{II} or La^{III} and Zn^{II}, have been reported.[53] It is noteworthy that such complexes increase the concentrations of neutral lipophilic complexes. A discussion of the principles of metal–metal stimulation phenomena *in vivo* has been presented.[54]

It should be noted that these highly complex systems require extremely precise data for their solution and the use of least-square assessments. In order to confirm that a minor complex occurs, normalized curve graphical analysis is advisable. A computerized curve comparison treatment which takes the tedium out of normalized curve analysis has been developed and used in studies of the binding of glycine to Zn^{II} and Pb^{II}.[55]

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Spectroscopic Studies.—I.r. spectroscopic studies of metal complexes of amino-acids are increasing in popularity. Band assignments are often a contentious topic. Although there is general agreement on the frequency ranges in which ligand vibrations occur, the assignment of metal–ligand stretching vibrations is often a matter for debate. Isotopic substitution of ligand atoms with ^{13}C, ^{15}N, and ^{18}O provides a suitable method for verifying band assignments. This technique has been used successfully with *trans*-$[Ni(Gly)_2(OH_2)_2]$.[56] Similar studies have been reported for the Ni^{II} and Cu^{II} complexes with L- and β-alanine.[57] The i.r. spectra of Mo^{VI} complexes of iminodiacetic acid, *N*-methyliminodiacetic acid, nitrilotriacetic acid, aspartic acid, and histidine indicate that they act as terdentate ligands, the complexes containing either a core of MoO_3 or di-μ-oxo-dioxo Mo_2O_4.[58]

Raman and i.r. studies of mercury(II) complexes of cysteine, cysteine methyl ester and methionine, have shown that in the solid state, bis(methionato)-mercury(II) has predominantly amino N– and carboxyl O–mercury bonds, whereas in solution sulphur–mercury bonding is preferred.[59] An i.r. investigation (with band assignments) of Hg^{II}, Zn^{II}, and Cd^{II} complexes of *N*-acetylglycine has also been published.[60]

Magnetic and i.r. measurements have been used to investigate Ni(L-arginine)-ClO_4,nH_2O complexes in which pressure induces ionic perchlorate to become a bidentate ligand.[61] The Faraday method has been used to estimate the molar magnetic susceptibilities of alkali-metal amino-acid salts.[62] Magnetic and e.p.r. studies have also been reported for bis(μ-hippurato-*O*)-bis(hippurato-*O*)-dicopper(II) tetrahydrate,[63] copper(II) complexes of hippurate and acetylglycine,[64] and bis(glycinato)copper(II).[65] Thermal stability and i.r. data for some edda-type polyaminocarboxylate complexes of nickel(II) have also been reported.[66]

Various n.m.r. studies have been carried out on amino-acid complexes. $[Co(acac)_2(L)]^{n+}$ (L = amino-acid or its ester) has been considered as a possible new type of *N*-terminal peptide bond-cleaving product.[67] Cobalt(III) complexes of L-aspartic acid and L-glutamic acid have been investigated using *d–d* spectra and c.d. and n.m.r. techniques.[68] N.m.r. techniques have been used to study (using D_2O solvent over a range of pD) nickel(II) complexes of alanine, valine, threonine, histidine, and cysteine.[69] A series of papers aimed at the reinterpretation of paramagnetic line broadening in the n.m.r. spectra of complexes of amino-acids and peptides has begun with studies of the copper(II) complexes of glycine and sarcosine.[70] Co-ordination sites of acetylhistamine and acetylhistidine in

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complexes with copper(II) are suggested as a result of n.m.r. studies.[71] Gergely and his co-workers[72] have studied the rates of proton exchange for 13 amino-acids with copper(II) using n.m.r. and equilibrium techniques. The rate-determining step in complex formation often appears to be ring closure.

The lability of the metal–nitrogen bonds in complexes of polyaminocarboxylic acids, $(HO_2CCH_2)_2N(CH_2)_nN(CH_2CO_2H)_2$ (n = 2, 3, or 4), with Cd^{II}, Zn^{II}, and Hg^{II} has been studied by n.m.r.[73] Various physical techniques, including n.m.r., have been used to investigate complexes of *S*-methyl-L-cysteine and 2-aminoethanethiol with Mo^{III}. There is a greater tendency to form sulphur-bridged species with Mo^{III} than with Mo^{IV}, the bridges being less labile than oxygen bridges.[74] Papers have also appeared dealing with the binding of methylmercury to methionine[75] and the effect of paramagnetic metal ion impurities on the ^1H spin–lattice relaxation times of L-histidine. The impurities can be removed by treatment with a chelating resin.[76] A spectroscopic investigation of ternary complexes of L- and D-aspartic acid, glutamic acid and arginine, lysine or ornithine with copper(II) has also been published.[77]

Some unusual octahedral complexes of nickel(II) with thioether derivatives of cysteine ethyl ester have been characterized; novel features arise due to steric interactions of bulky groups.[78] A number of new dinuclear and trinuclear complexes of μ-aminocarboxylates with cobalt(III) have been characterized.[79] Mixed ligand complexes of a wide range of amino-acids and related ligands with copper(II) have been investigated.[80] A particular aim of this work was to gain an insight into interactions between charged groups such as $-CO_2^-$, $-\overset{+}{N}H_3$, and guanidium.

The interaction of D-penicillamine with methylmercury and phenylmercury has been studied by a variety of physical techniques and the degree of σ-covalent and π-back bonding investigated.[81] The characteristic red-violet chromophore formed when D-penicillamine interacts with copper(II) in aqueous solution has been attributed to a polymeric anion of mixed oxidation states with $[Cu_2(\text{D-penicillamine})_2]_n$ repeating units.[82] It is intriguing that formation of this polymer is halide-ion dependent. The interaction of cysteine with silver(I) has also been studied.[83]

Diffraction Studies.—Several crystallographic investigations have been carried out, particularly of cobalt(III) and copper(II) complexes. The *X*-ray structure of the red (+)-*cis*-(O)*trans*(N_α)*cis*(N_δ)bis(L-ornithinato)cobalt(III) complex has been

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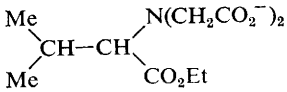
reported in conjunction with c.d. spectra.[84] Buckingham, Sargeson, and co-workers have determined crystal structures for Δ-*R*-[Co(ethylenediamine)$_2${*N*-methyl-(*S*)-alaninato}]I_2 [85] and [Co(trien)(glycinato)]Cl_2,H_2O,[86] and correlated the results to strain-energy minimization calculations.

The crystal structure of DL-(ethylvalinate-*NN*-diacetato)diaquocopper(II) has been determined in order to investigate the feasibility of hydrolysis mechanisms involving quadridentate bonding of the ligand (1). The structure is a distorted



(1)

octahedron, where the equatorial co-ordination consists of the tertiary nitrogen, the two acetate oxygens, and a water molecule.[87] The second water molecule occupies an axial position at a greater distance from the metal, while the ether oxygen of the ester group occupies the other axial position lying 2.84 Å from the metal. The ester carbonyl group is not involved in bonding.

Crystallographic, spectral, and magnetic studies of Cu(L-asparaginate)$_2$ and Cu(L-glutaminate)$_2$ have also been reported.[88]

Complexes of D-penicillamine have attracted the attention of crystallographers in both America [89] and Australia.[90, 91] The synthesis and *X*-ray structures of L-histidinyl-D-penicillaminatocobalt(III) and L-histidinyl-D-penicillaminochromium(III) have been described.[89] The central metal ion is approximately octahedral, the ligating atoms being N (imidazole), N (terminal), O (histidine), and N, O, and S (penicillamine). The stereochemical selectivity observed in these reactions is quite striking; reaction of [M(L-His)$_2$] (M = Co or Cr) with DL-penicillamine (pen) gave only [M(L-His)(D-pen)] and no diastereoisomeric [M(L-His)(L-pen)]. D-Penicillamine is used in chelation therapy for lead(II) and mercury(II), but not for cadmium(II) because it redistributes the metal to other tissues. The crystal structure of D-penicillaminatocadmium(II) establishes octahedral stereochemistry around cadmium with two S atoms, three O (carboxylate), and one N (amino) atoms from four ligand molecules.[90] This relatively weak and non-specific interaction is in contrast with the strong terdentate chelation of the analogous lead systems.[92]

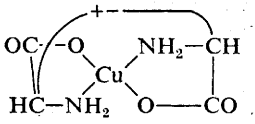
X-Ray crystallography has been carried out on the highly hydrated purple thallium(I) salt of the anion [$Cu^{I}_8Cu^{II}_6$-D-penicillaminato$_{12}$Cl]$^{5-}$. Eight S_3-co-ordinated Cu^{I} atoms surround a central chloride ion and each of the six

[84] Y. Nakayama, S. Ooi, and H. Kuroya, *Bull. Chem. Soc. Japan*, 1976, **49**, 151.
[85] B. F. Anderson, D. A. Buckingham, G. J. Gainsford, G. B. Robertson, and A. M. Sargeson, *Inorg. Chem.*, 1975, **14**, 1658.
[86] D. A. Buckingham, M. Dwyer, G. J. Gainsford, V. J. Ho. L. G. Marzilli, W. T. Robinson, A. M. Sargeson, and K. R. Turnbull, *Inorg. Chem.*, 1975, **14**, 1739.
[87] J. Rodgers and R. A. Jacobson, *Inorg. Chim. Acta*, 1975, **13**, 163.
[88] M. N. Srivastava, R. C. Tewari, U. C. Srivastava, G. B. Bhargava, and A. N. Vishnoi, *J. Inorg. Nuclear Chem.*, 1976, **38**, 1897.
[89] P. de Meester and D. J. Hodgson, *J.C.S. Chem. Comm.*, 1976, 280.
[90] H. C. Freeman, F. Huq, and G. N. Stevens, *J.C.S. Chem. Comm.*, 1976, 90.
[91] P. J. M. W. L. Birker and H. C. Freeman, *J.C.S. Chem. Comm.*, 1976, 312.
[92] H. C. Freeman, G. N. Stevens, and I. F. Taylor, *J.C.S. Chem. Comm.*, 1974, 366.

