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Amino-acids Peptides and Proteins

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Amino-acids, Peptides, and Proteins

Volume 13

A Specialist Periodical Report

Amino-acids, Peptides, and Proteins

Volume 13

A Review of the Literature Published during 1980

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Preface

This thirteenth Report reviews papers relevant to the chemistry of amino-acids, peptides, and proteins published in the main journals during 1980. Subject coverage is similar to that of previous volumes, except that the section on primary structures of proteins has, unfortunately, had to be omitted. This volume includes the customary biennial survey of metal complexes of amino-acids, peptides, and proteins (Chapter 6), which covers the two-year period 1979—1980.

This is the last volume which will be published under the present Senior Reportership. I should like, therefore, to thank most sincerely not only the contributors to the present volume but all those who in the past have made this series such a useful work of reference.

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 that have been used are listed here or are defined in the text and tables.

Ac	acetyl
Acm	acetamidomethyl
Ad	adamantyl
Adoc	adamantyloxycarbonyl
Adpoc	1-(1-adamantyl)-1-methylethoxycarbonyl
Aoc	t-amyloxycarbonyl
Aox	4,5-dianisoyl-4-oxazolin-2-one amino-acid derivative
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
$Bzl(NO_2)$	4-nitrobenzyl
$Bzl(2\text{-}NO_2)$	2-nitrobenzyl
Bzl(OMe)	4-methoxybenzyl
c.d.	circular dichroism
Cha	cyclohexylamine
Cm	carboxymethyl
Cmc	*S*-carboxymethylcysteine

Cox	4,5-di-(4-chlorophenyl-4-oxazolin-2-one) amino-acid derivative
CPh_2Py	diphenyl-4-pyridylmethyl
Dcha	dicyclohexylamine
Ddz	3,5-dimethoxy($\alpha\alpha$-dimethyl)benzyloxycarbonyl
DMCBzl	dimethylcarbamoylbenzyl
DMCZ	dimethylcarbamoylbenzyloxycarbonyl
DMF	*NN*-dimethylformamide
DMSO	dimethyl sulphoxide
Dnp	2,4-dinitrophenyl
2,4-Dnps	2,4-dinitrosulphenyl
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	ethylenediamine
e.p.r.	electron paramagnetic resonance
e.s.r.	electron spin resonance
Et	ethyl
Gal	galactose
g.c.–m.s.	gas chromatograph–mass spectrometer combination
g.l.c.	gas–liquid chromatography
Glc	glucose
Glp	2-pyrrolidone-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-methoxybenzenesulphonyl
Mds	4-methoxy-2,6-dimethylbenzenesulphonyl
Me	methyl
Mea	mercaptoethylamine
$MePh_2Peoc$	2-methyldiphenylphosphinioethyloxycarbonyl
Mhoc	1-methylcyclohexylcarbonyl
Moc	methoxycarbonyl
Msc	2-(methylsulphonyl)ethoxycarbonyl
Mtc	2-methylthioethyloxycarbonyl
NAD	nicotinamide–adenine dinucleotide (NAD^+ oxidized, NADH reduced)
NCA	*N*-carboxyanhydride

Nma	maleimido
Nmps	4-methyl-2-nitrosulphenyl
N.m.r.	nuclear magnetic resonance
Np	4-nitrophenyl
Nps	*o*-nitrophenylsulphenyl
Npys	3-nitropyridine-2-sulphenyl
Nsu	succinimido
OHFP	hexfluoroisopropyl esters
ONp	*p*-nitrophenoxy
ONp(*o*)	*o*-nitrophenoxy
ONSu	succinimido-oxy
OPcp	pentachlorophenoxy
OPfp	pentafluorophenoxy
OPic	4-picolyloxy
Opop	phenacyl-2-hydroxyphenyl ester
o.r.d.	optical rotatory dispersion
OTAT	thiazoline-2-thione esters
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
Pms	*p*-tolylmethylsulphonyl
PNp	4-nitrophenyl ester
Ppoc	phenylisopropoxycarbonyl
Ppt	diphenylphosphinothioyl
Pth-Gly	the phenylthiohydantoin derived from glycine, *etc.*
Pz	*p*-phenylazobenzyloxycarbonyl
SBu^t	t-butylthio
Scm	carboxymethylsulphenyl
SCB	t-butyloxycarbonylsulphenyl
SDS	sodium dodecyl sulphate
Spr^i	isopropylthio
Sub	5-dibenzosuberyl
SZ	benzyloxycarbonylsulphenyl
Tac	toluene-*p*-sulphonylaminocarbonyl
Tcp	2,4,5-trichlorophenyl
Tfa	trifluoroacetyl
Thp	tetrahydropyranyl
T.l.c.	thin layer chromatography
Tmeda	*NNN'N'*-tetramethylethylenediamine
Tnps	2,4,6-trinitrosulphenyl
Tos	toluene-*p*-sulphonyl
Troc	2,2,2-trichloroethyloxycarbonyl

Trt	triphenylmethyl
Tse	2-(toluene-*p*-sulphonyl)ethyl
U.v.	ultraviolet
Xan	9-xanthyl
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

This chapter continues to offer detailed coverage of the chemical and biochemical literature on the amino-acids, but with only superficial treatment of biological aspects (distribution of the common amino-acids, metabolism, and biosynthesis).

Textbooks and Reviews.—Several sources of up-to-date information have become available, dealing with biosynthesis,[1] stereochemical studies of metabolism,[2] toxic and other amino-acids with plant-defensive roles,[3] and a broader review of non-protein amino-acids.[4] Electrochemical synthesis of amino-acids has been surveyed.[5]

2 Naturally Occurring Amino-acids

Occurrence of Known Amino-acids.—Identification of four previously undetected leucine isomers (2-amino-2-ethylbutyric acid, both diastereoisomers of 2-methylnorvaline, *C*-t-butylglycine, and 2-amino-2,3-dimethylbutyric acid) in the Murchison meteorite[6] contributes further support to the hypothesis that a single one-carbon precursor can account for all amino-acids so far found in this sample.

A review of amino-acids present in marine algae has appeared.[7] Other α-amino-acids found in new locations are diaminopimelic acid from the cell wall of Legionnaires' disease bacterium,[8] L-2-amino-4,5-hexadienoic acid from *Amanita neooroidea*,[9] cyclopentenylglycine in Flacourtiaceae,[10] and 3-(2-furoyl)alanine from roots of *Rumex obtusifolius*[11] (this compound is now believed to be formed

[1] Biochemistry of Plants, Vol. 4, ed. P. K. Stumpf, Vol. 5, ed. B. J. Miflin, Academic Press, New York, 1980; L. Ninet and J. Renaut, *Bull. Soc. Chim. Fr., Part 2*, 1980, 80.

[2] D. J. Aberhart, *Recent Adv. Phytochem.*, 1979, **13**, 29.

[3] B. Unterhalt, *Dtsch. Apoth.-Ztg.*, 1980, **120**, 1093; 'Herbivores: Their Interaction with Secondary Plant Metabolites', ed. G. A. Rosenthal and D. H. Janzen, Academic Press, New York, 1979.

[4] E. A. Bell, in 'Encyclopaedia of Plant Physiology', Vol. 8 (Secondary Plant Products), New Series, ed. E. A. Bell and B. V. Charlwood, Springer Verlag, Berlin, 1980, p. 403; E. A. Bell, *Rev. Latino-am. Quim.*, 1980, **11**, 16; E. A. Bell, *Endeavour*, 1980, **4**, 102.

[5] I. A. Avrutskaya, in 'Elektrosint. Monomerov', ed. L. G. Feoktistov, Izd. Nauka, Moscow, 1980, p. 124 (*Chem. Abstr.*, 1981, **93**, 122 346).

[6] J. R. Cronin, W. E. Gandy, and S. Pizzarello, in 'Biogeochemistry of Amino-acids', ed. P. E. Hare, T. C. Hoening, and K. King, Wiley, New York, 1980, p. 153.

[7] E. Fattorusso and M. Piattelli, in 'Marine Natural Products: Chemical and Biological Perspectives', ed. P. J. Scheuer, Academic Press, New York, 1980, Vol. 3, p. 95.

[8] G. O. Guerrant, M. S. Lambert, and C. W. Moss, *J. Clin. Microbiol.*, 1979, **10**, 815.

[9] S. Hatanaka and K. Kawakami, *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo*, 1980, **30**, 147 (*Chem. Abstr.*, 1980, **93**, 61 778).

[10] U. Cramer, A. G. Rehfeldt, and F. Spener, *Biochemistry*, 1980, **19**, 3074.

[11] T. Kasai, M. Okuda, and S. Sakamura, *Agric. Biol. Chem.*, 1980, **44**, 2723.

from ascorbalamic acid during isolation from the plant [11]). An improved isolation procedure (3-hydroxyproline from seeds) gives an excellent account of modern methodology which is generally applicable.[12]

γ-Carboxyglutamic acid is a constituent of ovocalcin (hen eggshell),[13] and bovine teeth phosphoprotein contains α-aminoadipic acid,[14] probably derived from a lysine residue *via* the corresponding aldehyde ('allysine'). Several papers dwell on the possibility that crosslinking amino-acids previously located in proteins may be artifacts of the isolation procedures; although pyridinoline (see Vol. 11, p. 3), now structurally revised to (l; probably $n = 1$, $m = 2$),[15] has been established to be an *in vivo* component of collagen,[16] this has been disputed.[17] The tetrafunctional collagen crosslink, dehydrohistidinohydroxymerodesmosine, has also been shown not to be an artifact.[18]

Simple derivatives of the common protein amino-acids continue to be found, either in an uncombined form [*N*-methyl-L-alanine and *N*-methyl-L-serine in high concentrations in *Dichapetalum cymosum*;[19] *N*-(γ-L-glutamyl)ethanolamine in mushrooms;[20] *N*-*p*-coumarylglutamic acid in black tea;[21] and H·Leu·NHNMeP(O)(OH)OMe, as antibiotic FR-900137 from *Streptomyces unzenensis*[22]] or as protein constituents (*NNN*-trimethyl-L-alanine and $N^{\varepsilon}N^{\varepsilon}N^{\varepsilon}$-trimethyl-L-lysine in ribosomal protein L11 from *E. coli*,[23] and *NN*-dimethylproline at the *N*-terminus of a cytochrome[24]).

New Natural Free Amino-acids.—Plant sources and new free amino-acids are: *Caylusea abyssinica* (2 diastereoisomers of 4-carboxy-4-hydroxy-2-amino-adipic acid, with the (*S*)-configuration at C-2 assumed, as well as two diastereoisomers of 4-hydroxy-4-methylglutamic acid);[25] further information on mugineic acid (see Vol. 12, p. 3) from root-washings of Gramineae;[26] *Avena sativa* root washings as source of avenic acid A, (2), a new amino-acid with iron-chelating ability;[27,28] seeds of *Ateleia herbert smithii Pittier* are the source of the remarkable new cyclobutanes 2,4-methanoproline and 2,4-methanoglutamic acids [(3) and (4) respectively;[29] antibiotic SF-1836 (17) is a homologue of the former[167]]; and

[12] A. G. Szymanowicz, G. Poulin, N. Fontaine, J. P. Werquin, and J. P. Borel, *J. Chromatogr.*, 1980, **190**, 457.
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[15] Z. Deyl, K. Macek, M. Adam, and T. Vancskova, *Biochim. Biophys. Acta*, 1980, **625**, 248.
[16] D. Fujimoto, *Biochem. Biophys. Res. Commun.*, 1980, **93**, 948.
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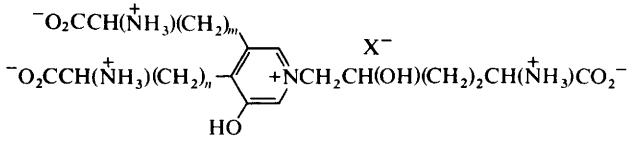
[27] S. Fushiya, Y. Sato, S. Nozoe, K. Nomoto, T. Takemoto, and S. Takagi, *Tetrahedron Lett.*, 1980, **21**, 3071.
[28] S. Fushiya, Y. Sato, and S. Nozoe, *Chem. Lett.*, 1980, 1215.
[29] E. A. Bell, M. Y. Qureshi, R. J. Pryce, D. H. Janzen, P. Lemke, and J. Clardy, *J. Am. Chem. Soc.*, 1980, **102**, 1409; synthesis M. C. Purring, *Tetrahedron Lett.*, 1980, **21**, 4577; P. Hughes, M. Martin, and J. Clardy, *ibid.*, p. 4579.



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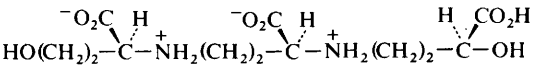

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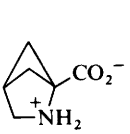

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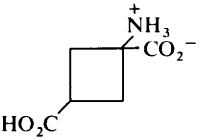

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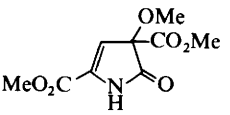
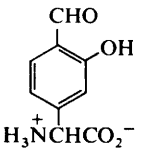


(5)

sargassumlactam, (5), a new $\beta\gamma$-unsaturated γ-lactam from the marine alga *Sargassum kjellmanianum*.[30] Shinorine,[31] claimed as a new amino-acid (from the red alga *Chondrus yendoi*), is identical with mytilin A (see Vol. 12, p. 4), a member of the palythine family (Vol. 11, p. 3).

Fungal and bacterial sources of new amino-acids are: *Streptomyces catenulae* (antibiotic FR-900130 is L-2-amino-3-butynoic acid);[32] unspecified *Actinomyces* [source of forphenicine, (6)];[33] *Streptomyces filamentosus* [antibiotic SF-1961, (7)];[34] 2-(3-alanyl)clavam, (8), from *Streptomyces clavuligerus*;[35] arogenic acid, (9), a biosynthetic precursor of phenylalanine and tyrosine (from a *Neurospora crassa* mutant).[36]



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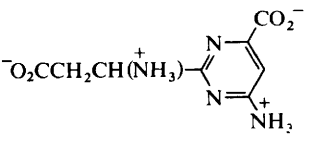

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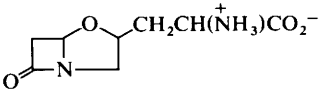

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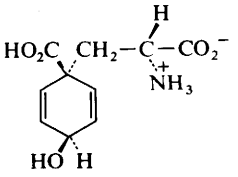

(9)

[30] H. Nozaki, Y. Fukuoka, A. Matsuo, O. Soga, and M. Nakayama, *Chem. Lett.*, 1980, 1453.
[31] I. Tsujino, K. Yabe, and I. Sekikawa, *Bot. Mar.*, 1980, **23**, 65.
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New Amino-acids from Hydrolysates.—One of the four possible stereoisomers of 3,4-dihydroxy-L-proline, the 2,3-*trans*-3,4-*trans* isomer, is a component of the virotoxins, toxic peptides of *Amanita virosa*.[37] Additional information on the chlorotyrosine derivatives from vancomycin (see Vol. 12, p. 5) has been published.[38]

3 Chemical Synthesis and Resolution of Amino-acids

General Methods of Synthesis of Amino-acids.—Standard syntheses of amino-acids have been applied to the synthesis of analogues of ibotenic acid,[39] including alkylation of diethyl acetamidomalonate (used in other laboratories;[40] see also refs. 75, 78, and 117). Alkylation of the potassium enolate of the Schiff base $(RS)_2C{=}NCH_2CO_2Et$ with alkyl halides illustrates a general synthesis of α-amino-acids from glycine derivatives which is of increasing importance.[41] As in other examples of this approach,[84] di-alkylation is feasible. The Bucherer-Bergs hydantoin synthesis (see refs. 120 and 121) and Strecker synthesis (see ref. 94) have been useful general procedures.

Yields of 21—84% have been claimed for the conversion of a primary amide into an α-acylamino-acid ($R^1CHO + CO + R^2CONH_2 \rightarrow R^2CONHCHR^1CO_2H$), catalysed by $Co_2(CO)_8$.[42] Effects of electron or radical scavengers on the amination of carboxylic acids induced by γ-irradiation have been studied.[43] Hydrogenolysis of 1-aryl-3-azido-azetidinones has been explored as a route to β-amino-acid amides.[44]

Examples of the applications of standard synthetic approaches to β- and higher homologous amino-acids are included later in this chapter.

Asymmetric Synthesis of Amino-acids.—Further development of previously established methods is illustrated in a synthesis of 2-t-butylglycine ('t-leucine') based on the asymmetric addition of HCN to the Schiff base derived from pivalic aldehyde and (*S*)-1-phenylethylamine, followed by hydrolysis and hydrogenolysis (see also Scheme 1);[45] asymmetric addition of $PhCH_2SH$ to α-phthalimidoacrylate catalysed by acrylonitrile–cinchona alkaloid co-polymers [to give an enantiomeric excess of the (*S*)-isomer of *N*-phthaloyl-*S*-benzylcysteine when quinine or cinchonidine are used];[46] asymmetric hydroformylation and hydrocarboxylation of enamides catalysed by hydridorhodium(II)carbonyl–chiral phosphine complexes[47] (use of a chiral aldehyde in the distantly related α-acylamino-acid synthesis[42] described in the preceding section led to no enantiomeric excess); and asymmetric hydrogenation processes of various types {alkylidene-oxazolinones

[37] A. Buku, H. Faulstich, T. Wieland, and J. Dabrowski, *Proc. Natl. Acad. Sci. U.S.A.*, 1980, **77**, 2370.
[38] Zh. P. Trifonova, G. S. Katrukha, A. B. Silaev, B. Diarra, B. V. Rozynov, and O. S. Reshetova, *Khim. Prir. Soedin.*, 1979, 875.
[39] J. J. Hansen and P. Krogsgaard-Larsen, *J. Chem. Soc., Perkin Trans. 1*, 1980, 1826.
[40] A. M. Kolodziejczyk and A. Arendt, *Pol. J. Chem.*, 1980, **54**, 1327.
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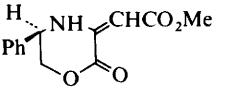
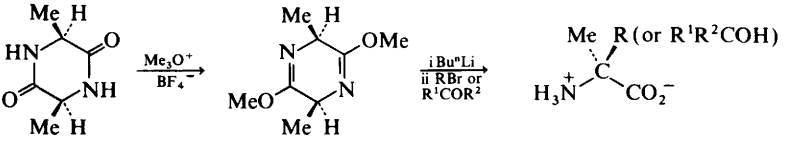
[42] J. J. Parnaud, G. Campari, and P. Pino, *J. Mol. Catal.*, 1979, **6**, 341.
[43] K. Ema and T. Masuda, *Technol. Rep. Osaka Univ.*, 1980, **30**, 313 (*Chem. Abstr.*, 1980, **93**, 168 566).
[44] I. Ojima, S. Suga, and R. Abe, *Chem. Lett.*, 1980, 853.
[45] J. L. Faucher and C. Petermann, *Helv. Chim. Acta*, 1980, **63**, 824.
[46] N. Kobayashi and K. Iwai, *J. Polymer Sci., Polym. Lett. Ed.*, 1980, **18**, 417.
[47] Y. Becker, A. Eisenstadt, and J. K. Stille, *J. Org. Chem.*, 1980, **45**, 2145.

with rhodium–chiral phosphine complexes[48] or with common hydrogenation catalysts in the presence of (*S*)-l-phenylethylamine[49] or Al–Hg,[50,51] or H_2–Raney Ni[50] hydrogenation of chiral 6-phenyl-2-alkylidene-oxazinones [(10) gives L-aspartic acid in 14—17% optical yield][50] and chiral dioxazepinones[51]}. The latter is an example of hydrogenation of a chiral Schiff base, related to the asymmetric synthesis of β-amino-acids by hydrogenation of (*Z*)-3-[(*R*)-1-phenylethylamino]-αβ-unsaturated esters.[52]

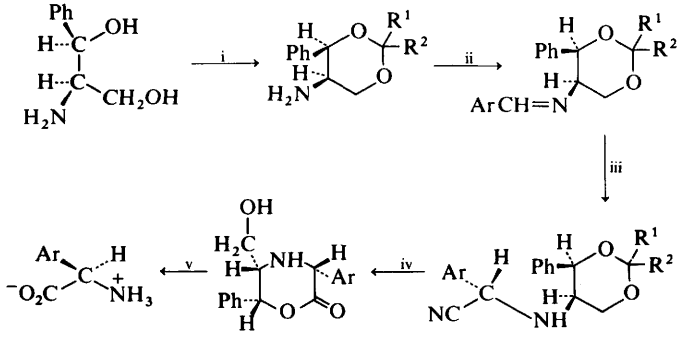


(10)



(11)

Enantioselective alkylation of the mono-anion of the L-alanine dioxopiperazine derivative (11) provides a route to α-methyl-α-amino-acids involving moderately high (41—74%) asymmetric induction.[53] The advantage of enclosing a chiral signal-centre in a ring in this area of asymmetric synthesis is further illustrated in a use of chiral 4-phenyl-5-alkylamino-1,3-dioxans (Scheme 1) leading to *C*-arylglycines.[54]

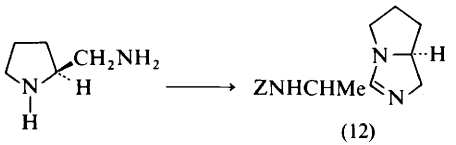


Scheme 1

Reagents: i, R^1R^2CO; ii, ArCHO; iii, HCN; iv, conc. HCl; v, $NaIO_3$ (aq)

[48] J. Koettner and G. Greber, *Chem. Ber.*, 1980, **113**, 2323.
[49] E. I. Karpeiskaya, G. V. Chel'tsova, E. I. Klabunovskii, and A. P. Kharchevnikov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1980, 1082.
[50] M. Tamura and K. Harada, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 561.
[51] J. Irurre Perez, J. Martin Juarez, and A. Bosch Rovira, *An. Quim.*, 1979, **75**, 958.
[52] M. Furukawa, T. Okawara, Y. Noguchi, and Y. Terawaki, *Chem. Pharm. Bull.*, 1979, **27**, 2223.
[53] U. Schoellkopf, W. Hartwig, and U. Groth, *Angew. Chem.*, 1979, **91**, 922; 1980, **92**, 205.
[54] K. Weinges, K. P. Klotz, and H. Droste, *Chem. Ber.*, 1980, **113**, 710; K. Weinges, G. Brune, and H. Droste, *Liebigs Ann. Chem.*, 1980, 212.

Useful asymmetric transformations are illustrated by the conversion (71.7%) of the (*R*)-1-phenylethylammonium salt of (*R,S*)-*N*-benzoyl-*C*-phenylglycine into the corresponding salt of the (*S*)-acid (overall 77% yield) by boiling in toluene solution,[55] and a related use of optically active cobalt(III)tetrammine-*N*-methyl-L-alanine complexes [56] (and see ref. 234). (*R*)-Alanine results from the hydrolysis of the imidazoline (12) formed from either (*R*)- or (*S*)-*N*-benzyloxycarbonylalanine imidate and (*S*)-2-(aminoethyl)pyrrolidine, as a result of auto-epimerization.[57]



(12)

Prebiotic Synthesis; Model Reactions.—A general review [58] and specific survey of results from studies of the formation of amino-acids from sugars and NH_3 in a model sea medium [59] indicate the broad scope of this topic. Most of the recent papers continue the themes established in earlier years [^{60}Co-γ-irradiation of O_2-free aqueous NH_4CN;[60] photolysis of NH_3 in propionic acid gives α- and β-alanines through $NH(^1\Delta)$ insertion of C—H bonds,[61] whereas atomic nitrogen attacks acetic or succinic acids in aqueous media, leading to glycine, aspartic acid, glutamic acid, serine, and threonine;[62] 254 nm irradiation of simple hydrocarbons, water, and NH_3 in the presence or absence of H_2S;[63] carboxylation of primary amines in aqueous solutions at various pH values;[64] and conversions of β-amino-acids into α-amino-acids[240] under contact glow discharge electrolysis conditions [64]]. The increasing emphasis on the involvement of hydrogen cyanide in putative mechanisms for abiogenic synthesis of amino-acids is further justified by the demonstration that this compound is the principal product of i.r.-laser photolysis of a methane–ammonia mixture.[65] Amino-acids are formed in aqueous KCN in the presence of montmorillonite or graphite oxide at 70 °C.[66]

The common feature of these model reactions is the involvement of an energy source to drive thermodynamically unfavourable processes. Matatov has shown that iron(III)-catalysed decomposition of H_2O_2 can facilitate the production of glycine, serine, threonine, and proline from formaldehyde and hydroxylamine hydrochloride in aqueous solutions.[67]

[55] K. Suzuki, S. Kiyooka, T. Miyagawa, and A. Kawai, *Nippon Kagaku Kaishi*, 1980, 287 (*Chem. Abstr.*, 1980, **93**, 95604).
[56] M. Yamagushi, S. Yano, M. Saburi, and S. Yoshikawa, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 691.
[57] S. Shibata, H. Matsushita, K. Kato, M. Noguchi, M. Saburi, and S. Yoshikawa, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2938.
[58] G. Sextl, R. Schwanker, and M. Eiswirth, *Biol. Unserer Zeit*, 1980, **10**, 123.
[59] H. Yanagawa, *Tanpakushitsu Kakusan Koso, Bessatsu*, 1980, 86 (*Chem. Abstr.*, 1981, **94**, 11660).
[60] Z. D. Draganic, V. Niketic, S. Jovanovic, and I. G. Draganic, *J. Mol. Evol.*, 1980, **15**, 239; I. G. Draganic, S. Jovanovic, V. Niketic, and Z. D. Draganic, *ibid.*, p. 261.
[61] S. Sato, T. Kitamura, and S. Tsunashima, *Chem. Lett.*, 1980, 687.
[62] M. A. Margulis, L. M. Grundel, and E. L. Girina, *Dokl. Akad. Nauk SSSR*, 1980, **251**, 639.
[63] E. Miyoshi, H. Ebisawa, T. Shirai, and S. Yanagisawa, *Nippon Kagaku Kaishi*, 1980, 1120.
[64] J. Terasawa and K. Harada, *Chem. Lett.*, 1980, 73.
[65] D. O. Davis, G. R. Smith, and W. A. Guillory, *Origins Life*, 1980, **10**, 237.
[66] F. Aragon de la Cruz and C. Viton Barbolla, *An. Quim.*, 1979, **75**, 820.
[67] Yu. I. Matatov, *Zh. Evol. Biokhim. Fiziol.*, 1980, **16**, 189 (*Chem. Abstr.*, 1981, **94**, 42850).

Protein Amino-acids and Other Naturally Occurring Amino-acids.—Little scope exists for thorough coverage of biosynthetic production of amino-acids, important though this topic has become in both commercial and mechanistic terms. The general field can be represented by selected references (reviews of enzymic synthesis;[68] fermentative production of L-glutamine by a *Flavobacterium rigense* mutant;[69] microbial conversion of glycine into L-serine,[70] and accumulation of *O*-methyl-L-homoserine in culture media of methanol-utilizing bacteria;[71] and conversion of *trans*-4-hydroxy-L-proline into L-proline *via* the 4,5-dehydro-analogue[72]).

A synthesis of L-α-aminoadipic acid from L-lysine involves treatment of the N^{α}-benzyloxycarbonyl derivative with NaOCl, elimination with DABCO, and hydrolysis of the resulting nitrile in refluxing 4M-HCl.[73] Cyclization of ornithine, lysine, or 5-hydroxylysine with nitrosylpentacyanoiron(II) gives proline, pipecolic acid, and 5-hydroxypipecolic acid, respectively.[74] Further new syntheses of γ-carboxy-L-glutamic acid involve either alkylation of diethyl benzyloxycarbonylamino-malonate with the Mannich reaction product of di-t-butyl malonate,[75] or carboxylation of *N*-trityl dibenzyl L-glutamate with benzyl chloroformate after carbanion formation with $LiNPr^{i}_{2}$, followed by de-protection with H_2–Pd.[76] Full details have been published[77] of the novel synthesis of kainic acid reported in Vol. 11 (p. 10). γ-Oxo-DL-homotyrosine has been prepared from *p*-methoxyphenacyl bromide and diethyl acetamidomalonate.[78]

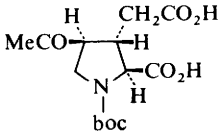
Syntheses of β-amino-acids reported in 1980 include (2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoic acid (present in amastatin), prepared from *N*-benzyloxycarbonyl-D-leucine methyl ester *via* $LiAlHBu^{i}_{2}$ reduction into the aldehyde, thence into the cyanohydrin,[79] and an alternative route to the same series of compounds from chiral oxiranes.[80] *threo*-γ-Hydroxy-L-β-lysine has been prepared by Arndt-Eistert extension of the corresponding lysine derivative.[81] A useful synthetic route to δ-amino-acids has been illustrated with a synthesis of δ-aminolaevulinic acid.[82]

Aliphatic Amino-acids.—*C*-t-Butylglycine ('t-leucine') is accessible through addition of MeMgI to 2-phenyl-4-isopropylidene-oxazolinone or to $Me_2C{=}C(CO_2Et)_2$ followed by hydrolysis or Curtius rearrangement, respective-

[68] N. Esaki, K. Soda, H. Kumagai, and H. Yamada, *Biotechnol. Bioeng.*, 1980, **22** (*Suppl. 1*), 127; Y. Hirose and H. Shibai, *ibid.*, p. 111.
[69] K. Nabe, T. Ujimaru, N. Izuo, S. Yamada, and I. Chibata, *Appl. Environ. Microbiol.*, 1980, **40**, 19.
[70] Y. Tanaka, K. Araki, and K. Nakayama, *J. Ferment. Technol.*, 1980, **58**, 417.
[71] Y. Tanaka, K. Araki, and K. Nakayama, *Biotechnol. Lett.*, 1980, **2**, 67.
[72] J. Varner, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 692.
[73] A. I. Scott and T. J. Wilkinson, *Synth. Commun.*, 1980, **10**, 127.
[74] M. T. Beck, A. Katho, and L. Dozsa, *Magy. Kem. Foly.*, 1980, **86**, 337.
[75] A. Juhasz and S. Bajusz, *Int. J. Pept. Protein Res.*, 1980, **15**, 154.
[76] R. K.-Y. Zee-Cheng and R. E. Olsen, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 1128.
[77] W. Oppolzer and H. Andres, *Helv. Chim. Acta*, 1979, **62**, 2282.
[78] W. Keller-Schierlein and B. Joos, *Helv. Chim. Acta*, 1980, **63**, 250.
[79] D. H. Rich, B. J. Moon, and A. S. Boparai, *J. Org. Chem.*, 1980, **45**, 2288.
[80] K. Kato, T. Saino, R. Nishizawa, T. Takita, and H. Umezawa, *J. Chem. Soc., Perkin Trans. 1*, 1980, 1618.
[81] T. Teshima, T. Ando, and T. Shiba, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 1191.
[82] G. Schulz and W. Steglich, *Chem. Ber.*, 1980, **113**, 787.

ly.[83] Unsaturated analogues of D-α-amino-adipic acid[84] and of 3-halo-4-aminobutanoic acids[85] have been prepared by alkylation of $Ph_2C{=}NCH_2CO_2Et$ with $EtO_2CCH{=}CHCH_2Br$, and from $ClCH_2C{\equiv}CCO_2H$, respectively, followed by straightforward elaboration. Kolbe reactions with mixtures of differently protected glutamic acids lead to 2,4-di-aminosuberates.[86]

Proline derivatives and analogues feature as synthetic objectives for several laboratories. A 90:10 *cis*:*trans*-mixture of 1-benzyl-2-methylazetidine-carboxylates emerges from condensation of methyl 2,4-dibromopentanoate with benzylamine;[87] an improved preparation of (*S*)-3,4-dehydroproline based on H_3PO_2–HI reduction of pyrrole-2-carboxylic acid involves resolution with (+)-tartaric acid,[88] which is not necessary in the apparently easier route from L-hydroxyproline involving protection and Chugaev elimination of the xanthate (formed with CS_2 and $Bu^n{}_4N^+HSO_4^-$);[89] 1,2-dehydroproline gives the 3-phenoxy-analogue through allylic bromination followed by treatment with thallium phenoxide, easily reduced to *cis*:*trans*-3-phenoxyproline.[90] Conversion of kainic acid into the strongly neuro-excitatory proline derivative (13) is achieved by ozonolysis of the *N*-boc-derivative.[91]



(13)

α-Alkyl Analogues of Protein Amino-acids.—Asymmetric synthesis of α-methyl-α-amino-acids has been illustrated earlier in the chapter,[53] and the same general objective, formation of the α-carbanion of a protected amino-acid followed by alkylation, has been used in a synthesis of α-methyltryptophan.[92] Synthesis of α-hydroxymethylserine from the reaction of formaldehyde with cobalt(III), copper(II), or nickel(II) complexed glycine Schiff bases,[93] and the synthesis of α-(hydroxymethyl)aspartic acid through the Strecker synthesis with $AcOCH_2COCH_2CO_2Et$[94] illustrate previously used routes. α-Vinyl analogues can be prepared through Michael addition of a 2-phenyloxazolin-5-one to $PhSO_2C{\equiv}CH$ followed by sulphone cleavage,[95] or by alkylation of a Schiff base with (*E*)- or (*Z*)-RCH=CHBr after carbanion formation with $LiNPr^i{}_2$.[96]

[83] T. Miyazawa, T. Nagai, T. Yamada, S. Kuwata, and H. Watanabe, *Mem. Konan Univ., Sci. Ser.*, 1979, **23**, 51 (*Chem. Abstr.*, 1980, **92**, 94681).
[84] R. D. Allan, *J. Chem. Res.* (*S*), 1980, 392.
[85] R. D. Allan, *Aust. J. Chem.*, 1979, **32**, 2507; R. D. Allan, G. A. R. Johnston, and B. Twitchin, *Aust. J. Chem.*, 1980, **33**, 1115.
[86] R. E. Nutt, R. G. Strachan, D. F. Veber, and F. W. Holly, *J. Org. Chem.*, 1980, **45**, 3078.
[87] D. S. Soriano, K. F. Podraza, and N. H. Cromwell, *J. Heterocycl. Chem.*, 1980, **17**, 623.
[88] J. W. Scott, A. Focella, U. O. Hengartner, D. R. Parrish, and D. Valentine, *Synth. Commun.*, 1980, **10**, 529.
[89] J. R. Dormoy, B. Castro, G. Chappuis, U. S. Fritschi, and P. Grogg, *Angew. Chem.*, 1980, **92**, 761.
[90] J. Hausler and U. Schmidt, *Liebigs Ann. Chem.*, 1979, 1881.
[91] O. Goldberg, A. Luini, and V. I. Teichberg, *Tetrahedron Lett.*, 1980, **21**, 2355.
[92] M. F. Brana, M. Garrido, M. L. Lopez, and A. M. Sanz, *J. Heterocycl. Chem.*, 1980, **17**, 829.
[93] L. Casella, A. Pasini, R. Ugo, and M. Visca, *J. Chem. Soc., Dalton Trans.*, 1980, 1655.
[94] J. J. Walsh, D. E. Metzler, D. Powell, and R. A. Jacobson, *J. Am. Chem. Soc.*, 1980, **102**, 7136.
[95] W. Steglich and H. Wegmann, *Synthesis*, 1980, 481.
[96] P. Bey and J. P. Vevert, *J. Org. Chem.*, 1980, **45**, 3249.

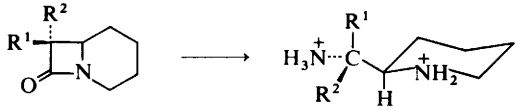
α-Heteroatom-substituted α-Amino-acids.—Good yields of α-methoxy-*N*-acetyl amino-acids are obtained through the reaction of an *N*-acetyl-*N*-benzyloxy-amino-acid ester with potassium t-butoxide and MeOH.[97] α-Bromination (NBS) and treatment with potassium thiolacetate places an acetylthio-grouping at the α-position of an *N*-acylamino-acid ester.[98]

Aliphatic Amino-acids Carrying Halogen Substituents in Side-chains.—Further examples of the use of aziridinecarboxylates for the preparation of β-fluoro-α-amino-acids, by treatment with HF–pyridine, have been reported (see Vol. 12, p. 8).[99a] The relative stereochemistry of the products has been defined[99b] by chemical correlations and *X*-ray analysis.