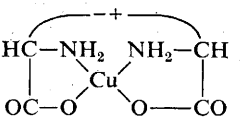
Cu^{II} atoms are N_2S_2-co-ordinated from two ligands.[91] Although when administered intravenously the half-life of the complex is sufficiently long to permit a significant proportion to be excreted through the kidneys,[82] it is unlikely that such mixed oxidation state cluster complexes are formed *in vivo* during therapy with D-penicillamine because of the relatively low concentrations of copper in blood plasma.

The stability of the dimolybdenum(II) unit, Mo_2^{4+}, has been noted in the structure of tetrakis(glycine)dimolybdenum disulphate tetrahydrate.[93] The structures of three Hg^{II} complexes of sulphur-containing amino-acids, $(HgCl_2)_2$-(DL-penicillamine),$2H_2O$, $HgCl_2$(DL-penicillamine)$_2$,H_2O, and $Hg(ClO_4)_2$-(methionine)$_2$,$2H_2O$ reveal polar, polymeric arrangement with chloride, sulphur, and carboxylate bridges which are absent in the corresponding methylmercury derivatives.[94] Trimethyltin glycinate has been found to be a linear polymer with amino-bridged, trigonal-bipyramidal structural units.[95] Two structural papers of general interest concern the crystal structures of imidazolium sulphate dihydrate [96] and tetraimidazolezinc(II) perchlorate.[97]

Stereochemistry and Stereoselectivity.—A novel optical resolution of racemic α-amino-acids by formation of mixed ligand copper(II) complexes with electrostatic ligand–ligand interactions has been reported.[98] Resolution of DL-aspartic acid and DL-glutamic acid (A) was achieved *via* the stereospecific formation of ternary copper(II) complexes composed of one of these amino-acids and a basic L-α-amino-acid (B = arginine, lysine, or ornithine). A *trans* structure (2) is suggested for Cu(L-A)(L-B) and a *cis* structure (3) for Cu(D-A)(L-B). The



Cu(L-A)(L-B)
(2)



Cu(D-A)(L-B)
(3)

resolution of DL-(A) implies that the resolution of DL-(B) is feasible by this method.

Fujii [99] has continued his work on the determination of the optical purity of amino-acids by complex formation. The method uses the fact that when an optically active amino-acid is added to a basic solution of $K[Co(acac_2en)(Gly)_2]$ the solution shows a much larger optical rotation than that of the free amino-acid in the visible region. About 0.1—0.05 g (*ca.* 1/50 to 1/100 of the usual amount) suffices to determine the optical purity of the amino-acid.

Copper(I) oxide has been reported to be an excellent initiator for the racemization of alanine in basic solution.[100] The metal-ion catalysed racemization

[93] F. A. Cotton and T. R. Webb, *Inorg. Chem.*, 1976, **15**, 68.
[94] A. J. Carthy and N. J. Taylor, *J.C.S. Chem. Comm.*, 1976, 214.
[95] B. Y. K. Ho, J. A. Zubieta, and J. J. Zuckerman, *J.C.S. Chem. Comm.*, 1975, 88.
[96] H. C. Freeman, F. Huq, J. M. Rosalky, and I. F. Taylor, jun., *Acta Cryst.*, 1975, **B31**, 2833.
[97] C. A. Bear, K. A. Duggan, and H. C. Freeman, *Acta Cryst.*, 1975, **B31**, 2713.
[98] T. Sakurai, O. Yamauchi, and A. Nakahara, *J.C.S. Chem. Comm.*, 1976, 553.
[99] Y. Fujii, *Bull. Chem. Soc. Japan*, 1974, **47**, 2856.
[100] A. Tai, K. Okada, T. Masuda, and Y. Izumi, *Bull. Chem. Soc. Japan*, 1976, **49**, 310.

and α-proton exchange of *NN*-dicarboxymethyl-D-phenylglycine have been studied kinetically.[101] When bound to metal ions, Pb^{II}, Cu^{II}, and Ni^{II}, the ligand undergoes racemization at rates up to 5000 times faster than for the free ligand.

A number of papers have appeared dealing with stereoselective effects in the formation of metal complexes of amino-acids. In the biological pH range marked stereoselectivity is present in the formation of ternary histidinato-copper(II) complexes containing ligands (A) with positively charged protonated side-chains,[102] with preferential formation of the complex containing ligands of the same chirality, *e.g.* Cu(L-His)(L-HA). Stereoselectivity in formation of mononuclear complexes of histidine with some bivalent metal ions has been observed.[103] Stereoselective binding of copper(II), zinc(II), cobalt(II), and nickel(II) to the optically active amino-acids β-(2-pyridyl)- and β-(6-methyl-2-pyridyl)-α-alanine, which can be regarded as analogues of histidine and phenylalanine, has also been observed.[104]

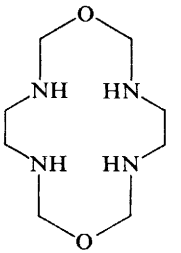
The *meso*-complex [Ni(D-Met)(L-Met)] is more thermodynamically stable than [Ni(D-Met)(D-Met)]. The stereoselectivity is attributed to terdentate binding of the methionine and indicates a weak interaction between nickel(II) and the thioether sulphur atom of the ligand.[105] It has been reported[106] that for the ligand *N*-benzyl-DL-valine (A), $Cu(D\text{-}A)_2$ and $Cu(L\text{-}A)_2$ are more stable than the *meso*-complex Cu(L-A)(D-A). Crystallographic results suggest that the observations can be rationalized in terms of steric hindrance to co-ordination of solvent water molecules in the axial positions of the *meso*-complex. The results of a study on the effect of solvent on stereoselective effects in copper(II) complexes of *N*-alkyl derivatives of bifunctional α-amino-acids have been published.[107] A number of theoretical investigations on the optical activity of copper(II) complexes of amino-acids have appeared.[108–110]

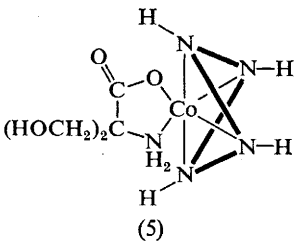
Several investigations with cobalt(III) complexes of amino-acids have been published. C.d. spectra of the *unsym-cis*(O) isomers of (L-alaninate-*N*-mono-acetato)(diethylenetriamine)cobalt(III) have been reported,[111] and stereoselectivity in formation of oxalato-L-aspartato-ethylenediaminecobalt(III), *i.e.* [Co(Ox)-(L-AspH)(en)], has been studied.[112] Stereoselectivity (>80%) was found in the *mer*-Δ and *fac*-Λ isomers. Biguanide-amino-acid complexes of the type $[Co(am)(Hbg)_2]^{3+}$, where am is the anion of glycine, sarcosine, L-alanine, L-valine, L-isoleucine, or L-proline, have been prepared and resolved.[113]

[101] L. G. Stadtherr and R. J. Angelici, *Inorg. Chem.*, 1975, **14**, 925.
[102] G. Brookes and L. D. Pettit, *J.C.S. Chem. Comm.*, 1975, 385.
[103] L. D. Pettit and J. L. M. Swash, *J.C.S. Dalton*, 1976, 588.
[104] P. R. Rechani, R. Nakon, and R. J. Angelici, *Bioinorg. Chem.*, 1976, **5**, 329.
[105] J. L. M. Swash and L. D. Pettit, *Inorg. Chim. Acta*, 1976, **19**, 19.
[106] V. A. Davankov, S. V. Rogozhin, Y. T. Struchkov, G. G. Alexandrov, and A. A. Kurganov, *J. Inorg. Nuclear Chem.*, 1976, **38**, 631.
[107] V. A. Davankov, S. V. Rogozhin, A. A. Kurganov, and L. Y. Zhuchkova, *J. Inorg. Nuclear Chem.*, 1975, **37**, 369.
[108] C.-Y. Yeh and F. S. Richardson, *Inorg. Chem.*, 1976, **15**, 682.
[109] A. Decinti and G. Larrazabal, *Inorg. Chim. Acta*, 1976, **18**, 121.
[110] L. Gil, E. Moraga, H. Bobadill, S. Bunel, and C. A. Bunton, *J. Inorg. Nuclear Chem.*, 1975, **37**, 2509.
[111] K. Okamoto, J. Hidaka, and Y. Shimura, *Bull. Chem. Soc. Japan*, 1975, **48**, 2456.
[112] M. Takeuchi and M. Shibata, *Bull. Chem. Soc. Japan*, 1974, **47**, 2797.
[113] H. Kawaguchi and B. Douglas, *J. Coordination Chem.*, 1976, **5**, 115.