Aliphatic Amino-acids Carrying Hydroxy-groups in Side-chains.—Free-radical chlorination of L-valine, and hydrolysis, gives a mixture of stereoisomers from which (2*S*,3*S*)- and (2*S*,3*R*)-4-hydroxyvaline have been isolated by crystallization and hydrolysis.[100] A 34:66 *erythro*:*threo*-mixture of γ-hydroxy-DL-ornithine formed through hydrolysis of 2,5-di-amino-4-pentanolide isomers has been separated and converted into corresponding γ-hydroxyarginines.[101]

α-Amino-acids with Unsaturated Side-chains.—A new synthesis of L-vinylglycine [(*S*)-2-amino-but-3-enoic acid] from L-methionine involves conversion into the sulphoxide, followed by pyrolytic elimination of methanesulphenic acid.[102] Another example of the dehydration of *N*-benzyloxycarbonylserine or threonine into the corresponding αβ-dehydro-amino-acids employing DCCI has been reported.[103]

Synthesis of Aromatic and Heterocyclic Amino-acids.—Most examples included in this section this year, as in previous years, concern simple derivatives of the protein aromatic and heteroaromatic amino-acids, but an interesting stereospecific synthesis of a saturated heterocyclic amino-acid (14) has also been described.[104]



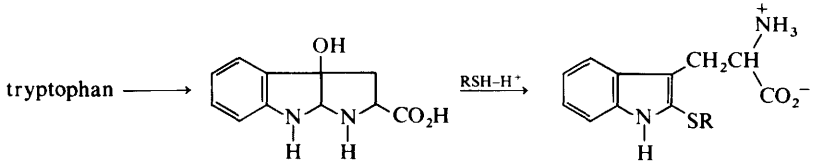
(14)
$R^1 = H$, $R^2 = CO_2^-$
$R^1 = CO_2^-$, $R^2 = H$

Tyrosine derivatives offering some interest in terms of routes for their synthesis are 3-fluoro- and 3,5-difluoro-L-tyrosine (from L-tyrosine methyl ester *via* nitration, reduction, and diazotization with $NaNO_2$–HBF_4),[105] and 3,4-dihydroxy-6-

[97] J. D. M. Herscheid, R. J. F. Nivard, M. W. Tijhuis, H. P. H. Scholten, and H. C. J. Ottenheijm, *J. Org. Chem.*, 1980, **45**, 1880.
[98] Z. Lidert and S. Gronowitz, *Synthesis*, 1980, 322.
[99] (*a*) A. Barama, R. Condom, and R. Guedj, *J. Fluorine Chem.*, 1980, **16**, 183; T. N. Wade and R. Kheribet, *J. Chem. Res.* (*S*), 1980, 210; (*b*) T. Tsuchima, T. Sato, and T. Tsuji, *Tetrahedron Lett.*, 1980, **21**, 3591; T. Tsuchima, J. Nishikawa, T. Sato, H. Tamida, K. Tori, and T. Tsuji, *ibid.*, p. 3593.
[100] J. J. Usher, *J. Chem. Res.* (*S*), 1980, 30.
[101] K. Mizusaki, H. Yamamoto, and S. Makisumi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2605.
[102] A. Afzali-Ardakani and H. Rapoport, *J. Org. Chem.*, 1980, **45**, 4817.
[103] M. J. Miller, *J. Org. Chem.*, 1980, **45**, 3131.
[104] B. T. Golding and A. J. Smith, *J. Chem. Soc., Chem. Commun.*, 1980, 702.
[105] K. K. Kirk, *J. Org. Chem.*, 1980, **45**, 2015.

fluorophenylalanine, formed in 25% yield from 3-methoxy-L-tyrosine ethyl ester and XeF_2.[106] Tyrosine or dopa can be converted into 5-hydroxydopa in the presence of tyrosinase.[107]

A novel route to 2-alkylthio-tryptophans [(15) → (16)][108] has been used in a synthesis of tryptathionine [16; R = $CH_2CH(NH_2)CO_2H$], a constituent of phalloidin. 5- and 7-bromotryptophans have been prepared by Fischer cyclization of the appropriate bromophenylhydrazones of 4-acetamido-4,4-bis(ethoxycarbonyl)butanals.[109]



(15) (16)

2-Trifluoromethylhistidines have been used in preparations of 2-carboxy-, 2-cyano-, and 2-ethoxycarbonyl-histidines.[110]

N-Benzoyl-α-hydroxyglycine, $PhCONHCH(OH)CO_2H$, continues to be used for the synthesis of *C*-arylglycines; a paper in the current literature describes condensations with benzimidazol-2-one and with benzo[*c*]-thiophen-2,2-dioxide.[111]

Synthesis of *N*-substituted Amino-acids.—Addition of HCN to an imine yields an α-amino-alkyl cyanide [$R^1CH{=}NR^2 + HCN \rightarrow R^1CH(CH)NHR^2$], from which *N*-mono-substituted α-amino-acids may be obtained.[112] A simple route to an *N*-mono-alkylamino-acid in which an *N*-benzyl-*N*-alkyl-L-amino-acid is debenzylated by hydrogenolysis[113] depends on the ready availability of *N*-benzyl-L-amino-acids.

Spontaneous conversion of L-lysine into N^{ε}-mono-, -di-, and -trimethyl-derivatives occurs with formaldehyde in aqueous solutions.[114]

Conversion of *N*-arylidene-amino-acid esters into oxaziridines using monoperphthalic acid provides a suitable intermediate for the synthesis of *N*-hydroxy-amino-acids through reaction with hydroxylamine.[115]

Synthesis of α-Amino-acids Containing Sulphur or Selenium.—Nucleophilic substitution of methyl α-acetamido-β-chloroacrylate with a thiol gives the corresponding β-alkylthioacrylate, whereas thiolacetic acid yields *N*-acetyl-ββ-bis(acetylthio)alanine.[116]

[106] G. Firnan, R. Chirakal, S. Sood, and S. Garnett, *Can. J. Chem.*, 1980, **58**, 1449.
[107] C. Hansson, H. Rorsman, and E. Rosengren, *Acta Derm.-Venereol.*, 1980, **60**, 281.
[108] W. E. Savige and A. Fontana, *Int. J. Pept. Protein Res.*, 1980, **15**, 102.
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[111] M. L. Edwards, *J. Heterocycl. Chem.*, 1980, **17**, 383.
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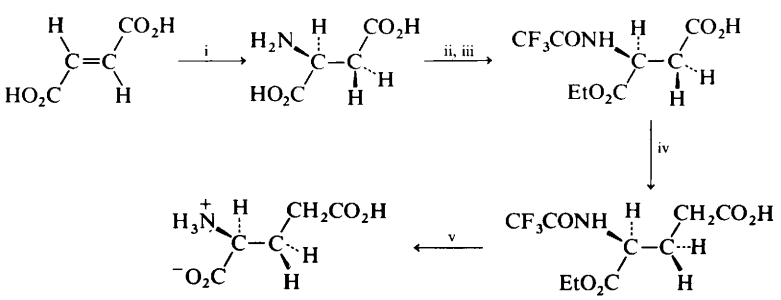
[116] A. J. Kolar and R. K. Olsen, *J. Org. Chem.*, 1980, **45**, 3246.

The acetamidomalonate route has been used for the synthesis of 2-selenienylalanine, using 2-chloromethylselenophen as alkylating agent.[117]

Synthesis of Phosphorus-containing α-Amino-acids.—Improved preparations of N^{ε}-phospholysine and N^{ω}-phospho-arginine starting from the α-amino-acids have been described.[118]

α-Amino-acids Synthesized for the First Time.—New α-amino-acids not mentioned elsewhere in this chapter are 2-amino-(4′-hydroxy-6′-benzothiazolyl)propanoic acid,[119] 1-amino-1-carboxy-3,4-benzocyclo[2.2.2]octane,[120] and D- and L-2-(1,2:5,6-di-*O*-isopropylidene-α-D-allofuranos-3-yl)glycine.[121]

Synthesis of Labelled Amino-acids.—L-[3′,5′-$^{13}C_2$]phenylalanine has been prepared from the correspondingly labelled tyrosine (synthesis displayed in Vol. 12, p. 16) by conversion into the *O*-(1-phenyltetrazol-5-yl)-derivative followed by catalytic transfer hydrogenolysis with cyclohexene.[122] Further examples of labelled amino-acids prepared for biosynthetic investigations are DL-[2-^{13}C]leucine,[123] DL-[*Me*-^{13}C]valine (starting from $^{13}CH_3OH$, thence to 2-[*Me*-^{13}C]methylthiazoline),[124] and DL-[2-^{14}C,2′,3′-$^{13}C_2$]tryptophan (prepared from [2-^{14}C]indole, [^{13}C]formaldehyde, and diethyl[2-^{13}C]malonate).[125] A route applied for the synthesis of (2*S*,3*S*)-[3-$^{2}H_1$]-, (2*S*,3*R*)-[2,3-$^{2}H_2$]-, (2*S*,3*S*,4*RS*)-[3-$^{2}H_1$, 4-$^{3}H_1$]-, and (2*S*,3*R*, 4*RS*)-[2,3-$^{2}H_2$, 4-$^{3}H_1$]-glutamic acids[126] is displayed in Scheme 2. ^{14}C-Labelled amino-acids described in recent papers include DL-[1-^{14}C]valine,[127] *N*-[*Me*-^{14}C]methyl-L-alanine,[113] N^{δ}-[*Me*-^{14}C]methylarginine,[128] β-[^{14}C]alanine from L-[^{14}C]aspartic



Scheme 2

Reagents: i, L-aspartase–NH_4Cl in $^{2}H_2O$ or $^{1}H_2O$; ii, trifluoroacetic anhydride; iii, EtOH; iv, $SOCl_2$ then CH_2N_2–Wolff rearrangement [*hν*–dioxan(aq)]; v, HCl(aq)

[117] T. Frejd, M. A. Davis, S. Gronowitz, and T. Sadeh, *J. Heterocycl. Chem.*, 1980, **17**, 759.
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acid mediated by aspartate 1-decarboxylase,[129] and 1-aminocyclobutanecarboxylic acid labelled in the carboxy-group.[130]

Exchange of aromatic ring protons of tyrosine using $^{2 \text{ or } 3}H_2O$, $^{2 \text{ or } 3}HCl$, and K_2PtCl_4 at 100 °C involves mainly the 3- and 5-positions,[131] and ^3H-atom bombardment is similarly specific as far as the aromatic ring is concerned but also brings about 84% exchange at the methylene protons;[132] the latter process with solid phenylalanine causes multiple exchange[133] but with alanine, predominantly α-substitution.[134] Less energetic methods have been used for the preparation of ^3H-labelled 1-(3,3-dimethylallyl)-L-tryptophan.[135]

Eighteen examples of ^{18}O-carboxy-group labelled amino-acids have been worked through, achieving 90 atom% incorporation by equilibration in $H_3{}^{18}O^+$–$H_2{}^{18}O$ at 60–70 °C during several days.[136]

Standard reactions have been used for the preparation of *m*- and *p*-[^{18}F]fluoro-DL-phenylalanines,[137] [3,5-$^{80m}Br_2$]dibromotyrosine,[138] [3,5-$^{125}I_2$]tri-iodo-L-thyronine,[139] and β-[^{131}I]iodo-D-alanine.[140] Conversion of 3-iodo- or 3,5-di-iodotyrosines into corresponding [^{211}At]astatotyrosines involves solid-state exchange reactions.[141]

[^{75}Se]Selenaproline has been prepared by the reaction of L-[^{75}Se]selenocysteine with formaldehyde.[142]

Resolution of Amino-acids.—Major areas of study have developed from long-established principles for the resolution of racemic amino-acids, employing various chiral stationary phases for liquid chromatography and exploiting the enantiospecificity of enzyme-mediated processes. At the same time, the usual methods based on separation of diastereoisomeric salts continue to be commonly used (*e.g.* resolution of DL-[1-^{14}C]lysine using L-glutamic acid[143]). A further example of the use of the principle of seeding a saturated solution with crystals of the desired enantiomer of an amino-acid has been described with a novel variation, in which *N*-acetyl-L-leucine of optical purity 92.6% is produced by asymmetric transformation of DL-leucine through seeding a reaction mixture in acetic anhydride–acetic acid with L-leucine.[144] Cram's major project on reciprocal chiral

[129] J. E. Cronan, *Anal. Biochem.*, 1980, **103**, 377.
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[131] M. Kanska and S. Drabarek, *Radiochem. Radioanal. Lett.*, 1980, **44**, 207.
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recognition by chiral crown ether hosts employs amino-acid perchlorates as guests, and further results have been described in the 1980 literature.[145]

Amino-acids have been resolved by ligand exchange chromatography, *e.g.* DL-[^{3}H]valine on polystyrene bonded to L-hydroxyproline, complexed with copper(II) ions;[146] DL-proline and DL-histidine, but not other amino-acids, on a similar system;[147] and related studies including uses of *N*-formyl-L-valylaminopropyl-silica.[148] More rapid hydrolysis of D-isomers of amides of DL-leucine or phenyl-alanine occurs on cross-linked polystyrene carrying L-hydroxyprolyl residues complexed with copper(II) ions.[149]

The variation of this procedure, in which the reversed-phase technique with a chiral metal chelate in the mobile phase is employed, has been applied to the resolution of DL-amino-acids [150] and dansyl-DL-amino-acids.[151]

Examples of the use of enzymes for 'resolution' of derivatives of DL-amino-acids include 5-chymotrypsin for the isolation of 5-fluoro-L-tryptophan from the DL-amino-acid methyl ester,[152] immobilized acylase for the liberation of L-methionine from the *N*-acetyl-DL-amino-acid,[153] and a related use of a D-aminoacylase from *Streptomyces olivaceus*,[154] mutant *Brevibacterium* strains for the liberation of L-amino-acids from DL-α-amino-alkyl cyanides,[155] and extensive work on the synthesis of D-amino-acids (*p*-hydroxyphenylglycine,[156] 2-thienylglycine,[157] amino-acids more generally [158]) from DL-hydantoins, *via* *N*-carbamyl derivatives, using microbial hydantoinase (*alias* dihydropyrimidinase [158]). D-α-Amino-adipic acid can be isolated after digestion of the racemate by *Pseudomonas putidea*.[159]

4 Physical and Stereochemical Studies of Amino-acids

Crystal Structures of Amino-acids and Their Derivatives.—Reports of *X*-ray analysis of protein and other natural amino-acids [α-L-glutamic acid,[160] α- and β-forms of DL-methionine,[161] DL-lysine hydrochloride,[162] palythene [163] and paly-

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[154] M. Sugie and H. Suzuki, *Agric. Biol. Chem.*, 1980, **44**, 1089.
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[156] S. Shimizu and K. Yoneda, *Hakko To Kogyo*, 1980, **38**, 937.
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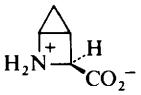
[160] M. S. Lehmann and A. C. Nunes, *Acta Crystallogr., Sect. B*, 1980, **36**, 1621.
[161] T. Taniguchi, Y. Takai, and K. Sakurai, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 803.
[162] D. Bhaduri and N. N. Saha, *J. Cryst. Mol. Struct.*, 1979, **9**, 311.
[163] D. Uemura, C. Katayama, A. Wada, and Y. Hirata, *Chem. Lett.*, 1980, 755.

thine trihydrate (see Vol. 11, p. 4),[164] and hydrochlorides of L-isoleucine, L-phenylalanine, and DL-methionine[165]] include a study of L-tyrosine found in a No. 1 Han Dynasty tomb, at Ma-Wang-Tui, China[166] (the word 'found' is tantalizingly used in the abstract source of this information). Antibiotic SF-1836 has been shown to be *trans*-2-azabicyclo[2.1.0]pentane-3*S*-carboxylic acid, (17),[167] related structurally to '2,4-methanoproline', (3), isolated recently[29] from a different source. *X*-Ray crystal analysis of D-α-amino-n-butyric acid has been reported.[168]



(17)

Derivatives of amino-acids studied by the *X*-ray technique include *N*-acetyl-L-cysteine,[169] *N*-(phosphonoethyl)glycine ('glyphosate'),[170] hydantoins of L-proline and D-allo-hydroxyproline,[171] L-arginine L-ascorbate,[172] and *N*-boc-L-phenylalanine.[173] The last-mentioned compound adopts the *E*-configuration in the solid state, although it is known to exist in the *Z*-form in solution in C^2HCl_3.[173]

After a spate of papers in the 1970's on neutron diffraction crystal analysis of amino-acids had appeared to subside, a study has been published on the γ-modification of glycine, studied at 83 K and 293 K.[174]

Nuclear Magnetic Resonance Spectrometry.—^{13}C N.m.r. studies continue to be developed to the point where routine laboratory studies can be carried out against a fully explored general background. However, scope still exists for non-routine ^1H n.m.r. studies, and pioneering work with other nuclei and new instrumentation.

Deprotonation of L-dopa as a function of p^2H is conveniently studied by ^1H n.m.r.[175] Conformational studies for amino-acids complexed to palladium(II)[176] or lanthanide cations[177] give information on rotamer equilibria concerning the C-α—C-β bond. Rotation of the amino-group has been detected in solid L-glutamic acid through ^1H n.m.r. spectrometry.[178] Wide-line n.m.r. studies have continued (see Vol. 12, p. 20), attention being paid to the solution behaviour of hippuric acid.[179]

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[165] B. Khawas, *Indian J. Phys. A*, 1979, **53**, 559.
[166] K.-S. Ting, *K'o Hsueh T'ung Pao*, 1980, **25**, 183 (*Chem. Abstr.*, 1980, **93**, 7014).
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Structural information with finer detail can often be obtained by approaching a problem in solution conformational behaviour with more than one physical technique, and this is well demonstrated in a ^{1}H–^{13}C n.m.r. study of *O*-acetylserine, *O*-phosphoserine and -threonine.[180] At all $p^{2}H$ values between 4 and 14, these derivatives adopt a planar W-type conformation through the H_{α}-C_{α}-C_{β}-O-P atom sequence. Multiple conformations, with similar behaviour in solution to that in the crystal state, are adopted by *N*-acetyl-D-allo-isoleucine, as shown by ^{13}C n.m.r. NT_1 values and proton–proton scalar coupling values.[181] This work establishes the potential of proton relaxation spectra for conformational analysis of amino-acids, and acyclic compounds more generally.