Reactivity and Kinetics.—A number of interesting papers have appeared in this area, and the topic of synthetic oxygen carriers of biological interest reviewed.[114] The two isomers of *NN*-bis-(2-aminoethyl)glycine react with cobalt(II) in the absence of dioxygen (O_2) to give a complex CoL^+ which, when exposed to dioxygen, forms a stable dimeric oxygen adduct $[CoLO_2OHCoL]^+$.[35] The complex does not react further with oxygen or rearrange to give a kinetically inert cobalt(III) complex. Purging at low pH with N_2 results in complete reversal of the reaction, giving CoL^+ and O_2. The oxygenation of the bis complexes of cobalt(II) with DL-2,3-diaminopropionate and L-ornithinate has also been studied. Equilibrium data for oxygenation were obtained using polarographic techniques.[115]

Chloroauric acid oxidizes methionine stereospecifically to methionine sulphoxide.[116] Condensation of formaldehyde with $[Co(en)_2(Gly)]^{2+}$ gives as the initial product [α-hydroxymethylserinebis(ethylenediamine)cobalt(III)]$^{2+}$, which is subsequently converted into the [α-hydroxymethylserine-1,4,8,11-tetra-aza-6,13-dioxacyclotetradecanecobalt(III)]$^{2+}$ ion containing the new macrocyclic ligand (4) which is co-ordinated *via* the nitrogen donors as in (5).[117] The acidity


(4)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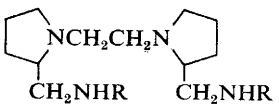

(6)

of the CH_2 group of co-ordinated glycine has been recognized for many years; thus $Cu(Gly)_2$ reacts with acetaldehyde to give complexes of threonine and allothreonine. Cooke and Dabrowiak [118] have studied the reaction of acetaldehyde with optically active $[Co(en)_2(Gly)]^{2+}$. The isolated threonine and allothreonine contain an excess of the *S*-enantiomer, indicating that the aldehyde prefers to attack the S 'side' of the co-ordinated glycine.

A number of studies of metal derivatives of amino-acid esters have appeared and the metal-ion promoted hydrolysis of amino-acid esters and peptides has been reviewed.[118a] The polymerization of the methyl esters of amino-acids *via* their copper(II) complexes has been reported.[119] The co-ordination of optically active amino-acids and their methyl esters to nickel(II) complexes of 1,2-bis-[2(*S*)-aminomethyl-1-pyrrolidinyl]ethane (6; R = H) and 1,2-bis-[2(*S*)-*N*-methyl-

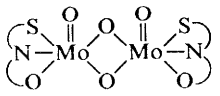
[114] F. Basolo, B. M. Hoffman, and J. A. Ibers, *Accounts Chem. Res.*, 1976, 384.
[115] K. T. Sew and H. K. J. Powell, *J.C.S. Dalton*, 1975, 2023.
[116] G. Natile, E. Bordignon, and L. Cattalini, *Inorg. Chem.*, 1976, **15**, 246.
[117] R. J. Geue, M. R. Snow, J. Springborg, A. J. Herlt, A. M. Sargeson, and D. Taylor, *J.C.S. Chem. Comm.*, 1976, 285.
[118] J. C. Dabrowiak and D. W. Cooke, *Inorg. Chem.*, 1975, **14**, 1305.
[118a] R. W. Hay and P. J. Morris, in ref. 16.
[119] A. Brack, D. Louembe, and G. Spach, *Origins Life*, 1975, **6**, 407.

aminomethyl-1-pyrrolidinyl]ethane (6; R = Me) has been studied.[120] Some amino-acidate ions co-ordinate stereoselectively, as do their methyl esters, so that base hydrolysis of the esters proceeds stereoselectively. The reactions of *cis*-$[MCl_2(PPh_2Cl)_2]$ (M = Pd or Pt) with α-amino-acid ester have been studied.[121] Complexes such as *cis*-$[MCl_2(PPh_2)_2NCH_2CO_2Me]$ and *trans*-$[MCl_2(PPh_2NHCH_2CO_2Me)_2]$ were prepared which may be useful catalysts for asymmetric induction.

The potential antitumour activity of some amino-acid complexes has been studied.[122] A number of copper(II) complexes of α-amino-acids have pronounced antiarthritic activity which is a function of their copper content.[18]

Efforts continue to be directed towards the simulation of the action of nitrogenase using systems containing a dimeric molybdenum(V) cysteine complex (7) and a reducing agent, and the area has been reviewed.[123] Such systems reduce acetylene to ethylene and dinitrogen (N_2) to ammonia in low yields. Ferredoxin model compounds such as $[Fe_4S_4(SR)_4]^{n-}$ (n = 2—4) apparently accelerate the transfer of electrons from reductant to molybdenum and improve ethylene yields.[124] Electrochemical reduction of the Mo^V cysteine dimer (7) shows that it undergoes a single four-electron reduction to Mo^{III} products, and it is suggested that a monomeric Mo^{III} species is the catalytically active complex.[125] A dextran-bound cysteine polymer (1.05 mmol cysteine per g dextran) forms a molybdenum complex analogous to (7) which does not appear to dissociate into a monomer analogous to (8) under basic conditions.[126] In the presence of borohydride the polymer-complex reduces acetylene 30 times faster than (7) possibly because the inert polymer support acts to keep apart reduced catalytically active species analogous to (9), thus preventing formation of inactive oxo-bridged dimers.

The metal-complex catalysed β-decarboxylation of L-aspartic acid has been reported.[127] Various other kinetic investigations have been carried out. The nitrogen to oxygen isomerization of the penta-ammineruthenium(III)glycine ion has been investigated,[128] as has the Cr^{II} reductions of amino-acid substituted



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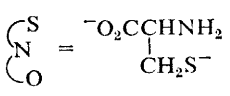

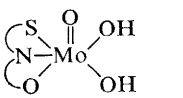

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[120] S. Kitagawa, T. Murakami, and M. Hatano, *Inorg. Chem.*, 1976, **15**, 1378.
[121] P. W. Lednor, W. Beck, and G. Thiel, *Inorg. Chim. Acta*, 1976, **20**, L11.
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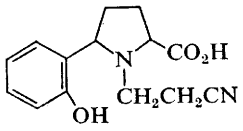
[125] D. A. Ledwith and F. A. Schultz, *J. Amer. Chem. Soc.*, 1975, **97**, 6591.
[126] H. Susuki, S. Meshitsuka, T. Tabashima, and M. Ichikawa, *Chem. Letters*, 1975, **4**, 285.
[127] N. Y. Sakkab and A. E. Martell, *Bioinorg. Chem.*, 1975, **5**, 67.
[128] S. E. Diamond and H. Taube, *J. Amer. Chem. Soc.*, 1975, **97**, 5921.

penta-amminecobalt(III) ions, *i.e.* $[Co(NH_3)_5OOCCHR\overset{+}{N}H_3]Cl_3$.[129] The reaction of $[HCrO_4]^-$ with L-cysteine (H_2L) has been studied by stopped-flow methods.[130] The transient orange species observed at 420 nm is the 1:1 chromate ester $[(HL)CrO_3]^-$. Oxygen-18 exchange studies of the *trans*(*fac*)-bis(*N*-methyliminodiacetato)chromate(III) anion have been carried out,[131] and studies made of the kinetics and mechanism of the elimination of chloride from *cis*-chlorobis(ethylenediamine)(glycinato-*N*)cobalt(III).[132]

The kinetics of complexing of nickel(II) by methionine, cysteine, penicillamine, cysteine ethyl ester, glycylmethionine, glutathionine, tyrosine, *m*-tyrosine, and *o*-tyrosine have been measured by stopped-flow methods. Initial complexing is at the carboxylate[133] group of the zwitterion; however, for methionine, penicillamine, and cysteine ethyl ester, a reaction path involving initial complexing at the thiol group occurs.