Characteristic ^{13}C chemical shift values as a function of solvent have been identified for *N*-acetylamino-acid methylamides[182] and corresponding esters.[183] Characteristic ^{13}C data have been carefully assembled for solutions at pH values 4.5 to 8.5, and an ambitious claim has been made that these data allow both qualitative and quantitative analysis of mixtures of the twenty common protein amino-acids.[184] A similar study[185] assesses the microscopic protonation behaviour of lysine and hydroxylysine. ^{13}C N.m.r. studies of amino-acids in the solid state have advanced significantly,[186,187] high-resolution data revealing splitting of the C-α resonance associated with nearby structural features, which may therefore possibly be identified.[187]

Natural abundance ^{15}N n.m.r. of α- and ω-amino-acids in protic solvents reveals an upfield shift for the N^{α}-resonance as a result of protonation.[188] The scope for natural abundance ^{17}O n.m.r. analysis of amino-acids has been explored.[189]

Optical Rotatory Dispersion and Circular Dichroism.—C.d. spectra of L-phenylalanine, and its *N*-acetyl and alkyl ester derivatives,[190] and interpretation of c.d. spectra of amino-acid alkyl esters in terms of conformational equilibria[191] extend studies described in earlier volumes of this *Specialist Periodical Report*, and need no further description here. In one of these studies, scope for magneto-c.d. study was offered and pursued.[190] The chromophores in these simple derivatives correspond to those in peptides and proteins, and their c.d. spectra are useful models for the contributions of individual amino-acid residues to the overall chirospectroscopic behaviour of disordered conformations of polypeptides. Long-chain *N*-acyl derivatives of L-glutamic acid and L-valine yield c.d. spectra in

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solution that are not typical of simple acyl derivatives, however, and have been interpreted to reveal the formation of chiral aggregates.[192]

The conversion of amino-acids into 'chromophoric derivatives' for the purpose of configurational or conformational assignments is also a long-established field of study, and the application of previously studied *N*-dithiocarbethoxy-β-amino-acids[193] and fluorescamine derivatives[194] to new configurational assignments has been described. Thus, (+)-(2-furyl- and -thienyl)-β-alanines have the L-configuration;[193] 1-pyrrolinones from L-amino-acids show a positive Cotton effect in the wavelength range 300—324 nm, and a negative Cotton effect in the range 263—290 nm.[194] A substantial study with the same objectives has been published for the chiral iso-indoles formed between D- or L-amino-acids and *o*-phthaldialdehyde with 2-mercaptoethanol.[195] A positive Cotton effect centred near 340 nm characterizes the L-configuration for all common amino-acids except alanine, tryptophan, aspartic acid, and histidine;[195] the same long path must now be trodden as in earlier studies of other chromophoric derivatives of amino-acids, to try to understand the reasons for exceptions to an empirical rule linking sign of Cotton effect with absolute configuration, but the relatively high sensitivity offered by the *o*-phthaldialdehyde derivatives (2×10^{-5}M) may be a sufficient encouragement to pursue these studies.

The c.d. of representative *N*-5- or -6-benzofuroxanyl-L-amino-acids has been reported.[196]

The use of c.d. or polarimetry for quantitative analysis is rarely considered, but a technique for the estimation of an amino-acid in the presence of its methyl ester, and simultaneous determination of the optical purity of the constituents of the mixture, has been worked out. Reaction with the cobalt complex of *NN'*-ethylenebis(acetylacetonimine) at pH 7 gives coloured species for the two constituents whose absorption spectra are sufficiently different to allow the separate contributions of the two species to the c.d. spectra to be measured.[197]

Mass Spectrometry.—The main content of this section in previous volumes has been a good indication of the advance of the frontiers of mass spectrometric analysis, year by year. This has been so because of the difficulty in obtaining spectra for amino-acids themselves, and the eagerness with which new instrumental techniques have been applied in this area. However, most of the analytical laboratories relying on commercially available spectrometers have continued to convert amino-acid mixtures into volatile derivatives, and new examples are 2,2-bis(difluorochloromethyl)oxazolidinones, formed from an amino-acid with bis-(difluorochloromethyl)ketone,[198] and fluorescamine derivatives, for which field

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[197] N. Spassky, M. Reix, M. O. Sepulchre, and J. P. Guette, *Analusis*, 1980, **8**, 130.
[198] R. Liardon, U. Ott-Kuhn, and P. Husek, *Biomed. Mass Spectrom.*, 1979, **6**, 381.

desorption techniques are well suited.[199] Mass spectrometric methods combined with g.l.c. separation would be resorted to for identification as well as quantitation of trace amounts, and brain tissue samples have been analysed in this way after conversion of their constituent amino-acids, with [1,2-$^{13}C_2$, ^{15}N]glycine as internal standard, into *N*-hexafluorobutyryl hexafluoroisopropyl esters.[200] Chemical ionization m.s. techniques can provide the same information for biological fluids containing ng or pg levels of amino-acids.[201]

Problems of interpretation of mass spectra of amino-acid derivatives have also featured in this section in previous volumes, and further study of the rearrangement of trimethylsilyl esters of *N*-acylglycines has been published.[202]

Other Physical and Theoretical Studies.—A number of i.r./Raman spectroscopic papers describe continuing studies of specifically deuteriated α-amino-acids (L-alanine,[203] L- and DL-cysteine[204]) aimed at assignments of vibrational modes. Polarized Raman spectra of α-glycine, L- and DL-alanine[205] continue recently described applications (see Vol. 12, p. 21) of this single-crystal variation of the standard technique. Conformational assignments to *N*-acetylamino-acid esters in different solvents based on i.r. data are usefully supported by n.m.r. studies.[206]

Physical adsorption of α-amino-acids on to clay (sodium montmorillonite) has formed the basis of a persistent theory of enantiomer discrimination leading to the present predominance of the L-isomers in proteins, and i.r. data for these adsorbates have been published.[207] No selective adsorption of protein amino-acids by clay from a solution containing also some non-protein amino-acids could be demonstrated, tending to dispose of a theory accounting for the relatively limited range of protein building blocks.[208] Bentonite has been shown to bind L-leucine, L-aspartic acid, and D-glucose through different contact geometry from that adopted for their respective enantiomers.[209]

Simple physical properties of amino-acid solutions continue to be determined, often by sophisticated methods, including solubilities in water–ethanol,[210] viscosities in water–MeCN,[211] dissociation constants in formic acid–butanone or acetic acid–butanone,[212] and Kerr effect studies of a series of eighteen amino-acids with an attempt to interpret the data in terms of conformational preferences of side-chains.[213] Thermodynamic properties that have been studied include heat

[199] K. E. Murray and D. I. Ingles, *Chem. Ind. (London)*, 1979, 476.
[200] A. Lapin and M. Karobath, *J. Chromatogr.*, 1980, **193**, 95.
[201] J. M. L. Mee, *Am. Lab. (Fairfield, Conn.)*, 1980, **12**, 55 (*Chem. Abstr.*, 1980, **93**, 91 335).
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[204] C. Madec, J. Lauransan, and C. Garrigou-Lagrange, *Can. J. Spectrosc.*, 1980, **25**, 47.
[205] K. Machida, M. Mori, and A. Kagayama, *J. Raman Spectrosc.*, 1980, **9**, 139.
[206] V. Slet, *Bio-org. Khim.*, 1979, **5**, 1319.
[207] Yu. I. Tarasevich, V. S. Rak, and E. G. Sivalov, *Teor. Eksp. Khim.*, 1980, **16**, 351.
[208] E. Friebele, A. Shimoyama, and C. Ponnamperuma, *J. Mol. Evol.*, 1980, **16**, 269.
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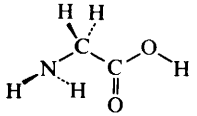
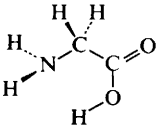
[212] A. P. Kreshkov, B. B. Tanganov, A. N. Yarovenko, and T. Kh. Batorova, *Zh. Fiz. Khim.*, 1980, **54**, 105; L. Pardeshi and R. A. Bhobe, *J. Indian Chem. Soc.*, 1980, 57, 442 (*Chem. Abstr.*, 1980, **93**, 81 570).
[213] G. Khanarian and W. J. Moore, *Aust. J. Chem.*, 1980, **33**, 1727.

capacities of transfer of amino-acids and peptides from water to aqueous urea,[214] heats of dilution of aqueous solutions of *N*-acetylamino-acid amides,[215] heats of mixing of aqueous solutions of glycine with corresponding solutions of alkali metal chlorides (Li^+ interacts with the amino-acid in a different manner compared with the other cations),[216] partial molar enthalpies of amino-acids in aqueous solutions,[217] and a useful discourse on the thermodynamic parameters (ΔG −7.67, ΔH −9.9, and ΔS −7.5 kcal mol^{-1}) for zwitterion formation $H_2NCHRCO_2H \rightleftharpoons H_3\overset{+}{N}CHRCO_2^-$.[218]

Molecular orbital calculations have received a substantial boost in persistently supporting a preferred conformation, (18), for glycine,[219] in spite of evidence from microwave spectroscopy favouring (19). The assignments, referring to behaviour in the gas phase, are important in terms of spectroscopic analysis of interstellar vapours, and more sensitive microwave techniques have now[220] detected (18) and possibly (20) for gaseous glycine. More routine m.o. calculations relating to



(18)



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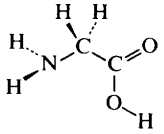

(20)

conformational energies have been reported for glycine, covering both zwitterionic and neutral forms,[221a] and for *N*-acetyldehydroalanine methylamide, revealing very different energy profiles for various conformations when compared with the alanine analogue.[221b] Comparisons have been made[221c] between the conformational behaviour of glycine and alanine with that of β-heteroatom-substituted homologues serine, cysteine, and threonine. The solvation structure around L-serine in aqueous solution provides an interesting challenge for energy calculations,[222] and mutual interactions of a different kind are explored for lattice energy calculations for α-, β-, and γ-glycine.[223]

5 Chemical Studies of Amino-acids

Racemization.—Although the two main strands of study, the use of racemization data for amino-acids in determinations of age for relatively recent fossils, and racemization mechanisms, are both well represented in the 1980 literature, there is increasing interest in mechanistic studies. Perhaps this exposes some shortcomings in knowledge of factors which influence the racemization kinetics of free or

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[215] G. M. Blackburn, T. H. Lilley, and E. Walmsley, *J. Chem. Soc., Faraday Trans. 1*, 1980, **76**, 915.
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[218] P. Haberfield, *J. Chem. Educ.*, 1980, **57**, 346.
[219] L. Schaefer, H. L. Sellers, F. J. Lovas, and R. D. Suenram, *J. Am. Chem. Soc.*, 1980, **102**, 6566.
[220] R. D. Suenram and F. J. Lovas, *J. Am. Chem. Soc.*, 1980, **102**, 7180.
[221] (*a*) P. Palla, C. Petrongolo, and J. Tomasi, *J. Phys. Chem.*, 1980, **84**, 435; (*b*) D. Ajo, G. Granozzi, E. Tondello, and A. Del Pra, *Biopolymers*, 1980, **19**, 469; (*c*) L. R. Wright and R. R. Borkman, *J. Am. Chem. Soc.*, 1980, **102**, 6207.
[222] S. Roman and E. Clementi, *Int. J. Quantum Chem.*, 1980, **17**, 1007.
[223] J. L. Derissen and J. Voogd, *J. Phys. Chem.*, 1980, **84**, 2035.

protein-bound amino-acids, which diminish the reliability of the age determinations.

D : L-Ratios for amino-acids, particularly aspartic acid, from human and animal protein sources, taken with racemization rate constants, could be most reliable for samples which are very young (on the fossil time scale), since for dental tissues [224] and other proteins with low turnover rates [225] from living sources, at least the racemization has taken place in constant temperatures. This area has been reviewed.[224–227] The problems with much older samples are starkly revealed in a study of the D : L-isomer ratio for aspartic acid from collagen of Dead Sea scroll parchment;[228] up to 60% of this amino-acid had racemized but D : L-ratios varied widely even for samples taken from the same scroll.

A substantial volume has appeared of conference proceedings concerned with studies of amino-acids from geological and biological sources.[6] Most of the papers cover deductions from racemization data, presented by all leading workers in the field.[229]

The racemization rates of amino-acids differ widely, but this variation does not reflect electronic effects relayed to the chiral centre.[230] Such a conclusion does not exclude a role for polar or polarizable groups in facilitating proton transfer from the chiral centre to an incoming base, and also leaves the reader in an unsatisfied state since the presumption that steric effects account for the variations in racemization rates does not seem to be related obviously to the facts.

Withdrawal of the proton from the chiral centre in L-histidine by the imidazole π-nitrogen atom has been concluded to be the cause of the pronounced racemization that accompanies DCCI-mediated coupling reactions of protected derivatives of this amino-acid, and which occurs after conversion of the derivatives into corresponding *O*-acyl iso-ureas.[231,232] Racemization kinetics for series of simple α-amino-acids in aqueous solutions over the pH range -1 to 12 at 142 °C are subject to three distinct influences: an acid catalysed process at pH values less than 1, a pH-independent region at pH values between 3 and 6.5, and a second pH-independent region between 9 and 12.[233]

New examples of racemizations of amino-acids in aqueous alkali through equilibration of Λ-β_2-[Co(tetra-ammine)(amino-acid)]$^{2+}$ complexes,[56,234] and a distantly related technique, the use of a polymeric salicylaldehyde capable of reversible Schiff base formation with copper(II) complexes of amino-acids,[235] have been published.

[224] B. Szabuniewicz, *Czas. Stomatol.*, 1980, **33**, 23 (*Chem. Abstr.*, 1980, **93**, 25278).
[225] F. Pautet, *Pathol. Biol.*, 1980, **28**, 325.
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[227] N. Hamda, *Kagaku To Seibutsu*, 1980, **18**, 678 (*Chem. Abstr.*, 1981, **94**, 60205).
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[229] *Inter alia*: T. C. Hoering, in ref. 6, p. 193; B. J. Katz and E. H. Man, *ibid.*, p. 215; K. A. Kvenvolden, *ibid.*, p. 223; J. L. Bada and M. Y. Shou, *ibid.*, p. 235; E. M. Jope, *ibid.*, p. 23; D. W. von Endt, *ibid.*, p. 297; K. M. Towe, *ibid.*, p. 65.
[230] G. G. Smith and B. Silva del Sol, *Science* (*Wash. D.C.*), 1980, **207**, 765.
[231] J. H. Jones, M. I. Ramage, and M. J. Witty, *Int. J. Pept. Protein Res.*, 1980, **15**, 301.
[232] J. H. Jones, Lecture at Meeting of the Peptide and Protein Group of The Chemical and Biochemical Societies, University of Sussex, 3 April 1981.
[233] P. M. Shou and J. L. Bada, *Naturwissenschaften*, 1980, **67**, 37.
[234] M. Yamaguchi, S. Yamamatsu, T. Furusawa, S. Yano, M. Saburi, and S. Yoshikawa, *Inorg. Chem.*, 1980, 2010.
[235] I. A. Yamskov, V. E. Tikhonov, and V. A. Davankov, *Bio-org. Khim.*, 1980, **6**, 885.

General Reactions of Amino-acids.—Following discussion of relatively drastic treatment, such as pyrolysis, reactions in strong mineral acid solutions, and oxidation, this section is divided, much as in previous volumes, between reactions at amino- and carboxy-groups, and uses in heterocyclic synthesis.

Formation of relatively large amounts of HCN during the pyrolysis of proline and glutamic acid at 850 °C, compared with other amino-acids, has been noted.[236] Hydrocarbons, CO, CO_2, and NH_3 are the major products. A kinetic study has been made [237] of the pyrolysis of a mixture of eight amino-acids at 178 °C through periods of up to 170 h, noting the accumulation of polymeric products which cannot be hydrolysed under peptide bond cleavage conditions (6M-hydrochloric acid at 105 °C during 12 h).[237] An important study [238] has shown that the presence of 0.01% NaN_3 during acid hydrolysis of proteins is responsible for destruction of tyrosine, phenylalanine, and histidine, and the generation of side-products overlapping arginine on the amino-acid analyser trace; in test mixtures, methionine sulphone was also destroyed. At the same time, the aspartic acid content was augmented by up to 15%.[238]

The sulphur-containing amino-acids, and tyrosine, tryptophan, and histidine, were the only protein amino-acids to undergo oxidation at a graphite anode.[239] Contact glow discharge electrolysis of β- and γ-amino-acids brings about their stepwise oxidative degradation, ascribed to the generation of hydroxy-radicals.[240] An interesting consequence is the formation of α-amino-acids; for example, the formation of glycine from β-alanine *via* isoserine and aminopyruvic acid. This observation is relevant to model reactions for the prebiotic synthesis of α-amino-acids, already known to be formed from simple precursors under contact glow discharge electrolysis,[64] since it is now conceivable that higher homologues of the protein amino-acids may have been formed first during the events leading to the genesis of life.

Labile *N*-hydroxymethyl derivatives that form at pH values above 9.2 in solutions of formaldehyde and amino-acids even at low concentrations are increasingly favoured at higher pH values,[241] and conditions for the condensation of 2 mol formaldehyde per mol amino-acid are eventually reached.

Condensation of pyridoxal with representative amino-acids (alanine, arginine, and methionine) is accelerated by reversed micelles.[242]

α-*N*-Nitroso-*N*-alkylamino-acids suffer decarboxylation under irradiation by u.v. light, yielding corresponding amidoximes, but β-amino-acid analogues are not photolabile.[243]

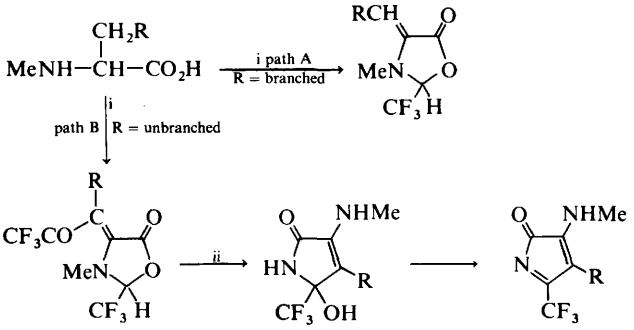
Nitrosation of alanine or α-aminobutyric acid with $NaNO_2$ and HF in pyridine yields the corresponding 2-fluoroalkanoic acids with retention of configuration, whereas phenylalanine, tyrosine, and threonine give the 3-fluoroalkanoic acids

[236] N. F. Haidar, J. M. Patterson, M. Moors, and W. T. Smith, *J. Agric. Food Chem.*, 1981, **29**, 163.
[237] G. A. Lavrent'ev, A. S. Timoshchenko, T. F. Strigunkova, and I. A. Egorov, *Dokl. Akad. Nauk SSSR*, 1980, **251**, 486.
[238] J. M. Walker, J. R. B. Hastings, and E. W. Johns, *J. Chromatogr.*, 1980, **189**, 106.
[239] V. Brabec and V. Mornstein, *Biophys. Chem.*, 1980, **12**, 159.
[240] K. Harada and J. Terasawa, *Chem. Lett.*, 1980, 441.
[241] Y. Kitamoto and H. Maeda, *J. Biochem.* (*Tokyo*), 1980, **87**, 1519.
[242] H. Kondo, H. Yoshinaga, and J. Sunamoto, *Chem. Lett.*, 1980, 973.
[243] Y. L. Chow, D. P. Horning, and J. Polo, *Can. J. Chem.*, 1980, **58**, 2477.

resulting from stereospecific 1,2-shift of the β-aryl or -hydroxy-group.[244] β-Branched aliphatic amino-acids (valine, isoleucine) give product mixtures indicating reluctant 1,2-methyl shift reactivity.

Isocyanides formed from *N*-formylamino-acid benzyl esters using $POCl_3$ and Et_3N undergo radical-induced reductive de-amination with tri-n-butyltin hydride in the presence of azo-bis-isobutyronitrile.[245] High-yield procedures for the *N*-acetylation of amino-acid esters have been described.[246]

Further examples of cycloaddition [247] and Michael addition [248] reactions of α-amino-acid ester imines, leading to pyrrolines and pyrrolidines, respectively, have been described. Continuing studies are also represented in cyclization of *N*-methylamino-acids by trifluoroacetic anhydride[249] (Scheme 3) in which an interesting pair of reaction pathways is revealed, which depend respectively on whether the amino-acid side-chain is branched (path A) at the β-carbon atom, or not (path B).

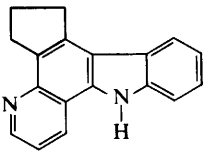


Scheme 3

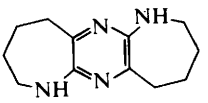
Reagents: i, trifluoroacetic anhydride; ii, NH_3

Specific Reactions of Natural Amino-acids.—Further indication of the importance of studying pyrolytic breakdown of protein amino-acids is provided in *X*-ray identification of (21) as the potent mutagen formed from L-lysine.[250a] The non-mutagenic pyrazine (22) has also been isolated from L-lysine hydrochloride pyrolysates.[250b] Pyrolysis of histidine and of 3-methylhistidine at 770 °C yields imidazole and 1-methylimidazole respectively.[251] Maillard reaction of L-lysine with D-glucose (reaction at 105 °C in aqueous solution during 6 h) gives the pyrrole (23).[252a] A further example of the conversion of one L-amino-acid into another is RuO_4 oxidation of an *N*-acyl proline to the pyroglutamic acid, thence to glutamic acid.[252b]

[244] R. Keck and J. Retey, *Helv. Chim. Acta*, 1980, **63**, 769.
[245] D. H. R. Barton, G. Bringmann, and W. B. Motherwell, *Synthesis*, 1980, 68.
[246] M. Dymicky, *Org. Prep. Proced. Int.*, 1980, **12**, 207.
[247] R. Grigg and J. Kemp, *Tetrahedron Lett.*, 1980, **21**, 2461.
[248] R. Grigg, J. Kemp, J. Malone, and A. Tangthougkum, *J. Chem. Soc., Chem. Commun.*, 1980, 648.
[249] U. Hess and W. A. Koenig, *Liebigs Ann. Chem.*, 1980, 611.
[250] (*a*) K. Yamaguchi, Y. Iitaka, K. Shudo, and T. Okamoto, *Acta Crystallogr., Sect. B*, 1980, **36**, 176; (*b*) V. S. Gann, A. L. Y. Lau, and H. H. Wassermann, *Tetrahedron Lett.*, 1980, **21**, 2679.
[251] R. M. Smith, G. A. Solabi, W. P. Hayes, and R. J. Stretton, *J. Anal. Appl. Pyrolysis*, 1980, **1**, 197.
[252] (*a*) T. Nakayama, F. Hayase, and H. Kato, *Agric. Biol. Chem.*, 1980, **44**, 1201; (*b*) S. Yoshifuji, H. Matsumoto, K. Tanaka, and Y. Nitta, *Tetrahedron Lett.*, 1980, **21**, 2963.



(21)



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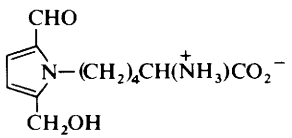


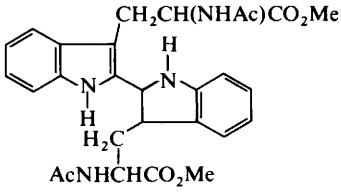
(23)

Liberation of ethylene from amino-acids has important botanical consequences, and its formation from 1-aminocyclopropanecarboxylic acid in tobacco leaves is inhibited by light.[253] Radiolytically produced oxygen radicals HO• and $O_2^{\cdot-}$ cause the formation of ethylene from methionine or *S*-adenosylmethionine.[254] Other detailed studies involving aliphatic amino-acids are decarboxylation kinetics of γ-carboxyglutamic acid in comparison with those of aminomalonic acid and β-carboxyaspartic acid ($t_{\frac{1}{2}}$ 8.6, 1.2, and 1.7 min, respectively),[255] and several studies of cysteine and its derivatives. Normal protein hydrolysis conditions convert cystine into 'thiocystine' [bis(2-amino-2-carboxyethyl) trisulphide], which is the source of sulphenyl cations RS^+ capable of initiating the breakdown of tryptophan in the protein hydrolysate.[256] Thiocystine has been detected in biological systems, and mechanisms for its breakdown into cystine have been described.[257] Enzyme systems capable of mediating the breakdown of sulphur-containing amino-acids have been discussed.[258] 'Cystine disulphoxide' is actually the thiolsulphonate $HO_2CCH(NH_2)CH_2SSO_2CH_2CH(NH_2)CO_2H$,[259] and the current position[260] in which 'α-disulphoxides' RS(O)S(O)R remain detectable only as transient intermediates in a limited number of organosulphur reactions remains intact. The common reagents used for the reductive cleavage of cystine are applicable for the corresponding reaction with selenocystine.[261]

Excepting a reference to the tyrosinase-catalysed oxidation of dopa and 5-*S*-cysteinyldopa to initiate the formation of pigments in higher species,[262] the other papers covering aromatic amino-acids which have been selected for citation deal with heterocyclic side-chain chemistry. Oxidation processes with tryptophan

[253] S. Gepstein and K. V. Thimann, *Planta*, 1980, **149**, 196.
[254] M. Saran, W. Bors, C. Michel, and E. F. Elstner, *Int. J. Radiat. Biol., Relat. Stud. Phys., Chem., Med.*, 1980, **37**, 521 (*Chem. Abstr.*, 1980, **93**, 128827).
[255] P. V. Hauschka, E. B. Henson, and P. M. Gallop, *Anal. Biochem.*, 1980, **108**, 57.
[256] T. Ohta and T. Nakai, *Agric. Biol. Chem.*, 1979, **43**, 2419.
[257] R. Abdolrasulina and J. L. Wood, *Bio-org. Chem.*, 1980, **9**, 253; in 'Natural Sulfur Compounds', Proceedings of the 3rd International Meeting 1979, ed. D. Cavallini, G. E. Gaull, and V. Zappia, Plenum Press, New York, 1980, p. 483 (*Chem. Abstr.*, 1980, **93**, 162967).
[258] D. Cavallini, G. Federici, S. Dupre, C. Cannella, and R. Scandurra, *Pure Appl. Chem.*, 1980, **52**, 147.
[259] T. Obuka, S. Yuasa, M. Kinuta, and R. Akagi, *Physiol. Chem. Phys.*, 1980, **12**, 45.
[260] G. C. Barrett, in 'Organic Compounds of Sulphur, Selenium, and Tellurium', ed. D. R. Hogg (Specialist Periodical Reports), The Chemical Society, London, 1980, Vol. 5, p. 67.
[261] J. N. Burnell, J. A. Karle, and A. Shrift, *J. Inorg. Biochem.*, 1980, **12**, 343.
[262] S. Ito, E. Novellino, F. Chioccara, G. Misuraca, and G. Prota, *Experientia*, 1980, **36**, 822.

(conversion into oxindolylalanine in dimethylsulphoxide–acetic acid media [263]) and its derivatives [dimer (24) and its stereoisomer are formed from *N*-acetyltryptophan methyl ester in TFA;[264] electro-oxidation of *N*-acetyltryptophanamide also brings about dimerization,[265] as does photo-oxidation of the amino-acid itself[266]]



(24)

are accompanied by descriptions of indole-substitution reactions (attack by sulphenyl cations,[256] and by t-butyl cations liberated during de-protection of N^{α}-boc-tryptophan derivatives in TFA–ethanedithiol [267]) as topics of recent papers. Kinetics of de-tritiation of C-2[^{3}H]histidine derivatives [268] and ^{1}H–^{2}H exchange at the same site [269] have been studied and interpreted as a reflection of the influence of nearby groupings.

Specific Reactions and Properties of Amino-acids Related to Biochemical Processes.—This section is intended to be read with the preceding and following sections if a general view is sought of recent literature on some biochemical aspects of the chemistry of the amino-acids. Interactions of L-tryptophan with nucleic acids induced by light [270] have been studied, an extension of one of several lines of inquiry on this general topic. Acetone-sensitized photo-coupling between *N*-acetyltryptophan methyl ester and 5-bromo-1,3-dimethyluracil leads to the corresponding 2-substituted indoles.[271] Apparent dissociation constants of AMP–amino-acid ester complexes in aqueous solutions correlate well with features of the genetic code and with the frequencies of occurrence of amino-acids as constituents of proteins;[272] another aspect of the same topic underlies a study of the relative rates of non-enzymic activation of hydrophobic amino-acids by ATP.[273]

Effects of Electromagnetic Radiation on Amino-acids.—Three major topics stand out from a broad view on the literature of this topic: a study of radicals formed through high-energy irradiation of amino-acids; the finer details of the absorption and re-emission of u.v. light by tryptophan, that archetypal 3-substituted indole;

[263] W. E. Savige and A. Fontana, *Int. J. Pept. Protein Res.*, 1980, **15**, 285.
[264] K. Hashizume and Y. Shimonishi, *Proceedings of 17th Peptide Symposium*, 1979, p. 77 (*Chem. Abstr.*, 1980, **93**, 168 577).
[265] C. Jakubowicz, R. Vallot, L. T. Yu, and J. Reynaud, *C. R. Seances Acad. Sci., Ser. C.*, 1980, **290**, 377.
[266] C. Sconfienza, A. Van de Vorst, and G. Jori, *Photochem. Photobiol.*, 1980, **31**, 351.
[267] Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 464.
[268] J. A. Elvidge, J. R. Jones, R. Salih, M. Shandala, and S. E. Taylor, *J. Chem. Soc., Perkin Trans. 2*, 1980, 447.
[269] J. H. Bradbury, B. E. Chapman, M. W. Crompton, R. S. Norton, and J. S. Teh, *J. Chem. Soc., Perkin Trans. 2*, 1980, 693.
[270] A. E. Reeve and T. R. Hopkins, *Photochem. Photobiol.*, 1980, **31**, 223, 413.
[271] S. Ito, I. Saito, and T. Matsuuro, *J. Am. Chem. Soc.*, 1980, **102**, 7535.
[272] J. Reuben and F. E. Polk, *J. Mol. Evol.*, 1980, **15**, 103.
[273] D. W. Mullins and J. C. Lacey, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 491.

and attempts to demonstrate differential degradation of enantiomers of amino-acids under irradiation.

γ-Irradiation of aqueous solutions of L-valine[274] and other simple aliphatic α-amino-acids glycine, alanine, leucine, and isoleucine[275] creates short-lived radicals whose breakdown products have been studied by h.p.l.c.[274] Spin-trapping using 2-methyl-2-nitropropane[275] has proved to be a useful method for locating the site of the unpaired electron in the initial products of irradiation, though e.s.r. and ENDOR techniques have been generally applied in related studies: γ-irradiation of L-alanine generates the radical cation structure $—\dot{C}(OH)O^-$ from the carboxy-group;[276] while the amino-group and the α-carbon atom are also implicated, for example in the pulse radiolysis of deuteriated glycine, alanine, and α-aminoisobutyric acid;[277] hydrated electrons liberated in aqueous solutions of phenylalanine react with the amino-acid at sites partly determined by the pH of the reaction mixture.[278] Solid samples can also suffer degradation under γ- or *X*-irradiation, and current studies have involved alanine single crystals doped with copper(II) salts,[279] L-aspartic[280] and L-glutamic acids,[281] *N*-acetyl-DL-alanine,[282] and L-proline hydrate and its thiazolidine analogue.[283] In the last-mentioned study, e.s.r. and ENDOR monitoring indicate the possibility of de-amination under *X*-irradiation, a process established previously with acyclic aliphatic amino-acids.

Although radiation at the levels to which meteorites must be exposed is sufficient to cause appreciable racemization of amino-acids,[284] as well as partial destruction or interconversions of some amino-acids (see earlier volumes of this *Specialist Periodical Report*), previous claims that enantiomeric amino-acids are degraded at different rates under irradiation now seem to be discounted by counter-claims generated by attempts to extend the topic. This leaves an open question; the fact that a wide variety of amino-acids is present in some meteorites but all in racemic form does not necessarily invalidate a hypothesis that these objects have travelled from some extra-terrestrial source on which one enantiomeric form of the amino-acids predominates. But the so-called Vester–Ulbricht theory, that the predominance of one enantiomeric form is associated with unequal rates of degradation of D- and L-isomers by electromagnetic radiation with dissymmetric characteristics, is not standing up well to experimental study. ^{32}P-β-Radiolysis of DL-tryptophan shows no evidence of asymmetric degradation,[285] in contrast to results reported in 1976, whose authors have offered comments in support of their original claims.[286] Radiolysis over a period in which complete destruction of

[274] K. Makino, *J. Phys. Chem.*, 1980, **84**, 1016.
[275] K. Makino, *J. Phys. Chem.*, 1980, **84**, 1968; F. Moriya, K. Makino, N. Suzuki, S. Rokushika, and H. Hatano, *J. Phys. Chem.*, 1980, **84**, 3085.
[276] L. Kevan, S. Schlick, K. Toriyama, and M. Iwasaki, *J. Phys. Chem.*, 1980, **84**, 1950.
[277] P. O. Samskog, G. Nilsson, A. Lund, and T. Gillbro, *J. Phys. Chem.*, 1980, **84**, 2819.
[278] R. F. Lakhary and P. Krebs, *Chem. Phys. Lett.*, 1980, **70**, 469.
[279] R. Calvo, S. B. Oseroff, and H. C. Abache, *J. Chem. Phys.*, 1980, **72**, 760.
[280] M. Ogawa, K. Ishigure, and K. Oshima, *Radiat. Phys. Chem.*, 1980, **16**, 289.
[281] M. Ogawa, K. Ishigure, and K. Oshima, *Radiat. Phys. Chem.*, 1980, **16**, 281.
[282] J. C. Haynes, S. Kuroda, K. Matsuki, and I. Miyagawa, *Radiat. Res.*, 1980, **84**, 426.
[283] W. H. Nelson and D. R. Taylor, *J. Chem. Phys.*, 1980, **72**, 524.
[284] R. M. Lemmon and W. A. Bonner, *Stud. Phys. Theor. Chem.*, 1979, **7**, 47.
[285] W. A. Bonner, N. E. Blair, and J. J. Flores, *Nature (London)*, 1979, **281**, 150.
[286] W. Darge, I. Laczko, and W. Thiemann, *Nature (London)*, 1979, **281**, 151.

tryptophan is brought about causes destruction to the extent of only 20—30% when applied to DL-leucine, and careful analysis has established that there is no asymmetric bias in the case of leucine.[287] However, less energetic (u.v.) irradiation of DL-tryptophan derivatives and determination of the equilibrium constants for excimer formation reveal a chiral discrimination energy of 0.7 kcal mol^{-1},[288] thus providing a new basis for speculation on mechanisms accounting for the predominance of L-amino-acids.

The remarkable result stated in the preceding sentence is one culmination of a vigorous field of photochemical study of aromatic and heteroaromatic amino-acids. Recent papers cited here all concern tryptophan solutions, whose u.v. chemiluminescence, phosphorescence, and fluorescence have been studied,[289] and whose light instability has been described in precautionary terms for the attention of those engaged in clinical research.[290] Kinetics of fluorescence decay of photo-excited tryptophan in aqueous solutions have provided useful detailed information,[291-293] notably [293] an interpretation of data consistent with double exponential kinetics, originating in two distinct conformations of the amino-acid.