Schiff Bases.—Compared with previous years there has been some resurgence of interest in the general field of Schiff base complexes of amino-acids. The oxidative deamination of alanine by atmospheric dioxygen in bis(*N*-salicylidenealaninato)cobaltate(III) has been reported,[134] as has the asymmetric synthesis of threonine and allothreonine by the condensation of acetaldehyde at pH 11 with dissymmetric bis(*N*-salicylideneglycinato)cobaltate(III) complexes.[135] A novel threonine aldolase model has been described.[136] The Al^{III} pyridoxal-catalysed threonine aldolase model reactions of threonine and a new substrate, β-hydroxyvaline, were followed by quantitative n.m.r. in D_2O solution. Formation of acetone in the β-hydroxyvaline reaction provides the first quantitative evidence for C—C bond fission of an α-amino-acid having an α-proton in the presence of pyridoxal and metal ions. The reaction of acrylonitrile with *N*-salicylideneglycinatocopper(II) gives the copper(II) complex of *N*-(2-cyanoethyl)-4-cyano-5-*o*-hydroxyphenylproline (10).[137]

The crystal and molecular structures of iron(III) complexes of the sexadentate ligand *NN'*-ethylenebis-(*o*-hydroxyphenylglycine) have been reported.[138] Structures of the metal complexes of *N*-salicylidenecysteine have also been



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[129] R. Bembi and W. U. Malik, *J. Inorg. Nuclear Chem.*, 1975, **37**, 570.
[130] J. P. McCann and A. McAuley, *J.C.S. Dalton*, 1975, 783.
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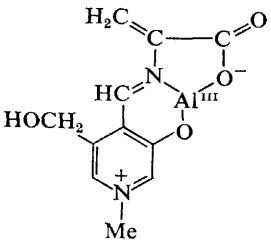
[132] A. C. Dash, R. K. Nanda, and S. K. Mohapatra, *J.C.S. Dalton*, 1975, 897.
[133] J. E. Letter and R. B. Jordan, *J. Amer. Chem. Soc.*, 1975, **97**, 2381.
[134] N. G. Faleev, Y. N. Belokon, V. M. Belikov, and L. M. Mel'nikova, *J.C.S. Chem. Comm.*, 1975, 85.
[135] Y. N. Belokon, V. M. Belikov, S. V. Vitt, M. M. Dolgaya, and T. F. Savel'eva, *J.C.S. Chem. Comm.*, 1975, 86.
[136] J. A. Marcello, A. E. Martell, and E. H. Abbott, *J.C.S. Chem. Comm.*, 1975, 16.
[137] L. Casella, M. Gullotti, A. Pasini, G. Ciani, M. Manassero, M. Sansoni, and A. Swoni, *Inorg. Chim. Acta*, 1976, **20**, L31.
[138] N. A. Bailey, D. Cummins, E. D. McKenzie, and J. M. Worthington, *Inorg. Chim. Acta*, 1976, **18**, L13.

described.[139] Various spectroscopic investigations have been carried out on Schiff-base derivatives. The absorption and c.d. spectra of pyridoxylidenalanato-aluminium(III) have been studied,[140] as have the i.r. spectra of nickel(II) and cobalt(II) *N*-salicylideneglycinates.[141] A series of Cu^{II} and Cu^{II}–Zn^{II} complexes of X-salicylaldehyde-amino-acid Schiff bases (X = 4-OH, H, 5-OH, 5-Cl) with glycine, alanine, and phenylglycine have been prepared and their magnetic and spectral properties investigated.[142] The condensation of salicylaldehyde or acetylacetone with *S*-(+)-cysteine esters in the presence of nickel(II) gives diamagnetic, dinuclear, planar Schiff-base complexes. Their c.d. spectra show negative Cotton effects due to the stereospecific co-ordination of the chiral ligand.[143] Stereoselective interactions between amino-acids and optically active diketones in copper(II) complexes of their Schiff bases have been described.[144] The structures of some transition-metal complexes of *N*-glycyl-α-picolylamine have been studied, and their mechanism of formation was investigated.[145] Karube and Matsuschima [146] have carried out work on non-enzymatic pyridoxal catalysis and describe a species absorbing at 467 nm which they suggest to be the Al^{III} complex of the Schiff base *N*-methylpyridoxylidene-α-aminoacrylate (11). The



(11)

species is considered as a possible model for an intermediate in pyridoxal-catalysed α,β-elimination and β-replacement reactions of amino-acids.

A review dealing with Schiff base complexes of copper(II) has appeared.[147]

3 Peptides

Structural Aspects.—There appears to have been an increase in activity in this area, particularly in using metal-peptides as models for metalloproteins. Thus Lau and Sarkar [148] have studied the copper(II) complexes of diglycyl-L-histidine as a peptide mimicking the copper(II)-transport site of albumin. In addition there has been a significant increase in the volume of work dealing with the formation constants of metal complexes of simple peptides.

[139] F. Baykut, A. Aydin, and A. Uren, *Chim. Acta Turc.*, 1975, **3**, 105.
[140] A. E. Martell and A. F. Eidson, *Bioinorg. Chem.*, 1975, **4**, 277.
[141] G. C. Percy, *J. Inorg. Nuclear Chem.*, 1975, **37**, 2071.
[142] L. A. Zyzyck, H. Frummer, and J. F. Villa, *J. Inorg. Nuclear Chem.*, 1975, **37**, 1653.
[143] F. Jursik and B. Hajek, *Inorg. Chim. Acta*, 1975, **13**, 169.
[144] L. Casella, M. Gullotti, A. Pasini, and M. Visca, *Inorg. Chim. Acta*, 1976, **19**, L9.
[145] O. Bekaroglu, *Chim. Acta Turc.*, 1975, **3**, 23.
[146] Y. Karube and Y. Matsushima, *J. Amer. Chem. Soc.*, 1976, **98**, 3725.
[147] H. S. Maslen and T. M. Waters, *Coordination Chem. Rev.*, 1975, **17**, 137.
[148] S. Lau and B. Sarkar, *Canad. J. Chem.*, 1975, **53**, 710.

The thermodynamics of formation of complexes of copper(II) and nickel(II) with glycylhistidine, β-alanylhistidine, and histidylglycine have been studied potentiometrically at 25 °C and $I = 0.10$ mol dm^{-3} (KNO_3).[149] The equilibria are very complicated but polynuclear species are insignificant below pH 10 in dilute solution. Suggestions are made as to the probable co-ordination sites as a function of pH. A further paper by the same group [150] deals with the formation constants of nickel(II) and copper(II) complexes of dipeptides containing non-co-ordinating substituent groups. The structures of the complexes are discussed and the possible equilibria in the nickel–dipeptide systems considered. Several metallo-proteins contain more than one copper ion per protein molecule, and in some cases a pair of copper ions may be close enough to interact with each other. Model amino-acid and peptide complexes containing two or more neighbouring copper(II) ions are rare. Carnosine (β-alanyl-L-histidine) forms a dimer in equimolar solutions of copper(II) and ligand at pH 6.[151] L-Histidylglycine is also reported to form a dimer in equimolar solutions with copper(II) at pH 7,[152] and this conclusion has now been confirmed by Boggess and Martin [153] who carried out a spectrophotometric–pH study over an eight-fold concentration range.

A considerable number of potentiometric studies have been carried out on copper(II) and nickel(II) complexes of peptides, although it is now becoming more common for a variety of other physical techniques to be employed to study these problems. Thus Scheinblatt [154] has investigated the interaction of copper(II) with acetylglycine using potentiometric titration, e.s.r., n.m.r., and spectrophotometric techniques. At pH < 6.5 the copper is bound to the carboxylate group to give a 'blue' complex. Precipitation of copper occurs at pH *ca.* 7, but at pH > 9 in the presence of excess ligand, soluble complexes are formed due to binding *via* deprotonated amide groups.

Sigel [155] has carried out a detailed investigation of the influence of alkyl side-chains on the stability of binary and ternary copper(II)–dipeptide complexes. Protonation constants and formation constants have been determined for the dipeptides: glycylglycine, glycyl-L-alanine, L-alanylglycine, L-alanyl-L-alanine, glycyl-L-leucine, L-leucylglycine, glycyl-L-isoleucine, L-isoleucylglycine, glycylsarcosine, sarcosylglycine, glycyl-L-proline, and L-prolylglycine. Besides the binary complexes CuL^+ and Cu(L − H) the mixed ligand complexes with 2,2′-dipyridyl Cu(dipy)L^+ and Cu(dipy)(L − H) were also investigated. Depending upon the position of the alkyl group either the stability or the acidity of the complexes is altered.

Highly polynuclear species are formed in equilibrium solutions of copper(II) and glycyl-L-histidylglycine.[156] Histidine-containing peptides have received considerable attention. Formation constants have been determined [157] for zinc(II) and cobalt(II) complexes of glycyl-L-histidine, glycyl-L-histidylglycine,

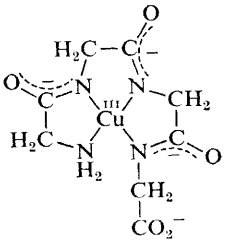
[149] G. Brookes and L. D. Pettit, *J.C.S. Dalton*, 1976, 2112.
[150] G. Brookes and L. D. Pettit, *J.C.S. Dalton*, 1976, 2106.
[151] E. W. Wilson, M. H. Kasperian, and R. B. Martin, *J. Amer. Chem. Soc.*, 1970, **92**, 5365.
[152] H. Aiba, A. Yokoyama, and H. Tanaka, *Bull. Chem. Soc. Japan*, 1974, **47**, 136.
[153] R. K. Boggess and R. B. Martin, *J. Inorg. Nuclear Chem.*, 1975, **37**, 1097.
[154] M. Scheinblatt, *Bioinorg. Chem.*, 1975, **5**, 95.
[155] H. Sigel, *Inorg. Chem.*, 1975, **14**, 1535.
[156] R. Osterberg and B. Sjoberg, *J. Inorg. Nuclear Chem.*, 1975, **37**, 815.
[157] R. P. Agarwal and D. D. Perrin, *J.C.S. Dalton*, 1975, 1045.

and L-histidylglycine from pH–titration data, using a range of metal ion and ligand concentrations. Dinuclear complexes are the predominant species in solutions of zinc(II) and glycylhistidine. Mixed-ligand complexes of the type MAL are also formed by zinc(II) and cobalt(II) with histidine (HA) and the peptides. Comparisons are made of the zinc(II)-complexing abilities of the peptides in competition with α-amino-acids under physiological conditions. Formation constants have also been determined [158] for the copper(II) complexes of glycylhistidine, glycylhistidylglycine, histidylglycine, and carnosine by pH–titration, and comparisons made with the copper-complexing abilities of these peptides in competition with α-amino-acids under physiological conditions.

Diglycyl-L-histidine is a peptide molecule designed to mimic the specific copper(II)-transport site of human albumin. The equilibria and structures of the complexes formed in the ternary system of L-histidine, copper(II), and diglycyl-L-histidine have been studied by Kruck and Sarkar.[159] In addition, kinetic studies of the copper(II)-exchange from L-histidine to human serum albumin and diglycyl-L-histidine have been made.[148] The exchange rates from L-histidine to albumin and the tripeptide are 0.67 and 0.42 s^{-1} at pH 7.53, respectively. Possible mechanisms for these reactions are considered.

Only limited work has been carried out on stereoselective effects in the co-ordination of peptides to metal ions. For complexes of copper(II) with Gly-Val, Gly-Phe, and Leu-Leu there is no stereoselectivity when only one of the amino-acids is optically active.[160] When both residues contain chiral centres there is no stereoselectivity in the complexes formed prior to ionization of the amide hydrogen, but after ionization occurs, dipeptide complexes containing amino-acid residues of the same chirality (*e.g.* [Cu(L-Leu-L-Leu]) are more thermodynamically stable than those of mixed chirality (*e.g.* [Cu(L-Leu-D-Leu]).

Probably the most significant development in the area of copper–peptide complexes has been the characterization of a readily accessible copper(III)–peptide complex by Margerum and his co-workers.[161] Copper(II) occurs in a number of compounds, many of which are not stable in aqueous solution. Thus, crystalline $NaCuO_2$ can be prepared but it decomposes in solution in a few seconds. Cu^{II}-tetraglycine can be oxidized quantitatively to $[Cu^{III}(H_{-3}G_4)]^-$ (12) by $IrCl_6^{2-}$. If the other species are removed by anion exchange, the resulting



(12)

[158] R. P. Agarwal and D. D. Perrin, *J.C.S. Dalton*, 1975, 268.
[159] T. P. A. Kruck and B. Sarkar, *Inorg. Chem.*, 1975, **14**, 2383.
[160] G. Brookes and L. D. Pettit, *J.C.S. Dalton*, 1975, 2302.
[161] D. W. Margerum, K. L. Chellappa, F. P. Bossu, and G. L. Burce, *J. Amer. Chem. Soc.*, 1975, **97**, 6894.

solutions of $[Cu^{III}(H_{-3}G_4)]^-$ are slow to decompose in weakly acidic media. Visible spectra, e.p.r. measurements, and substitution kinetics (d^8-square planar complex) are consistent with the presence of copper(III). The E^0 value of the complex is 0.892 V at 25 °C and I = 0.1M-$NaClO_4$. The low potential and relatively high stability of the complex are of special interest in biological redox reactions. Molecular oxygen reacts with Cu^{II}-tetraglycine in neutral solution to give $[Cu^{III}(H_{-3}G_4)]^-$, provided photochemical inhibition is avoided.[162] This reaction provides a pathway for the activation of molecular oxygen in its oxidation of peptides. In addition, the attractive possibility of two electron transfer reactions between Cu^I and Cu^{III} is of interest as a biological redox pathway which would avoid high-energy free radical intermediates. Hamilton has proposed a Cu^{III}/Cu^I couple in the reactions of the copper enzyme galactose oxidase.[163]

In view of the importance of thiol groups of cysteine residues in the active sites of metalloproteins, it is surprising that only a few studies have been reported concerning the interaction of the thiol-containing peptides with biologically interesting metal ions. Thiol co-ordination has been suggested by *p*-mercuribenzoate binding studies on azurin, plastocyanin, and stellacyanin. There is now evidence that this lack is being rectified. Sugiura and Hirayama [164] have investigated the copper(II) and nickel(II) complexes of a variety of thiol peptides using potentiometric and visible spectral measurements. The formation constants increase in the order 6–6 < 6–5 < 5–6 < 5–5 for the chelate rings. The nickel(II) complex of α-mercaptopropionylglycine has also been studied by visible spectra, potentiometric, and ^{13}C n.m.r. techniques.[165] The co-ordination sites are sulphur, the peptide nitrogen, and the carboxylate group. Unlike the glycylglycine–nickel(II) system, ionization of the peptide proton occurs in the neutral pH region with the formation of a diamagnetic square-planar complex.

Complexes of α-glutamylcysteinylglycine (glutathione) with some transition-metal ions have been isolated and characterized.[166] Glutathione ($GluH_3$) is an extremely versatile ligand complexing as the mononegative $GluH_2^-$ species in $Pd(GluH_2)Cl,3H_2O$, the dinegative $GluH^{2-}$ species in $Cu(GluH),4H_2O$ and the trinegative species in $Li[Ni(Glu)],2H_2O$ and $Li[Co(Glu)],2H_2O$. The mercapto-group ionizes most readily, and subsequent ionizations involve the carboxylate groups.

The iron complexes of several sulphur-containing peptides such as mercapto-propionylglycine, glycylcysteine, and glutathione have similar absorption spectra to those of 2Fe–2S ferredoxins and show a dramatic e.p.r. signal change from g = 4.4 to g = 2.0 upon addition of sulphide.[167]

Various investigations have been carried out using glycyl peptides. Formation constants obtained using PSEUDOPLOT are reported for zinc(II) and lead(II)

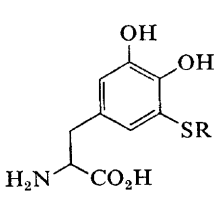
[162] G. L. Burce, E. B. Paniago, and D. W. Margerum, *J.C.S. Chem. Comm.*, 1975, 261.
[163] G. A. Hamilton, R. D. Libby, and C. R. Hartzell, *Biochem. Biophys. Res. Comm.*, 1973, **55**, 333.
[164] Y. Sugiura and Y. Hirayama, *Inorg. Chem.*, 1976, **15**, 679.
[165] Y. Sugiura, Y. Hirayama, H. Tanaka, and H. Sakurai, *J. Inorg. Nuclear Chem.*, 1975, **37**, 2367.
[166] S. T. Chow, C. A. McAuliffe and B. J. Sayle, *J. Inorg. Nuclear Chem.*, 1975, **37**, 451.
[167] Y. Sugiura, M. Kunishima, H. Tanaka, and H. H. Dearman, *J. Inorg. Nuclear Chem.*, 1975, **37**, 1511.

complexes of glycine, glycylglycine, and triglycine.[168] Kaneka and Martell have studied the solution equilibria of copper(II) and nickel(II) polyglycine complexes by magnetic susceptibility, optical spectra, and pH–titration.[169] O.r.d. and c.d. measurements have been used to investigate the copper(II) complexes of various optically active di- and tri-peptides.[170] The aqueous solution equilibria of the copper(II) and nickel(II) complexes of glycinamide, glycylglycinamide, and glycylglycylglycinamide have been determined by pH–titration at 25 °C.[171] Protons are ionized from terminal amide nitrogens in addition to peptide hydrogens. The nickel(II) complex of glycylglycinamide has two amide proton ionizations with pK_a values of 8.52 and 9.34. A third ionization, pK_a 10.5, is attributed to a co-ordinated water molecule.

The polarographic behaviour of nickel(II) complexes of di- and tri-glycine has been studied,[172] as have the formation constants of biuret with a variety of bivalent metal ions.[173]

Wilson's disease is a genetic defect resulting in an excessive accumulation of copper(II) ions in the liver, kidney, and brain. Several metal-binding reagents have been studied for their effectiveness in removing the excess copper, notably 2,3-dimercaptopropanol (BAL) and D-penicillamine. Laurie and co-workers[174] have investigated the ternary systems Cu^{II}–Gly–Gly–D-penicillamine and Cu^{II}–L-His–D-penicillamine in aqueous solution by electronic spectra and e.s.r. Evidence is presented indicating the formation of dimeric ternary complexes. The possible role of ternary complexes in the mobilization of the excess copper in Wilson's disease is discussed.