6 Analytical Methods

Gas–Liquid Chromatography.—While the g.l.c. technique in itself is rapid and accurate after calibration for a particular purpose, the derivatization procedure which is an essential precursor to the analysis of amino-acid mixtures may introduce errors of precision. This is because yields in the reactions used to convert amino-acids into volatile derivatives may vary from one amino-acid to the next, or even from one procedure to a repetition on the same sample. This is overstating the case somewhat, but clearly there is scope for uncertainty in the accuracy of quantitative analysis of amino-acids by g.l.c., and attempts have been made to introduce modifications which compensate for artifactual errors. In the 'enantiomer labelling' technique,[294] an aliquot of a solution of known amounts of *the enantiomers* of the amino-acids present in the sample is added, in the form of their isopropyl esters, to act as internal standards. Esterification, clean-up, and *N*-acylation are then followed by g.l.c. on a chiral stationary phase, noting variations in proportions of the internal standards from their actual concentrations. The accuracy and precision of the novel variation is claimed to be equal to, or better than, the performance of the ion-exchange analyser,[294] although much preliminary work is required to establish conditions leading to fully resolved g.l.c. peaks.

Routines for derivatization of amino-acids have been described,[295] and many examples of the favoured combinations of *N*-acyl and esterifying groups have

[287] N. E. Blair and W. A. Bonner, *J. Mol. Evol.*, 1980, **15**, 21.
[288] C. D. Tran and J. H. Fendler, *J. Am. Chem. Soc.*, 1980, **102**, 2923.
[289] J. Slawinski, M. Elbanowski, and D. Slawinska, *Photochem. Photobiol.*, 1980, **32**, 253.
[290] M. Kenney, R. F. Lambe, D. A. O'Kelly, and A. Darragh, *Clin. Chem.* (*Winston-Salem, N.C.*), 1980, **26**, 1511.
[291] G. S. Beddard, G. R. Fleming, G. Porter, and R. J. Robbins, *Philos. Trans. R. Soc. London, Ser. A*, 1980, **298**, 321; R. J. Robbins, G. R. Fleming, G. S. Beddard, G. W. Robinson, P. J. Thistlethwaite, and G. J. Woolfe, *J. Am. Chem. Soc.*, 1980, **102**, 6271.
[292] K. P. Ghiggino, G. R. Mant, D. Phillips, and A. J. Roberts, *J. Photochem.*, 1979, **11**, 297.
[293] A. G. Szabo and D. M. Rayner, *J. Am. Chem. Soc.*, 1980, **102**, 554.
[294] H. Frank, A. Rettenmeier, H. Weicker, G. J. Nicholson, and E. Beyer, *Clin. Chim. Acta*, 1980, **105**, 201.
[295] I. M. Moodie, *Lab. Pract.*, 1980, **29**, 1074.

again been reported. *N*-Trifluoroacetyl amino-acid n-butyl esters[296-298] and corresponding n-propyl,[299] isopropyl,[300] or hexafluoroisopropyl esters[301] have been used, as have *N*-heptafluorobutyryl n-propyl esters,[302] their isopropyl esters,[303] and particularly their isobutyl esters.[304-306] Users of the g.l.c. method are tending to quote the precedent source from which they obtained practical details for the preparation of derivatives (*e.g.* ref. 307 quoted in 306 for the conversion of amino-acid mixtures into *N*-heptafluorobutyryl isobutyl esters), and this tends to encourage moves towards uniformity of operations. Trimethylsilyl derivatives are less favoured now, although trimethylsilylation has been advocated for the quantitative analysis of 3-methylhistidine,[308] and trimethylsilyl esters of *NO*-bis(trifluoroacetylated)hydroxyamino-acids have been used.[309] Amino-acids yield 2-trifluoromethyloxazolin-5-ones on treatment with trifluoroacetic anhydride, and these are useful in g.l.c.–m.s. studies.[310]

Points of interest from these derivatization procedures are the problems arising with sulphur-containing amino-acids,[296, 298, 305] and three independent approaches to the g.l.c. analysis of 3-methylhistidine.[299, 304, 308] δ-Amino-n-valeric acid has been advocated as an internal standard for g.l.c. studies.[301]

Resolution of amino-acid enantiomers by g.l.c. methods can be accomplished either by the conversion of the D : L-mixture into a pair of diastereoisomers, for example using an optically active *N*-acyl or ester grouping, or through the use of a chiral stationary phase. An example of the former approach, esterification of *N*-isobutoxycarbonyl-DL-histidine using (+)-pantoyl-lactone, yields diastereoisomers which can be separated completely.[311] Grafting L-valine t-butylamide on to a cyanosilicone for use as a stationary phase, a further example of a well investigated methodology, has been found to be satisfactory for resolution of derivatized D : L-amino-acid pairs,[312] and other examples can be found in papers cited earlier in this chapter,[300] including several papers published in the form of Conference Proceedings.[6, 229]

The success of the derivatization approach for the g.l.c. of amino-acids has meant that the pyrolysis–g.l.c. technique has been largely ignored. In any case, the complex mixture of simple pyrolysis products which can arise (*cf.* ref. 236) renders this approach of little value in diagnostic work, although it is the only g.l.c.

[296] J. Kvalraag and T. Tjoernhom, *Nord. Jordbrugsforsk.*, 1980, **62**, 281 (*Chem. Abstr.*, 1980, **93**, 200284).
[297] C. Perier, M. C. Ronziere, A. Rattner, and J. Frey, *J. Chromatogr.*, 1980, **182**, 155.
[298] E. Bailey, F. B. Farmer, and J. H. Lamb, *J. Chromatogr.*, 1980, **200**, 145.
[299] L. Cotellessa, F. Marcucci, D. Corni, P. Sfondrini, L. Colombo, E. Mussini, and F. Poy, *J. Chromatogr.*, 1980, **221**, 149.
[300] N. Oi, O. Hiroaki, H. Shimida, M. Horiba, and H. Kitahara, *Bunseki Kagaku*, 1980, **29**, 270.
[301] T. Asakura and M. Matsuda, *Jikeikai Med. J.*, 1980, **27**, 63.
[302] T. Yoneda, *Anal. Biochem.*, 1980, **104**, 247.
[303] M. A. Kirkman, M. M. Burrell, P. J. Lea, and W. R. Mills, *Anal. Biochem.*, 1980, **101**, 364.
[304] T. W. Larsen and R. F. Thornton, *Anal. Biochem.*, 1980, **109**, 137.
[305] S. L. MacKenzie and A. J. Finlayson, *J. Chromatogr.*, 1980, **187**, 239.
[306] H. Sedova and M. Kahler, *Kvasny Prum.*, 1980, **26**, 193.
[307] S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 1979, **171**, 195; 1979, **173**, 53.
[308] H. Vielma and J. Mendez, *J. Chromatogr.*, 1980, **196**, 166.
[309] G. Michael, *J. Chromatogr.*, 1980, **188**, 251.
[310] V. Ferrito, R. Borg, J. Eagles, and G. R. Fenwick, *Biomed. Mass Spectrom.*, 1979, **6**, 499.
[311] M. Makita, Y. Ohkaru, and S. Yamamoto, *J. Chromatogr.*, 1980, **188**, 408.
[312] T. Saeed, P. Sandra, and M. Verzele, *J. Chromatogr.*, 1979, **186**, 611.

approach available for amino-acid betaines,[313] many of which (*e.g.* glycine betaine $Me_3\overset{+}{N}CH_2CO_2^-$) occur in plants.

Ion-exchange Chromatography.—As in previous volumes, no comprehensive coverage is attempted for this topic. While it is considered to be appropriate to exclude routine acquisition of results of a type familiar to readers, novel work concerning instrumentation (including microprocessor control and computer-assisted operations) is also felt to be beyond the scope of this report.

The opportunity has been taken to comment on textbook errors on the methodology of amino-acid analysis by ion-exchange, and to describe improvements in buffer composition.[314] Less common amino-acids for which ion-exchange separation techniques have been established are aminomalonic, β-carboxyaspartic, and γ-carboxyglutamic acids,[255] diaminopimelic acid,[315] and the group of sulphonic acids taurine, *S*-sulphocysteine, cysteic acid, and *S*-sulphothiocysteine.[316] Identification of hydroxylysine, and its glycoside, and 3-methylhistidine in urine has been studied.[317]

Thin-layer and Paper Chromatography.—The current literature on this topic amounts to consolidation of established techniques with minor modifications. Two multi-author books include chapters on t.l.c. of amino-acids.[318, 319]

Rapid t.l.c. methods have been established for the identification of hydroxylysine and hydroxyproline in mixtures,[320] the separation on cellulose of tyrosine from its mono-, di-, tri-, and tetra-iodo derivatives,[321] and the estimation of tryptophan in human plasma.[322] Solvent systems and the effects of pH have been investigated for the t.l.c. on silica gel[323] and on DEAE-cellulose[324] of representative amino-acids. Dansylamino-acids,[325] phenylthiohydantoins[326, 327] and their *p*-(*NN*-dimethylaminophenylazo)-analogues,[328] and methylthiohydantoins[327] are described in a small selection from a larger number of papers dealing with t.l.c. identification of commonly used amino-acid derivatives. It is becoming more common to perform parallel t.l.c. and h.p.l.c. analyses with these derivatives, since resolutions of groups of close-running amino-acid phenylthiohydantoins, for example, which are not possible by one technique are often achieved by the other.[327, 328]

[313] W. D. Hitz and A. D. Hanson, *Phytochemistry*, 1980, **19**, 2371.
[314] J. Svasti, *Trends Biochem. Sci. (Pers. Ed.)*, 1980, **5**, p. viii.
[315] S. Pongor and K. Baintner, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 1980, **15**, 1.
[316] T. Ubuka, M. Kinuta, K. Akagi, and S. Kiguchi, *J. Chromatogr.*, 1980, **188**, 442.
[317] D. T. Di Ferrante, N. Y. Wilson, and C. S. Leach, *J. Chromatogr.*, 1980, **187**, 271.
[318] J. G. Heathcote, in 'Densitometry in Thin-Layer Chromatography', ed. J. C. Touchstone and J. Sherma, Wiley, New York, 1979.
[319] T. Omori, in 'Instrumentation for H.P.T.L.C., Proceedings of 1st International Symposium', ed. W. Bertsch, S. Hara, and R. E. Kaiser, Huethig, Heidelberg, 1980, p. 275.
[320] Z. Buzas, B. Polyak, and L. Boross, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 1980, **15**, 173.
[321] M. Lederer, *J. Chromatogr.*, 1980, **194**, 270.
[322] H. K. L. Hundt, E. C. Clark, and H. C. Van der Linde, *J. Chromatogr.*, 1980, **182**, 110.
[323] I. Kalnina, L. Krauja, and T. M. Sheveleva, *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.*, 1980, 76 (*Chem. Abstr.*, 1980, **92**, 226127).
[324] I. Kalnina and L. Krauja, *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.*, 1980, 71 (*Chem. Abstr.*, 1980, **92**, 226126).
[325] J. C. Wesenberg and J. E. Walpole, *Mikrochim. Acta*, 1980, **2**, 1.
[326] C.-Y. Yang, *Hoppe-Seyler's Z. Physiol. Chem.*, 1980, **361**, 1599.
[327] M. J. Horn, P. A. Hargrave, and J. K. Wang, *J. Chromatogr.*, 1979, **180**, 111.
[328] K. J. Wilson, K. Rodger, and G. J. Hughes, *FEBS Lett.*, 1979, **108**, 87.

Microcrystalline cellulose can effect the resolution into enantiomers of the protein aromatic and heteroaromatic amino-acids.[329] Although this is a well known attribute of cellulose, it is as well to have this reminder that a pair of spots on a t.l.c. plate may originate in this way.

Two-dimensional paper chromatography of amino-acids has been described [330] and, though not in itself a new method of course, full details are presented of development in one direction, a second development in the same direction with a different solvent, and finally the use of a third solvent system in the second direction. This method, together with the use of strontium nitrate–ninhydrin spray reagent, has been claimed to give good separations of mixtures of common amino-acids.

High-performance Liquid Chromatography.—Many more papers year by year are devoted to analysis of amino-acids and their derivatives by h.p.l.c., and some novel variations of standard methodology accompany a substantial body of routine work. Scope continues to exist for improved procedures for detection of constituents emerging from h.p.l.c. columns, and this aspect is represented in the 1980 literature.

Detailed description is given of techniques for h.p.l.c. analysis of free amino-acids,[331] including tryptophan [332–335] (detection based on its fluoresence) and γ-amino-butyric acid [336] (detection based on derivatization with *o*-phthaldialdehyde–mercaptoethanol leading to a one pmole sensitivity limit). Prior conversion of a sample into fluorescent derivatives has been used for the h.p.l.c. analysis of amino-acids in synaptosomal extracts [337] and for 3-methylhistidine estimation [338, 339] (using the iso-indoles formed from amino-acids with *o*-phthaldialdehyde and a thiol). The same approach has been used for the estimation of dopa at 45 pmole sensitivity, but using fluorescamine as the reagent.[340] Alternative derivatization procedures involve *N*-substitution reactions of an equally familiar kind, illustrated in h.p.l.c. analysis of lysine as its 2,4-dinitrophenyl derivative,[341] dansylamino-acids,[342–345] and a less commonly used relative, dabsyl derivatives [346] (useful for the estimation of amino-acids in urine based on their maximal absorbance at 425 nm).

Derivative formation of a different kind has been used for the determination of

[329] S. Yuasa, A. Shimada, K. Kameyama, M. Yasui, and K. Adzuma, *J. Chromatogr. Sci.*, 1980, **18**, 311.
[330] A. Abbasi, R. Ali, and Z. H. Zaidi, *J. Biochem. Biophys. Methods*, 1980, **3**, 311.
[331] R. Schuster, *Anal. Chem.*, 1980, **52**, 617.
[332] O. Beck and T. Hesselgren, *J. Chromatogr.*, 1980, **181**, 100.
[333] T. Flatmark, S. Wahlstroem Jacobsen, and J. Haavik, *Anal. Biochem.*, 1980, **107**, 71.
[334] H. R. McKim and W. G. Dewhurst, *Proc. West. Pharmacol. Soc., 23rd Meeting*, 1980, p. 291 (*Chem. Abstr.*, 1980, **93**, 14 565).
[335] H. F. Baker, M. H. Joseph, and R. M. Ridley, *J. Pharmacol.*, 1980, **70**, 133P.
[336] T. A. Hare and N. V. B. Manyam, *Anal. Biochem.*, 1980, **101**, 349.
[337] K. Lenda and G. Svenneby, *J. Chromatogr.*, 1980, **198**, 516.
[338] Z. Friedman, H. W. Smith, and W. S. Hancock, *J. Chromatogr.*, 1980, **182**, 414.
[339] S. J. Wassner, J. L. Schlitzer, and J. B. Li, *Anal. Biochem.*, 1980, **104**, 284.
[340] B. Tabakoff and R. F. Black, *J. Neurochem.*, 1980, **34**, 1707.
[341] N. Muhammad and J. A. Bodnar, *J. Liq. Chromatogr.*, 1980, **3**, 529.
[342] S. Kobayashi and K. Imai, *Anal. Chem.*, 1980, **52**, 424.
[343] S. K. Lam and F. K. Chow, *J. Liq. Chromatogr.*, 1980, **3**, 1579.
[344] H. Engelhardt and S. Kromidas, *Naturwissenschaften*, 1980, **67**, 353.
[345] W. Lindner, *Naturwissenschaften*, 1980, **67**, 354.
[346] J.-K. Liu and C.-H. Wang, *Clin. Chem.* (*Winston-Salem, N.C.*), 1980, **26**, 579.

the enantiomeric purity of tri- and tetra-iodothyronines, in which these amino-acids are converted into diastereoisomer mixtures through coupling with L-leucine,[347] then separated by h.p.l.c. An alternative resolution procedure, in which a chiral stationary phase is used (L-prolinamide [345] or L-valinamide [346] bonded to silica gel) for the separation of D : L-dansylamino-acid pairs, is not the only other variation of the chromatographic resolution technique, since dansylamino-acids can be resolved using an eluent containing a copper(II)–L-proline complex.[343] An example of the determination of enantiomeric purity concerning one of the less common amino-acids has been reported for penicillamine.[348]

Substantial studies continue to be reported on h.p.l.c. analysis of phenylthiohydantoins [327,349–352] and *p*-(*NN*-dimethylaminophenylazo)-analogues.[328,353] Although the mention here of a few points of interest does not do justice to the useful details to be found in each of the papers, attention to the separation of glutamic and aspartic acid derivatives,[351] and a maximum time of 30 min for the separation of all 20 protein amino-acid derivatives,[350] can be highlighted. Use of the dimethylaminoazobenzenethiohydantoins allows sensitivity levels of 5—10 pmole to be reached.[353]

Fluorimetry.—Ammonia released from glutamine by heating in dilute sulphuric acid at 100 °C can be assayed by absorption into an *o*-phthaldialdehyde–mercaptoethanol reaction mixture, yielding fluorescence in proportion to the glutamine content of the sample.[354] The reagent system is extremely sensitive, and careful cleaning of glassware is essential for accurate results.[355]

Mention has been made in preceding sections of analytical exploitation of fluorescence-forming reactions. Further examples are reported for the fluorimetric estimation of phenylthiohydantoins using the pyridoxamine–lead(II) acetate reagent,[356] for the formation of fluorescent spots on thin-layer chromatograms by thiamine and sodium hypochlorite for the detection of these compounds,[352] and for the estimation of γ-aminobutyric acid or glutamic acid based on the formation of a fluorescent chelate with ninhydrin in the presence of a copper(II) salt.[357]

Other Separation Methods.—Low-voltage electrophoresis in acidic media, in combination with concurrent chromatographic separation on cellulose layers, has been advocated [358] for identification of mixtures containing 1—10 nmole levels of

[347] E. P. Lankmayr, K. W. Budna, and F. Nachtmann, *J. Chromatogr.*, 1980, **198**, 471.
[348] F. Nachtmann, *Int. J. Pharm.*, 1980, **4**, 337.
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[350] J. Fohlman, L. Rask, and P. A. Peterson, *Anal. Biochem.*, 1980, **106**, 22.
[351] J. U. Harris, D. Robinson, and A. J. Johnson, *Anal. Biochem.*, 1980, **105**, 239.
[352] T. Kinoshita, K. Murayama, and A. Tsuji, *Chem. Pharm. Bull.*, 1980, **28**, 1925.
[353] J. Y. Chang, A. Lehmann, and B. Wittman-Liebold, *Anal. Biochem.*, 1980, **102**, 380.
[354] T. Z. Liu and H. Khayam-Bashi, *Clin. Chem. (Winston-Salem, N.C.)*, 1980, **26**, 700.
[355] D. J. Shute, *Med. Lab. Sci.*, 1980, **37**, 173.
[356] T. Kinoshita and K. Murayama, *Jpn. Kokai Tokkyo Koho* 80 36 740 (*Chem. Abstr.*, 1980, **93**, 91 531).
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[358] R. L. Munier and S. Mennier, *Anal. Biochem.*, 1979, **100**, 254.

amino-acids. High-voltage electrophoresis following conventional procedures has been used for the determination of proline and its 3- and 4-hydroxy-derivatives in biological samples.[359] Isotachophoresis techniques are suitable for the estimation of *S*-(carboxymethyl)cysteine in urine.[360] Reverse osmosis across a DDS-cellulose acetate membrane from binary aqueous solutions containing L-alanine, and the effects of other amino-acids on the permeation of this compound have been described.[361]

Determinations of Specific Amino-acids.—Nearly all the citations in this section refer to specific enzyme-based procedures, but this is not a realistic picture of this topic, since colorimetric assays are still fashionable, and have been largely located in earlier sections of this chapter.