Iron(II) and cobalt(II) complexes of Boc-(Gly-L-Cys-Gly)$_4$-NH_2 have been synthesized in dimethyl sulphoxide solution.[175] It is suggested that the iron complex is the first rubredoxin model incorporating a polypeptide backbone which has a tetrahedral FeS_4 core. Adenochrome, the iron(III)-containing pigment from the bronchial heart of *Octopus vulgaris*, has been characterized[176] and shown to consist of a group of closely related peptides derived from glycine and two novel iron-binding amino-acids, adenochromines A (13) and B (14), which may arise biologically from L-dopa and L-histidine-5-thiol.



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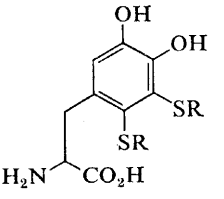
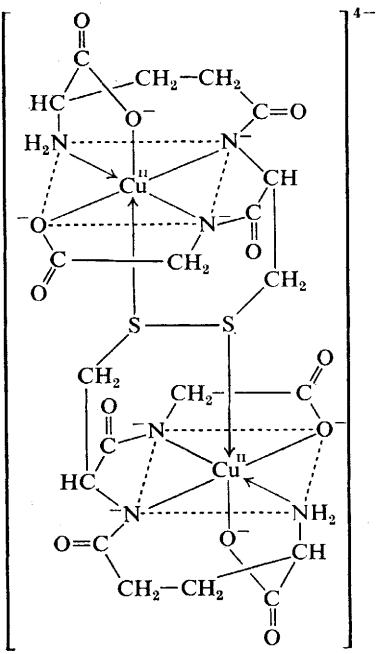


(14)

[168] A. M. Corrie, G. K. R. Makar, M. L. D. Touche, and D. R. Williams, *J.C.S. Dalton*, 1975, 105.
[169] A. Kaneda and A. E. Martell, *J. Coordination Chem.*, 1975, **4**, 137.
[170] A. E. Martell, M. K. Kim, and A. Kaneda, *J. Coordination Chem.*, 1975, **4**, 159.
[171] T. F. Dorigatti and E. J. Billo, *J. Inorg. Nuclear Chem.*, 1975, **37**, 1515.
[172] K. Nag and P. Bannerjee, *J. Inorg. Nuclear Chem.*, 1975, **37**, 1521.
[173] R. M. Sanyal, P. C. Srivastava, and B. K. Banerjee, *J. Inorg. Nuclear Chem.*, 1975, **37**, 343.
[174] S. H. Laurie, T. Lund, and J. B. Raynor, *J.C.S. Dalton*, 1975, 1389.
[175] J. R. Anglin and A. Davidson, *Inorg. Chem.*, 1975, **14**, 234.
[176] S. Ito, G. Nardi, and G. Prota, *J.C.S. Chem. Comm.*, 1976, 1042; see also G. Nardi and H. Steinberg, *Comp. Biochem. Physiol.*, 1974, **48B**, 453.

Sites of copper(II) binding to molecules such as histidine and peptides have often been characterized by selective broadening in ^1H n.m.r. spectra of ligand hydrogens located nearest to copper(II). Typically the ligand is present in a 100-fold excess over copper(II). Martin and co-workers [177] have suggested that two criteria must be met before this method can be applied, (i) there must be rapid exchange of copper(II) among all sites and (ii) there must be no scalar coupling to contribute to line-broadening. An interesting dinuclear copper(II) complex of oxidized glutathione has been characterized.[178] The suggested structure (15) involves metal–metal interaction *via* the disulphide bridge, and it



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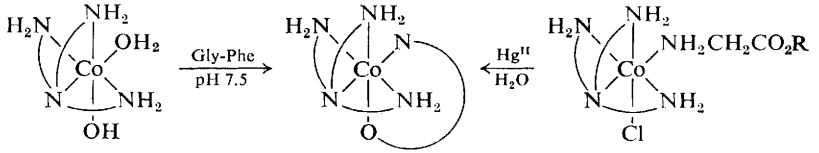
may be regarded as a model for the non-e.p.r.-detectable copper in the 'blue oxidases'. The reaction of a variety of di- and tri-peptides with Zeise's salt, $K[PtC_2H_4Cl_3]H_2O$, has been studied.[179] Uni-, and bi-, and ter-dentate complexes involving the amino N, peptide N or O, and the carboxylate O–donor atoms have been characterized and their i.r. spectra studied.

Several interesting crystal structures have been published. In glycylglycinato-(1,10-phenanthroline)copper(II)trihydrate,[180] glycylglycine acts as a terdentate ligand *via* amino N-ionized amide and carboxylate O-donors. The fourth tetragonal position about copper(II) is occupied by one phen N, while the other

[177] W. G. Espersen, W. C. Hutton, S. T. Chow, and R. B. Martin, *J. Amer. Chem. Soc.*, 1974, **96**, 8111.
[178] P. Kroneck, *J. Amer. Chem. Soc.*, 1975, **97**, 3839.
[179] L. E. Nance and H. G. Frye, *J. Inorg. Nuclear Chem.*, 1976, **38**, 637.
[180] M. C. Lim, E. Sinn, and R. B. Martin, *Inorg. Chem.*, 1976, **15**, 807.

N-donor occupies a tilted apical position resulting in a distorted square-pyramidal geometry about copper(II). Visible spectra suggest that the structure found in the solid persists in aqueous solution near pH 9. The crystal structure of aqua-(glycyl)-L-tryptophanatocopper(II) dihydrate has been established.[181] The peptide ligand behaves as a terdentate ligand *via* the terminal amino-group, deprotonated peptide nitrogen, and the carboxylate group; a single water molecule completes an approximate square (four-co-ordinate copper). The crystal structure[182] of the orange isomer of $[Co(tren)(gly)]Cl,ClO_4$ [tren = tris-(2-aminoethyl)amine] establishes that the glycine is co-ordinated with its nitrogen atom *trans* to the tertiary amino-group of tren, and that the tren chelate rings have two *k* and one *k'* conformations. This result is important since the hydroxoaquotris-(2-aminoethyl)aminocobalt(III) ion (16) promotes the hydrolysis of glycylphenylalanine to give the orange glycinato complex (17), which is also



(16) (17) (18)

obtained in the Hg^{II}-promoted reaction of $[Co(tren)(glyOR)Cl]^{2+}$ (18). It is probable that the red isomer[183] has the oxygen *trans* to the tertiary nitrogen of tren.

Reactivity.—There seems to have been some decline in the amount of work carried out in this area. The interaction of dioxygen with cobalt(II) complexes of glycylglycine in solution has been investigated.[184] It has previously been generally assumed that only the bis-glycylglycine complex interacted with dioxygen, since it has been accepted[185, 186] that only complexes which contain three nitrogens in the co-ordination sphere interact with dioxygen. The '3*N*' rule has been questioned as a result of studies on the oxygenation of cobalt complexes of the ethylenediaminediacetic acids.[187] The oxygenation equilibria of a large variety of cobalt(II) complexes of amino-acids and dipeptides have been investigated.[188] The stoicheiometries of the oxygenation complexes have been determined and thermodynamic equilibrium constants obtained. The actual oxygenated species is the 'brown intermediate' observed by some early investigators at high pH. This intermediate eventually loses the bridging peroxo-group to form red, unoxygenated mononuclear cobalt(III) complexes.

The binding of zinc(II) to L-aspartyl-L-phenylalanine methyl ester has been studied.[189] Hydrolysis of the ester is promoted by the zinc. N.m.r. (100-MHz)

[181] M. B. Hursthouse, S. A. A. Jayaweera, H. Milburn, and A. Quick, *J.C.S. Dalton*, 1975, 2569.
[182] Y. Mitsui, J. Watanabe, Y. Iitaka, and E. Kimura, *J.C.S. Chem. Comm.*, 1975, 280.
[183] E. Kimura, S. Young, and J. P. Collman, *Inorg. Chem.*, 1970, **9**, 1183.
[184] G. McLendon and A. E. Martell, *Inorg. Chem.*, 1975, **14**, 1423.
[185] M. S. Michailidis and R. B. Martin, *J. Amer. Chem. Soc.*, 1969, **91**, 4683.
[186] S. Fallab, *Angew. Chem. Internat. Edn.*, 1967, **6**, 496.
[187] R. G. Wilkins, in 'Bioinorganic Chemistry' (Advances in Chemistry Series No. 100). The American Chemical Society, Washington, 1971, p. 100.
[188] W. R. Harris, G. McLendon, and A. E. Martell, *J. Amer. Chem. Soc.*, 1976, **98**, 8378.
[189] M. L. D. Touche and D. R. Williams, *J.C.S. Dalton*, 1976, 2001.

measurements were used to identify the binding sites. A detailed investigation of the metal-promoted hydrolysis of glycylglycine has appeared,[190] in conjunction with an i.r. investigation of the structures of the copper(II) and zinc(II) complexes in D_2O solution.[191] The results confirm the earlier observations[192] that in the presence of copper(II) the pH–rate profile has a maximum at pH 4.4 (the rate decrease at high pH being associated with ionization of the amide proton), whereas this effect is not observed with zinc(II).

Several kinetic investigations have dealt with metal–peptide complexes. The proton-transfer kinetics of cyano(glycylglycinato)nickelate(II) and cyano-(glycylglycinamido)nickelate(II) have been studied.[193] Margerum and co-workers[194] have established that the rates of proton transfer at the nitrogen atom in metal peptide complexes are several orders of magnitude slower than the reaction rates of typical nitrogen acids with similar pK_a values. The Brönsted plots for the metal–peptides undergo a relatively rapid transition from α values of unity to zero without reaching the diffusion-limiting rates. The acid decomposition and proton-assisted nucleophilic displacement by triethylene-tetramine of copper(II)–glycylglycyl-L-histidine has been studied kinetically.[195] The presence of histidine as the third amino-acid residue in tripeptide complexes of copper(II) drastically decreases their susceptibility to nucleophilic attack.

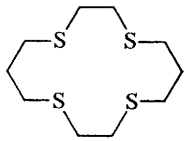
4 Proteins

Interest in this area increases from year to year. One point of considerable importance is that jackbean urease (the first enzyme to be crystallized) has been established to be a nickel metalloenzyme.[196] Until now nickel(II) metalloenzymes were unknown, with the exception of procarboxypeptidase A. Study of copper–sulphur interactions in relation to copper-containing proteins such as oxidases has been an active field. The results of an *X*-ray photoelectron spectroscopic study of bean plastocyanin have been interpreted in terms of delocalized Cu^{II}–cysteine-sulphur binding.[197]

Freeman's[198] group in Sydney have reported preliminary crystallographic data for plastocyanin from poplar leaves. The metalloprotein was crystallized in the oxidized (*i.e.* Cu^{II}) state. The deep-blue crystals are rhombic prisms with space group $P2_12_12_1$ and cell dimensions $a = 29.6$, $b = 46.9$, $c = 57.6$ Å. The molecular weight is 10 500 with four molecules of protein in the unit cell. An n.m.r. study (at 270 MHz) of oxidized and reduced French bean plastocyanin has appeared.[199] Copper complexes of cyclic or linear poly-thioethers show an

[190] T. Nakata, M. Tasumi, and T. Miyazawa, *Bull. Chem. Soc. Japan*, 1975, **48**, 1599.
[191] M. Tasumi, S. Takahashi, T. Nakata, and T. Miyazawa, *Bull. Chem. Soc. Japan*, 1975, **48**, 1595.
[192] I. J. Grant and R. W. Hay, *Austral. J. Chem.*, 1965, **18**, 1189.
[193] G. K. Pagenkopf and V. T. Brice, *Inorg. Chem.*, 1975, **14**, 3119.
[194] C. E. Bannister, D. W. Margerum, J. M. T. Raycheba, and L. F. Wong, 'Proton Transfer' Faraday Society Symposium No. 10, The Chemical Society, London, 1975.
[195] L. F. Wong, J. C. Cooper, and D. W. Margerum. *J. Amer. Chem. Soc.*, 1976, **98**, 7268.
[196] N. E. Dixon, C. Gazzola, R. L. Blakeley, and B. Zerner, *J. Amer. Chem. Soc.*, 1975, **97**, 4131.
[197] P. J. Clendening, H. B. Gray, F. J. Grunthaner, and E. I. Solomon, *J. Amer. Chem. Soc.*, 1975, **97**, 3878.
[198] G. V. Chapman, P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, J. A. M. Ramshaw, and M. P. Venkatappa, *J. Mol. Biol.*, in press.
[199] J. K. Beattie, D. J. Fensom, H. C. Freeman, E. Woodcock, H. A. O. Hill, and A. M. Stokes, *Biochim. Biophys. Acta*, 1975, **405**, 109.

absorption band (600 nm) similar to that of 'blue' proteins, assigned in both complexes and proteins to an S → Cu^{II} charge-transfer band.[200] The complex of the macrocyclic thioether ligand (19) has a planar arrangement about copper,



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suggesting that distorted site symmetry about copper need not occur in the proteins. A further extrapolation from this work is that thioether sulphurs of methionine groups could be the copper-binding site of 'blue' proteins. A kinetic study of the reduction of 'blue' proteins by $[Fe(edta)]^{2-}$ has been discussed in terms of an outer-sphere mechanism for all such proteins but laccase, which requires a specific protein activation to accept the reductant.[201] McLendon and Martell [202] have examined the sequence data for the 'blue' proteins plastocyanine and azurin, and suggested a copper binding site of one cysteine, one methionine, and one or two histidine residues.

Sigel [203] has presented a very useful summary of metal binding sites in naturally occurring metal complexes of 3*d* ions (see Table, p. 518). Various investigations of carboxypeptidase A have appeared. Cobalt(III) carboxypeptidase A has been prepared by oxidation of cobalt(II) carboxypeptidase A with hydrogen peroxide at pH 7.5.[204] The new derivative has a band at 503 nm (ε 500) and its catalytic activity was investigated. ^{35}Cl N.m.r. linewidth measurements have been used to probe the active site zinc chemistry of carboxypeptidase A.[205] The arsanilazotyrosine-248 zinc complex of carboxypeptidase A has been used to monitor the catalytic events at the active site.[206] Cobalt(III) complexes of azophenols have been synthesized and their relevance to azotyrosine-modified enzymes, which may form exchange-inert cobalt(III) complexes, has been discussed.[207] Electronic spectra and magnetic susceptibility studies of nickel(II) and cobalt(II) carboxypeptidase A have been reported.[208] Comparison with known complexes suggests an N_2O_4 or NO_5 donor set in the nickel derivative. Spectral studies of copper(II) carboxypeptidase A and related model complexes have also been reported.[209] Oxidase activity is displayed by the copper(II)

[200] T. E. Jones, D. B. Rorabacher, and L. A. Ochrymowycz, *J. Amer. Chem. Soc.*, 1975, **97**, 7485; L. C. Zimmer and L. L. Diaddario, *ibid.*, p. 7163, and references therein.
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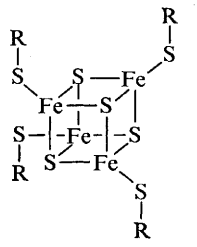
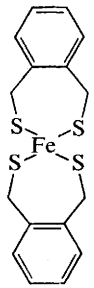
enzyme.[210] Breslow and co-workers[211] have studied the very fast zinc(II)-promoted hydrolysis of an anhydride which provides a model for the rate and mechanism of carboxypeptidase A catalysis.

Non-haem iron–sulphur proteins are implicated in biological processes as diverse as photosynthesis and nitrogen fixation. They can be classified according to the number of iron atoms present: Fe, 2Fe, 4Fe, and 8Fe proteins. *X*-Ray crystal structures of the 4Fe protein from *Chromatium* and the 8Fe protein from *P. aerogenes* show them to contain one and two $[Fe_4S_4(S\text{-Cys})_4]$ clusters, respectively. The complexes $[Fe_4S_4(SR)_4]^{2-}$ (R = alkyl or aryl), containing structurally and spectrally analogous clusters, were synthesized some time ago, and work in this area up to the end of 1974 has been reviewed.[212] The general area of electron-transfer proteins is the subject of a current review by Moore and Williams.[3]

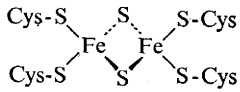
The synthetic clusters are prepared in high yields from $FeCl_3$, thiol, and NaHS in the presence of base, and an *X*-ray crystal structure (20) indicates that they are somewhat distorted from cubic symmetry.[213] Polarography shows that the $[Fe_4S_4(SR)_4]^{2-}$ clusters are members of the electron-transfer series $[Fe_4S_4(SR)_4]^{n-}$ (n = 1—4).[213] The structural unit of the Fe—S moiety of the 2Fe proteins has not been established by *X*-ray diffraction, but the synthetic model (21) has similar spectroscopic properties to the biological system.[214] Critical assessment of the physicochemical data and further structural work on synthetic analogues[215] suggests (22) as the minimal formulation of the 2Fe proteins.



(20)



(21)



(22)

The $[Fe_4S_4(SR)_4]^{2-}$ clusters undergo ready thiol exchange reactions, and these reactions have now been studied kinetically.[216] This reaction provides the basis for a method for the removal of intact iron–sulphur clusters from the active sites of ferredoxin proteins.[217] Any subtle distortions within the clusters caused by a variation of the peripheral ligands should be reflected in the Fe—S stretching

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frequencies within the cluster. These vibrations can be enhanced in intensity by use of resonance Raman spectroscopy, and some recent results [218] suggest that the overall symmetry of the biological clusters is lower than that of the models. A number of dithiol–iron–sulphur and –selenium complexes have been prepared and compared with iron–sulphur proteins.[219]

Various investigations dealing with carbonic anhydrase have been made. The three-dimensional crystal structure of human erythrocyte carbonic anhydrase B to 2.2 Å resolution has been announced.[220] The essential metal ion, zinc, is firmly bound to the enzyme *via* three histidyl ligands and is located at the bottom of a 12 Å deep conical cavity. Several mechanistic studies of the action of the enzyme have been reported.[221, 222] The preparation of an Fe^{II} bovine carbonic and human carbonic anhydrase has been reported.[223] Mössbauer spectroscopy indicates that Fe^{II} (low spin) is bound at the enzymatically active site. The interaction of cobalt(II) complexes [224] and metal-directed inhibitors [225] with the enzyme have been investigated.