Modified ninhydrin colour-forming reactions have been established for the assay of mixtures containing proline, hydroxyproline, and hydroxylysine.[362] Further development of spectrophotometric assay of hydroxyproline in tissue (see also Vol. 11, p. 2) has been reported,[363] and an improvement of established nitroprusside colorimetry of cystine in urine has been developed.[364]

Enzyme-catalysed degradations of amino-acids which have been applied for specific estimation procedures include: a bacterial ω-amino-acid–pyruvate amino-transferase together with lactate dehydrogenase for estimation of L-alanine;[365] leucine dehydrogenase used for the quantitative determination of branched-chain amino-acids;[366] lysine decarboxylase in immobilized form for an automated assay of L-lysine;[367] nmole level assay of L-ornithine employing ornithine amino-transferase with Δ^1-pyrroline-5-carboxylate reductase;[368] and microassay of cysteinesulphinic acid through enzymic conversion into lactate with glutamate oxalacetate transaminase with α-ketoglutarate and NADP(H).[369]

Microbioassay of L-leucine[370] or L-phenylalanine[371] through metabolism by *Leuconostoc mesenteroides* followed by use of an immobilized lactate oxidase electrode illustrates the continuing development of potentiometric sensor methods. The lactate sensor used in these studies is based on an oxygen electrode which is coated with the immobilized enzyme. A related example applied to the assay of L-histidine uses an ammonia-sensing electrode coated with immobilized *Pseudomonas*, and is based on the stoicheiometry 2 mol $NH_3 \equiv$ 1 mol histidine.[372] This technique has been refined through the isolation of the enzyme (histidine ammonia-lyase) and its immobilization on the electrode.[373]

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All these enzyme-mediated assays depend on a quantitation stage, and a spectrometric determination of NADP(H) released through the degradation of meso-αε-diaminopimelate by the specific D-amino-acid dehydrogenase has been adopted in this case; alternatively, the conversion of the NADP(H) into a formazan preceding spectrophotometry may be considered.[374]

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2

Structural Investigations of Peptides and Proteins

BY M. D. SCAWEN, A. J. GARMAN, R. A. G. SMITH, W. D. MERCER, R. H. PAIN, AND OTHERS

PART IA: Protein Isolation and Characterization *By M. D. Scawen, R. F. Sherwood, D. A. P. Small, P. M. Hammond, P. Hughes, A. Electricwala, S. Alwan, and T. Atkinson*

1 Introduction

This year's report follows closely on the patterns set in previous years. The ever increasing number of publications coupled with limitations of space means that a considerable degree of selectivity is necessary in virtually all aspects of protein isolation and characterization methodology. As a result the tabulated data are restricted to certain classes of protein and the emphasis, as before, is on proteins isolated by means of affinity techniques.

2 Protein Isolation Methodology

Affinity Chromatography.—*General Comments.* The development and recent extensive exploitation of biospecific adsorbents of the group specific type used in affinity chromatography has proved a most valuable addition to the range of techniques available in enzyme purification.[1,2]

The most widely used ligands for application of this type of affinity chromatography have been various analogues of adenosine mononucleotides and dinucleotides substituted on the base, ribose, or phosphate.[3] Interest in immobilized adenine nucleotides and coenzymes is derived both from their broad application in the purification of complementary enzymes (*e.g.* kinases, dehydrogenases, flavoproteins, and coenzyme A-dependent enzymes) and because immobilized coenzymically active adenine nucleotides are becoming increasingly important in enzyme technology.[4,5] New nucleotide matrices have recently been synthesized. For example, the synthesis and characterization of two IMP analogues 8-(6-aminohexyl)inosine 5-monophosphate and inosine 2',3-*O*-[(6-aminohexyl)-levulinic acid amide]acetal 5-monophosphate have been recently described.[6] These analogues were attached to CNBr-activated agarose through the terminal amino-

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group of the spacer molecule. *E. coli* IMP dehydrogenase could be eluted biospecifically from the immobilized 8-substituted and ribose substituted IMP absorbents with IMP, XMP, or GMP.[6]

With the range of potential absorbents for any enzyme rapidly increasing it is worth noting that theoretical considerations[7] dictate that the adsorbent which binds an enzyme with lower affinity is that which is most likely to be responsive to biospecific desorption. This consideration has been widely used in many of the affinity chromatography purifications listed in Table 1.[8–12] A number of proteins other than those shown in Table 1 have been purified by affinity or pseudo-affinity chromatography including human low-density lipoproteins,[13,14] sex-steroid-binding proteins,[15] initiation factor eIf-2 from rat-liver microsomes on rRNA cellulose,[16] cortisol-binding globulin,[17] poly A-containing ribonucleoproteins on oligo (dT)-cellulose,[18] plant carbohydrate-binding proteins,[19] serine acetyl transferase by immunoadsorption,[20] arylsulphatase A sub-units,[21] plasma membrane proteins on concanavalin A–agarose,[22] bovine lens aldolase reductase,[23] plant cytokinin-binding protein,[24] pregnancy associated plasma protein-A[25] and plasma urate-binding protein,[26] liver ribosomal tRNA-binding proteins,[27] bovine-liver NADH–cytochrome b_5 reductase,[28] plasma fibronectin,[29] and cytochrome *c* oxidase on cytochrome *c*–thiol–Sepharose.[30]

Coupling Methods. The typical bioaffinity adsorbent is prepared by coupling an affinity ligand to an insoluble hydrophilic support. Although direct coupling to CNBr-activated Sepharose remains one of the most common methods of immobilizing ligands, it has the disadvantage that cationic charges are introduced into the matrix which can cause non-specific adsorption.

Tosylated polysaccharides have recently been applied as alternative supports for ligand immobilization.[31] It is known from the chemistry of soluble saccharides

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that the reaction of hydroxy groups with toluene-*p*-sulphonyl chloride (tosyl chloride) gives the corresponding esters (tosylates), which have excellent leaving properties in reactions with nucleophiles, giving stable linkages between ligand and saccharide carbon. The reactions involved in the binding of biomolecules to polysaccharides using tosyl chloride are shown in the Figure. Soybean trypsin inhibitor (75 mg g^{-1} dry support) has been immobilized to tosylated agarose, tested as affinity chromatography material, and shown to bind 60 mg trypsin g^{-1} dry gel.[31]

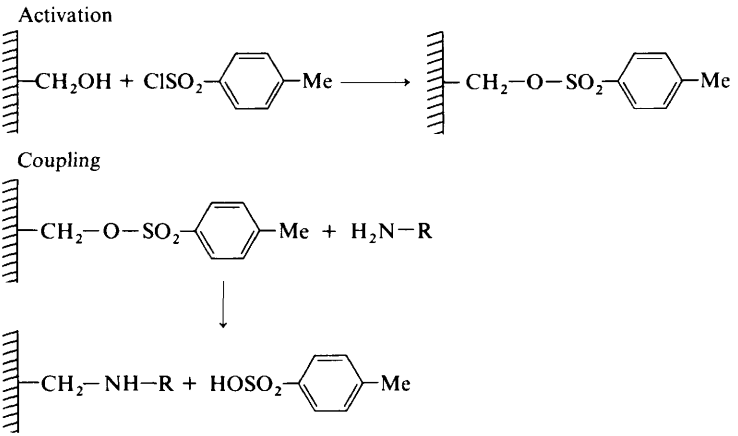

Figure *Immobilization of ligands to Sepharose using tosyl chloride*

A further reagent which is capable of forming strong covalent bonds, 2,4,6-trichloro-*s*-triazine, has been used for coupling enzymes and affinity ligands to supports like cellulose and agarose. Thus, in the purification of purine nucleoside phosphorylase from human red cells the substrate analogue 6-hydroxy-9-*p*-aminobenzylpurine was coupled to Sepharose using 2,4,6-trichloro-*s*-triazine to give an efficient biospecific affinity gel.[32]

Sequential Affinity Chromatography and Affinity Elution. The application of sequential affinity columns as an aid to protein purification has been further demonstrated by the purification of larval acetyl cholinesterase.[33] Concanavalin A–Sepharose chromatography provided the initial purification and was followed by chromatography on columns to which competitive inhibitors of acetyl cholinesterase had been attached. The most efficient of those used, *m*-carboxyphenylmethylammonium iodide, has been coupled to Sepharose 4B *via* a hydrophobic C_6 spacer arm. This combination of affinity steps gave a purification of 5000—7000-fold.

Affinity elution, rather than affinity chromatography, has been applied to the purification of type II hexokinase from rat skeletal muscle. The procedure entailed initial chromatography on DEAE cellulose, affinity elution from phosphocellulose, and gel filtration on Sephadex G-200. The key to the preparation of

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homogenous enzyme is the affinity elution step in which an effector molecule, glucose-6-phosphate, is used as the eluting ligand.[34] A 400-fold purification was obtained in the affinity elution step alone.

Triazine Dye Affinity Chromatography. The purification of proteins by affinity chromatography is the broadest and best documented application of the use of dyes in protein studies.[35] The low capital cost, general availability, and ease of coupling to matrix materials represent a major advantage of triazine dyes over chemically defined nucleotide adsorbents. The capacity of dye columns for complementary proteins are 10—100 times higher than for immobilized nucleotide columns and the triazine linkage less prone to 'leakage' than the isouronium linkage introduced during CNBr activation of polysaccharides.[35]

The selectivity of dye chromatography is illustrated by the binding of troponin to a Cibacron Blue F3G-A agarose column and its selective release from the gel in the presence of 0.5 M KCl. This has provided the basis for a new purification method [36] for this protein. Adsorption of troponin to the immobilized dye appears to occur through the troponin T sub-unit. Troponin I and troponin C do not bind to this matrix whereas troponin T binds very tightly.

The application of a number of immobilized Procion dyes to the purification of inosine-5-monophosphate dehydrogenase has been described.[11] The enzyme was eluted by a salt gradient with a substantial increase in specific activity. Adsorption of the enzyme from a crude cell-free extract of *E. coli* to immobilized Procion Yellow MX-8G in the presence of 15% (v/v) ethylene glycol and subsequent elution with a linear gradient of NaCl have 90% recovery with 14-fold increase in specific activity.

The interactions of several triazine dyes with two enzymes from purine metabolism, IMP dehydrogenase and adenylsuccinate synthetase, have been investigated.[9] Evidence from kinetic inhibition studies, enzyme inactivation with specific affinity labels, and specific elution techniques from agarose-immobilized dyes indicate that triazine dyes such as Procion Blue H-B (Cibacron Blue F3G-A), Red HE-3B, and Red H-3B are able to differentiate between the nucleotide binding sites of these enzymes. This information has been exploited to design specific elution techniques for the purification of these enzymes by affinity chromatography.

6-Phosphogluconate dehydrogenase from *B. stearothermophilus* has been purified approximately 260-fold by tandem affinity chromatography on two dye columns in series, Red HE-3B and Cibacron Blue F3G-A respectively, linked to agarose.[8]

Interferon Purification. The purification of interferon has been a primary goal of interferon research, yet the purification to homogeneity of any interferon has been difficult and only very small amounts of pure interferon have been produced. An improved purification procedure for human fibroblast interferon utilizing chromatography on Blue Sepharose has been achieved with a 2900-fold purification.[37] This procedure is suitable for the purification of large volumes of crude

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interferon and yields of homogeneous protein range from 20—40%. Interferon, prepared in serum-free medium, has also been purified to homogeneity by a method involving a combination of affinity chromatography and h.p.l.c.[38] Affinity chromatography on Blue Sepharose provides a high purification factor but results in a dilute solution of interferon in 50% ethylene glycol. The final product is obtained in concentrated form free of ethylene glycol and buffer salts by h.p.l.c. on Lichrosorb RP8, with a stepwise elution employing 32% (v/v) propan-l-ol.

Other Applications and Techniques. A process termed transition state affinity jump chromatography, a double selection method for isolating catalytically active enzymes, has been described.[39] This technique uses substrate analogue affinity chromatography and elution with transition state analogues. The method may be applied to the separation of enzymes and other catalysts according to their molecular turnover number.

Affinity chromatography has been applied in an assay system to determine phosphorylase kinase activity in crude homogenates using 5'-AMP Sepharose.[40] This is a sensitive method for measuring enzyme activity, which involves incorporation of ^{32}P from [γ-^{32}P]ATP into phosphorylase in the presence of other phosphorylating reactions. The kinase reaction is carried out in crude homogenates, the reaction stopped, and a portion of the reaction mixture applied to a 5'-AMP Sepharose column. Phosphorylated protein and [γ-^{32}P]ATP are washed out and phosphorylase is eluted by 10 mM ATP and the radioactivity incorporated is counted.

Passing chromatin fragments of rat-liver nuclei through DNA–agarose columns results in the removal of all histones of the H1 class and almost all (95%) non-histone proteins from the chromatin, and thus leads to the separation of DNA molecules containing nucleosomal histones only.[41] Elution of the proteins bound to DNA agarose by salt gradients leads to a fractionation of chromosomal proteins indicating that they bind with differing affinities to single stranded DNA. This simple and fast procedure is suitable for both the isolation of histone H1 depleted chromatin and the fractionation of non-histone proteins.

Some of the important controlling factors involved in the electrophoretic desorption of material from affinity matrices, *viz.* the electrophoresis current, matrix thickness, and electrophoresis buffer molarity and temperature, have been investigated.[42] Thus, optimum conditions have been developed to desorb human serum albumin from Cibacron Blue F3G-A Sepharose. Electrophoretic desorption has been developed as a mild, non-chaotropic technique for the removal of charged material from affinity matrices and in particular immunoadsorbents.

A combination of affinity chromatography and high performance liquid chromatography (h.p.l.c.) is currently being developed in several laboratories.[43] This technique (h.p.l.a.c.), which combines the speed and resolving power of h.p.l.c. with the biological specificity of affinity chromatography, is likely to

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Table 1 *Proteins purified by affinity chromatography*

Protein	*Source*	*Matrix**	*Ligand*	*Eluant*	*Ref.*
Dihydrofolate reductase	*E. coli*	S	2,4-Diamino-5(3,5-dimethoxy)4-substituted pyrimidines	0.02% folate, 0.5 M NaCl	44
L-Lysine 6-amino-transferase	*Flavobacterium lutiscence*	S	L-Lysylacetamido-dodecyl	1.0 M NaCl	45
Asparagine synthetase	Rat liver	S	Cibacron Blue F3G-A	0.1 mM ATP, 1 mM L-aspartate	46
Phosphotransferase	Wheat shoots	S	Cibacron Blue F3G-A	0.15 M sodium acetate, pH gradient 4.0—5.5, or 25 mM uridine pH 5	47
Acetylcholinesterase	*Electrophorus electricus*	S	9-(5-Carboxypentyl)-aminoacridine	10 mM decamethonium bromide	48
Acetylcholinesterase	*Ciona intestinalis*	S	Concanavalin A	α-Methyl-(+)-mannoside	33
Troponin	Rabbit muscle	S	Cibacron Blue F3G-A	0.5 M KCl	36
α-D-Glucosidase	Horse kidney	S	*p*-Aminophenyl α-D-maltoside	0.25—0.5 M NaCl gradient	49
Glyoxalase II	Mouse liver		Glutathione	2 mM *S*-octylglutathione	50
Oestrogen receptor	Chicken liver	S	1,7-β′-Oestradiol-17-hemisuccinyl-ovalbumin	40 μM oestradiol	51
Adenosine kinase	Rat heart	S	N^6-(6-Amino-hexyl) 5′ AMP	1 mM ATP, 1 mM Mg^{2+}, 0.1 mM adenosine	52
3-Hydroxy-3-methyl glutaryl coenzyme A reductase	Rat liver	S	Blue Dextran	0.5 M KCl	53

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Table 1 (*cont.*)

Protein	*Source*	*Matrix**	*Ligand*	*Eluant*	*Ref.*
Ornithine transcarbamylase	Rat liver	S	δ-*N*-(Phosphonacetyl) L-ornithine	0—10 mM carbamyl phosphate gradient	54
Luciferase	Firefly lanterns	CH-S	Benzylamine	0.15 M sodium phosphate pH 7.8	55
Glutamate synthase	*E. coli*	S	2′,5′-ADP	1 mM L-glutamine, 100 μM NADPH	55*a*
Bile salt activated lipase	Human milk (whey)	S	Concanavalin A	2% sodium cholate	56
Fructose 1,6-bisphosphatase	Rabbit liver	S	Blue Dextran	25 μM AMP	57
Fructose 1,6-bisphosphate aldolase	Rabbit liver	S	Blue Dextran	Fructose 1,6-bisphosphate 0.1 mM	57
Phenylalanine hydroxylase	Rat liver	S	2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine	0.1 M phenylalanine	58
Phenylalanine hydroxylase	Rat liver	S	6,7-Dimethyl-5,6,7,8-tetrahydropterin	50% ethanediol, 1 M KCl	59
Carboxypeptidase A	Bovine pancreatic tissue	S	*p*-Aminobenzyl-succinic acid		60
Tubulin	Rat brain	Affigel 10	Lactoperoxidase	1 M NaCl	61
Purine nucleoside phosphorylase	Human red cells	–	6-Hydroxy-9-(*p*-aminobenzyl)-purine	4 mM inosine, 50 mM sodium phosphate pH 7.6	32
Purine nucleoside phosphorylase	Human leukaemic granulocytes	S	Formycin B	10 mM inosine	62
Histones	Calf thymus	S	Organo-mercurial	10 mM dithiothreitol	63
Diol dehydrase	*Klebsiella pneumonia*	–	Adenosyl cobalamin	Propan-1,2-diol (2%)	64
Flavokinase	Rat liver	S	Flavinyl	0.1 mM riboflavin	65
Ribonuclease F1 and F2	*Fusarium moniliforme*	AH-S	5′-GMP	1 mM 2′(3′)-GMP	66
DNase I	Porcine pancreas	S	d-DNA	1 M KCl	67