Many other investigations have been carried out. A discussion of albumin as the major metal transport agent in blood has appeared,[226] and the copper(II)-induced polymerization of human albumin studied.[227] The ^1H n.m.r. spectra of peroxidases from turnip and horseradish have been studied,[228] and the function and mechanism of peroxidases reviewed.[229] Gadolinium and calcium binding to bovine serum albumin has been studied by temperature-jump techniques.[230] For the reaction of $CaOH^+$ and bovine serum albumin the formation rate constant is 1×10^8 mol^{-1} dm^3 s^{-1} and the dissociation rate 9.3 s^{-1} at 7 °C. The kinetics of oxidation of horse heart ferrocytochrome *c* by tris-(1,10-phenanthroline)cobalt(III) have been investigated.[231] Inhibition of leucine aminopeptidase by platinum complexes has been discussed,[232] and iron–sulphur bonding in cytochrome *c* studied by *X*-ray photoelectron spectroscopy.[233]

A polarographic study of the interaction of copper(II) and cadmium(II) with poly(α,L-glutamic)glutamic acid has appeared [234] and the interaction of Ag^I with

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polypeptides studied.[235] Oxygen binding to iron porphyrins has been investigated [236, 237] and the topic of inorganic oxygen carriers reviewed.[10, 238] The kinetics of reduction of *Rhus vernicifera* laccase by $Fe(CN)_6^{4-}$ have been studied,[239] and the reversible uncoupling of the copper and cobalt spin systems of cobalt bovine superoxide dismutase at low pH investigated.[240] Conversion of the exchange labile cobalt(II) in *E. coli* alkaline phosphatase into exchange-inert cobalt(III) has been carried out by H_2O_2 oxidation.[241] Oxidation leads to a single absorption band at 530 nm and loss of the characteristic e.p.r. signal and of enzymatic activity. A zinc(II) protein has been isolated from human parotid saliva (mol. wt. 37 000) and contains two moles of zinc per mole of protein.[242] Zinc plays an important role in taste perception and patients with hypogeusia (decreased taste acuity) exhibit lower than normal zinc in parotid saliva. A calcium-binding protein has been identified as a calcium-dependent regulator of brain adenylate cyclase.[243] Selenium appears to induce redistribution of calcium binding to tissue proteins and may provide a possible mechanism against cadmium toxicity.[244] The single polypeptide chain of conalbumin strongly binds two iron(III) or two copper(II) to give intense absorption in the visible region similar to that of the related protein transferrin.[245] The interaction between uranyl ion (UO_2^{2+}) and cocarboxylase (thiamine pyrophosphate) leads to 1 : 1 or 3 : 2 UO_2^{2+}/TPP complexes which may be related to the uranyl inhibition of TPP-catalysed reactions.[246]

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Table *Compilation of binding sites in naturally occurring metal–ion complexes of 3d-ions* [a]

Enzyme/protein Amino-acid	M^{n+}	*Binding sites* 1	2	3	4	5	6	*Methods/Comments*	*Ref.*
Concanavalin A [b]	Mn^{2+}	O (Glu)	O (Asp)	O (Asp) N (Im)	N (Im) N (Im) ?	H_2O	H_2O	*X*-ray	247
								titrat./chem. modif./ dialys.	248, 249
Pyruvate kinase	Mn^{2+} (Mg^{2+} ?)	N (Im)	O (phosphate/substrate)					n.m.r./chem. modif.	250, 251
Hemerythrin [c]	Fe^{3+}/Fe^{2+}	four N Im) and two O (Tyr) are bound to two Fe						*X*-ray (5.5 Å resolution)	252
								chem. modif.	253
Transferrin [d] (conalbumin is closely related) [63]	Fe^{3+}	O (Tyr)	O (Tyr)	O (Tyr)	N (Im)	N (Im)	HCO_3^-	chem. studies (Tyr role more emphasized, His involvement more uncertain)	249, 254, 255
Urease (jack bean)	Ni^{2+}	S(Cys)						chem. modif.	256
Ceruloplasmin [e]	Cu^{+}/Cu^{2+}	N (Im) moieties and thiol groups						chem. modif./titrat.	249, 257
Haemocyanin [f]	Cu^{2+}	$Cu^{II}—O_2^{2-}—Cu^{II}$, and Cu—N (Im) bonds						spectroscopic exper.	258
Plastocyanin (bean) [g]	Cu^{2+}	N (Im)	S (Cys)	N (Im)	$\overline{N}$ (deprot. amide)			spectroscopic exper.	259
L-Histidinate and L-threoninate [h]	Cu^{2+}	N (Im)	NH_2 (His)	O (Thr)	NH_2 (Thr)	O (His) [i]	H_2O	*X*-ray [i]	260
								rather stable in aq. sol.	249, 261, 262
Albumin (human)	Cu^{2+}	NH_2 (Asp)	$\overline{N}$ (amide, Ala)	$\overline{N}$ (amide, His)	N (Im)			chem. studies	249, 262, 263
Galactose oxidase	Cu^{2+}	N_2O_2 square-planar system with a protein sulphur in one axial site/N (Im) seems to be involved						spectral studies, chem. modif.	264
Superoxide dismutase [j]	Cu^{2+}	N (Im)	N (Im)	N (Im)	N (Im)			*X*-ray	265
	Zn^{2+}	N (Im)	N (Im)	N (Im)	O (Asp)				
Insulin	Zn^{2+}	N (Im)	N (Im)	N (Im)	H_2O	H_2O	H_2O	*X*-ray	266
Thermolysin [b]	Zn^{2+}	N (Im)	N (Im)	O (Glu)	H_2O			*X*-ray	267, 268
Alkaline phosphatase (*E. coli*)	Zn^{2+}	three N (Im) appear to be involved and O (phosphate/substrate)						photo-oxidation	269
								e.p.r. of Cu^{2+} enzyme	268, 270
								^{31}P n.m.r.	

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Table (*cont.*)

Enzyme/protein Amino-acid	M^{n+}	*Binding sites* 1	2	3	4	5	6	*Methods/Comments*	*Ref.*
Carbonic anhydrase [k]	Zn^{2+}	N (Im)	N (Im)	N (Im)	H_2O			*X*-ray	268, 271
Carboxypeptidase A	Zn^{2+}	N (Im)	O (Glu)	N (Im)	H_2O			*X*-ray	268, 272
					O (carbonyl/substrate)			*X*-ray	268, 272
		O (phenolate of Tyr) co-ord. to Zn^{2+} in solution						chem., spect., kinet. data	273
Alcohol dehydrogenase	Zn^{2+}	S (Cys)	S (Cys)	N (Im)	H_2O [l]			*X*-ray [l]	274
	Zn^{2+}	S (Cys)	S (Cys)	S (Cys)	S (Cys)				

[a] Im = imidazole moiety of a histidyl residue; the abbreviations of the amino-acid residues are those generally used. [b] Ca^{2+} is also present, co-ordinated to O donors. [c] Oxygen-carrying non-haem protein.[249] [d] Conalbumin is closely related in its binding sites with transferrin.[249] [e] Half of the copper atoms are copper(II) and the other half copper(I).[263] [f] Oxygen-carrying 'Cu^{I}' protein.[249] [g] ^1H n.m.r. studies of plastocyanins from spinach and a blue-green alga 'suggests that the imidazole groups of two histidine residues are liganded directly to the copper.... The single copper of plastocyanin may exist in either the oxidized Cu^{II} or reduced Cu form'. [h] Present in human serum and in equilibrium with albumin bound Cu^{2+}.[263] [i] The distance of the O (carbonyl) atom of L-histidine from Cu^{2+} is 2.58 Å, compared with only 1.97 Å for the O (carbonyl) atom of L-threonine; moreover, the O (carbonyl) atom of histidine is in an irregular axial position [angle N (amino)—Cu—O (carboxyl) = 68.3°]. Thus, it seems that this latter bond is not very strong, and in aqueous solution the axial position (at least in equilibrium) may well be occupied by water. [j] The active site contains an imidazolate-bridged Cu^{2+}–Zn^{2+} moiety; one of the axial directions of the Cu is open to solvent access.[268] [k] Human carbonic anhydrase B and C are two isoenzymes which differ in the position of the third histidine residue.[268] [l] There is evidence that this (*i.e.* the first) Zn^{2+} with its co-ordination sphere is at the active site and that H^2O is replaced by an O of alcohol/aldehyde in the active enzyme.[268] The function of the second Zn^{2+} is not clear, it appears there is no direct functional role in catalysis, or in maintaining the secondary or quaternary structure.[268]

The authors thank Dr. H. Sigel for the prepublication information used in the Table.

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