Phospholipase A_2	Cobra venom	S	Cibacron Blue F3G-A	50 mM NH_4HCO_3 pH 8 50 mM $(NH_4)_2CO_3$ pH 10.5	68
Cytochrome P450	Rabbit-liver microsomes	S	Cytochrome b_5	0.25 M KCl, 0.2% Triton X-100, 0.2 mM EDTA, 20% glycerol	69
Haemoglobin	Blood and bone marrow	S	Haptoglobin	5% formic acid	70
Pyridoxal kinase	Rat brain	AH-S	Pyridoxyl	10 mM pyridoxine	71
Cholic acid-binding protein	Rat liver	AH-S	Cholic acid	5 mM cholic acid	72
Pyridoxamine-5-phosphate oxidase	Rat brain	AH-S	Phosphopyridoxyl	5 mM pyridoxal phosphate	71
6-Phosphogluconate dehydrogenase	*B. stearothermophilus*	S	Procion Red HE-3B	0—0.8 M KCl gradient	8
6-Phosphogluconate dehydrogenase	*B. stearothermophilus*	S	Cibacron Blue F3G-A	0.5 mM NADP	8
Cyclic nucleotide phosphodiesterase	Rat heart	AH-S	Phenylbutenolide	0.4—1.8 M KCl gradient	73

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Table 1 (*cont.*)

Protein	*Source*	*Matrix**	*Ligand*	*Eluant*	*Ref.*
Heparin	Porcine gastric mucosa	S	Antithrombin	0.5 M KCl	74
Propionyl CoA carboxylase	Human liver	S	Monomeric avidin	0—12 mM biotin gradient	75
Thymidine kinase	Rat liver	S	Rat kidney glycoprotein	1 M NaCl, 300 μM thymidine	76
Pgro E	*E. coli*	S	Blue Dextran	1 M KCl	77
Glutamine synthetase	*E. coli*	S	Blue Dextran	5 mM ADP	77
Cytochrome *c* oxidase	Beef-heart mitochondria	S	Cytochrome *c*	50 mM NaCl	78
Valy tRNA synthetase	*Euglena*	S	Blue Dextran	Yeast $tRNA_2^{Val}$	79
Thymidylate synthetase	*E. coli*	S	Tetrahydromethotrexate	0.2 M sodium phosphate pH 7.2	80
IMP dehydrogenase	*E. coli*	S	Procion Red HE-3B	AMP, NAD 0—20 mM gradient	9
IMP dehydrogenase	*E. coli*	S	Cibacron Blue F3G-A	IMP, 0—50 mM gradient	9
Dopamine β-mono-oxygenase	Bovine adrenal medulla	S	Cibacron Blue F3G-A	0.2 M NaCl, 20 mM phosphate pH 7	81
Dopamine β-mono-oxygenase	Bovine adrenal medulla	S	Procion Red HE-3B	0.8 M NaCl	81
Dihydrofolate reductase	Walker 256 carcinoma	S	Cibacron Blue F3G-A + other Procion dyes	0—1 M KCl gradient	10
Phosphoglycerate kinase	*Lactobacillus*	S	Blue Dextran	1 M KCl	82
IMP dehydrogenase	*E. coli*	S	Procion Yellow MX-8G	0—1 M KCl gradient	11
Interferon	Human fibroblasts	S	Cibacron Blue F3G-A	20 mM sodium phosphate pH 7.2 50% ethanediol	37
Succinyl CoA synthetase	Rat liver	S	GDP	2 mM GDP-Mg^{2+}	83

IMP dehydrogenase	*E. coli*	S	8-(6-Aminohexyl) AMP	XMP 0—40 mM gradient	84
Glycerokinase	*B. stearothermophilus*	S	Procion Blue MX-3G	5 mM ATP	82, 35
Malate dehydrogenase	*R. spheroides*	S	Procion Red H-3B	2 mM NADH in 1 M KCl	12, 35
Malate dehydrogenase	*R. spheroides*	S	Procion Blue MX-4GD	0—1 M KCl gradient	12, 35
β-Hydroxybutyrate dehydrogenase	*R. spheroides*	S	Procion Red H-3B	1 M KCl	12, 35
β-Hydroxybutyrate dehydrogenase	*R. spheroides*	S	Procion Blue MX-4GD	2 mM NADH in 2 M KCl	12, 35
D-Glyceraldehyde 3-phosphate dehydrogenase	*T. aquaticus*	S	NAD	2 mM NAD^+	85
D-Glyceraldehyde 3-phosphate dehydrogenase	*B. stearothermophilus*	S	NAD	2 mM NAD^+	85
Serum albumin	Several	S	Cibacron Blue F3G-A	0.5 M NaSCN	86

[a] S = Sepharose; CH-S = ω-carboxyhexyl Sepharose; AH-S = ω-aminohexyl Sepharose.

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provide a powerful new tool in protein purification and characterization studies in the future. The technique has already been applied to the rapid separation of serum albumin, liver alcohol dehydrogenase, and lactate dehydrogenase on silica-immobilized 5'-AMP.[43]

Hydrophobic Chromatography.—Hydrophobic interaction chromatography is used to separate protein molecules on the basis of the differing strength of their hydrophobic bonding to a stationary matrix. The interaction between protein and matrix results from an increase in the hydrogen bonding of water molecules when hydrophobic molecules bind with each other. The procedure is now considered a standard technique in protein purification and has found widespread application in many disciplines besides the traditional field of pure enzymology. In particular, recent uses have been reported in the field of clinical chemistry. These have included the purification of the human complement components C3 and C5[87] and the sub-component C3d,[88] human serum inter-α-trypsin inhibitor,[89] and human erythropoietin.[90] Hydrophobic chromatography has been successfully used to separate fragments of diphtheria toxin[91] and to separate heparin into fractions with differing anticoagulant activity.[92] Its application to the purification of albumin has also been considered.[93] The technique has been applied to the separation of isoenzymes[94,95] and several closely related aminoacyl tRNA synthetases,[96] to study the purification of membrane proteins,[97,98] and to separate carrier ampholytes from various peptides.[99]

The types of ligand used in hydrophobic interactions are also becoming more diverse. Recently investigated matrices include trityl Sepharose and trityl cellulose,[100] palmitoyl cellulose,[101] tannin Sepharose,[102] and Lipidex 1000, a substituted hydroxyalkoxypropyl derivative of Sephadex G25.[103]

To date, however, little work has been devoted to the study of the nature of the hydrophobic interaction. One recent investigation is that of Chang *et al.*[104] who examined the interaction of *E. coli* galactosidase and 3,3'-diaminodipropylamine-substituted Sepharose 4B. These workers found, amongst other factors, that the

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equilibrium constants exhibited a hysteresis effect during adsorption of the enzyme to, and desorption from, the hydrophobic matrix. A less detailed study of the interaction of *Micrococcus lysodeikticus* ATP-ase with various hydrophobic matrices has also been reported.[105]

Table 2 lists examples, from the literature of 1980, of proteins isolated using hydrophobic interaction chromatography.

Immunoaffinity Chromatography.—Immunoaffinity chromatography has been used successfully over the past few years for protein purification. The use of an immunoaffinity ligand allows the separation of specific proteins from a complex mixture by a one-step procedure resulting in increased yield and purity compared to the conventional 4—5-step purification procedure. In recent years, this technique has been refined in many ways to reduce non-specific binding of proteins without affecting specific recoveries. The conditions for the binding and specific elution of protein from an immunoaffinity column, as in the case of rabbit anti-albumin antibodies from Ultrogel AcA34 immunosorbent,[120] were examined in detail in relation to yield and biological activity. It was found that buffers of low pH or even low ionic strength, for example water in the case of phosphoenol-pyruvate carboxylase purification,[121] were most effective in disrupting the complex and eluting specific proteins from immunosorbents, relative to the harsh conditions used previously.

The question of homogeneity of the eluted protein has also been largely solved by the use of monoclonal antibodies. This has been well illustrated during the purification of mouse F_c receptors from a macrophage cell line.[122] The resultant purified preparation was then used directly for further biochemical and biophysical characterization and sub-class specificity. In another analytical technique, the eluate from an immunosorbent column prepared from monoclonal antibodies was analysed directly on two-dimensional gel electrophoresis, as a means for screening large numbers of myeloma hybrids in a complex mixture.[123]

One of the main advantages of using an immunoaffinity ligand is that only one protein need be purified to raise the antiserum and this can then be used to prepare either the same protein from a variety of species, for example the isolation of neurofilament protein from bovine brain using anti-neurofilament antisera raised against chicken-brain antigen,[124] or to isolate several related proteins, as in the purification of serine acetyl transferase, a component of a multienzyme complex, using antibodies to the other component of the complex, namely acetylserine sulphhydrylase.[125]

Recently a technique designated as high performance immunoaffinity chromatography has been developed using silica-immobilized antibodies.[126] Although this method is still in its infancy it is likely that it will prove useful both for studying the kinetics of antigen–antibody reactions and for the rapid fractionation of heterogeneous antibody populations.

Table 3 lists proteins purified by immunoaffinity chromatography.

Covalent Chromatography.—Covalent chromatography using ampicillin, cephalexin, or 6-aminopenicillanic acid immobilized on agarose has been used to purify penicillin-binding proteins from *E. coli* and *Bacillus megaterium*.[138–140] Thiol

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Table 2 *Proteins purified by hydrophobic interaction chromatography*

Protein	*Source*	*Ligand and support*	*Loading buffer**	*Elution buffer**	*Ref.*
Complement C3 and C5 (C2, C4, C8, and C9)	Human	ω-Aminohexyl Sepharose	50 mM KP, pH 7.5	50 mM KP, pH 7.5 + 200 mM NaCl (C2, C4, C8, and C9 complement eluted with 700 mM NaCl)	87
			10 mM Tris–HCl, pH 7.5 + 1.25 M $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$ with 50% ethanediol	
Complement C3d	Human	Phenyl Sepharose	10 mM NaP, pH 7.2, 0.15 M NaCl, 0.8 M $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$, increasing gradient of ethanediol to 50%	88
Inter-α-trypsin inhibitor	Human serum	Phenyl Sepharose	10 mM (Na_2HPO_4 + KH_2O_4), pH 6.85 + 0.8 M $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$	89
Erythropoietin	Human	Phenyl Sepharose	10 mM NaP, pH 6.8 + 4 M NaCl	10 mM NaP, pH 6.8 + 0.5 M NaCl followed by 10 mM NaP, pH 6.8 + 20% ethanediol + 4 M guanidine	90
Diphtheria toxin	*Corynebacterium diphtheriae*	Aminohexyl Sepharose	50 mM Tris–HCl, pH 8.0	1. 50 mM NaCl 2. 200 mM NaCl	91
Fragment A	*Corynebacterium diphtheriae*	Aminobutyl Sepharose	50 mM Tris–HCl, pH 8.0	Not retarded	91
Fragment B	*Corynebacterium diphtheriae*	Aminobutyl Sepharose	50 mM Tris–HCl, pH8.0	100 mM NaCl	91
Methionine adenosyl-transferase	Rat liver	Phenyl Sepharose	10 mM Tris, pH 7.5 + 10 mM β-MSH + 25% $(NH_4)_2SO_4$	*Isoenzyme 1*: 10 mM Tris, pH 7.5 + 10 mM β-MSH *Isoenzyme 2*: 10 mM Tris, pH 7.5 + 10 mM β-MSH + 40% DMSO	94
Aspartate amino-transferase	Porcine heart	Alkyl agarose	20 mM phosphate, pH 6.8	*Cytosolic*: not retarded *Mitochondrial*: detail not given	95

Lysyl tRNA synthetase	Rat liver	Diamino-octyl Sepharose	50 mM Tris, pH 7.5 + 25 mM KCl + 5 mM Mg-acetate + 2 mM DTE	0.3 M KCl + 40% ethanediol	96
Phospholipase B	*Penicillium notatum*	Palmitoyl cellulose	1 mM phosphate + 0.2 M EDTA	0.2% Adekatol SO-120	101
ATP-ase	*Micrococcus lysodeikticus*	Ethyl and butyl agarose	30 mM Tris–HCl, pH 7.5	Step gradient of LiCl at 20, 30, 50, 70, 100, and 150 mM	105
Proteinase A	Yeast	Phenyl Sepharose	Batch-bind in demineralized water	1. 100 mM KP, pH 6.0 + 2 M NaCl 2. 100 mM KP, pH 6.0 + 2 M NaCl + 50% ethanediol 3. 100 mM KP, pH 6.0 + 85% ethanediol	106
Eukaryotic eEF-Ts elongation factor	*Artemia salina*	Phenyl Sepharose	20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM Mg-acetate, 10 mM MSH, 10% propanol	Not retarded	107
Cytochrome oxidase	*Nitrobacter agilis*	Octyl Sepharose	100 mM Tris–SO_4, pH 7.8 + 1 mM EDTA + 1.5% DCHO + 20% $(NH_4)_2SO_4$ + 0.1 M KCl	1. 100 mM Tris–SO_4, pH 7.8, 1 mM EDTA, 5% DCHO, 5% $(NH_4)_2SO_4$ 2. 100 mM Tris–SO_4, 1 mM EDTA, 0.1 M KCl, 0.25% Tween 80 3. 50 mM Tris–SO_4, 1 mM EDTA, 1% Triton	108
Peroxidase	Tomato	Phenyl Sepharose	Phosphate, pH 6.0, 2 M $(NH_4)_2SO_4$	1. Reducing gradient of $(NH_4)_2SO_4$ (step) 2 M, 1.5 M, 1 M, 0.5 M 2. Phosphate, pH 6.0 + 50% ethanediol	109

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Table 2 *(cont.)*

Protein	*Source*	*Ligand and support*	*Loading buffer**	*Elution buffer**	*Ref.*
Hexosaminidase B	Human liver	Phenyl Sepharose	0.1 M Na-citrate, 0.2 M NaP, pH 4.4	1. Load buffer 2. Demineralized water 3. 0.5% Triton X100	110
		Valine Sepharose	0.9 M KH_2PO_4–KOH, pH 7.5	Decreasing gradient of load buffer	
Lysophospholipase	Rat intestine	Phenyl Sepharose	0.1 M phosphate, pH 7.0	1. 40% ethanediol 2. 50% ethanediol	111
Polyphenoloxidase	Grapes	Phenyl Sepharose	50 mM KH_2PO_4, pH 6.5 + 1 M KCl + 1 M $(NH_4)_2SO_4$	Batch elution of decreasing strength. 50% ethanediol. Demineralized water	112
Cytochrome P450	Rabbit	Octyl and phenyl Sepharose	200 mM phosphate + 0.1 mM DTT + 0.1 mM EDTA + 20% glycerol	100 mM load buffer + 0.4% Na-cholate, followed by Emulgen 911	113
Cyclic nucleotide phosphodiesterase	—	Octyl Sepharose	10 mM Tris–HCl, pH 7.2 + 1 mM EDTA + 1 mM DTE + 25% saturation $(NH_4)_2SO_4$	1. Reducing gradient $(NH_4)_2SO_4$ 20—0% 2. Increasing ethanediol gradient 0—40% 3. 1% Triton X-100	114

Serum proteins	Human	Phenyl Sepharose	35 mM KP buffer, pH 6.5 + 0.8 M $(NH_4)_2SO_4$	1. Decreasing gradient $(NH_4)_2SO_4$ 0.8—0 M 2. Increasing gradient ethanediol 0—8 M	115
Venom cardiotoxins	Elapids	Phenyl Sepharose	2 M $(NH_4)_2SO_4$	Reducing gradient $(NH_4)_2SO_4$ 2 M—20mM	116
Polyphenyl oxidase	Peaches	Phenyl Sepharose	0.05 M KP, pH 6.2 + 1 M KCl + 1 M $(NH_4)_2SO_4$	5 mM KP, pH 6.2, followed by 50% ethanediol	117
Colony stimulating factor (CSF)	Human urine	Propylamine Sepharose	0.03 M phosphate, pH 7.0	1 M NaCl	118
Peroxidase	Tomato	Phenyl Sepharose	0.05 M phosphate, pH 6.0 + 2 M $(NH_4)_2SO_4$	Reducing gradient $(NH_4)_2SO_4$ (step) 2 M, 1.5 M, 1 M, 0.5 M, followed by 0.05 M phosphate, pH 6.0, followed by ethanediol	119

*KP = Potassium phosphate; NaP = sodium phosphate; MSH = 2-mercaptoethanol; DMSO = dimethyl sulphoxide; DCHO = Deoxycholate; DTT = Dithiothreitol; DTE = Dithioerythreitol.

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Table 3 *Proteins purified by immunoaffinity chromatography*

Protein	*Source*	*Eluant/Comment*	*Ref.*
Phosphoenol-pyruvate carboxylase	Sorghum leaves	10 mM sodium phosphate buffer, pH 7.0 followed by water	121
Angiotensin-I converting enzyme	Baboon lung	2 M $MgCl_2$, pH 5.8	127
Anti-*Pseudomonas* IgG	Serum and lung leakage fluid	0.5 M glycine-HCl buffer, pH 2.4	128
Anti-albumin antibodies	Rabbit serum	Various elution buffers	120
Complement C5	Human serum	2 M potassium bromide using goat anti-human C5	129
Complememt C1q	Human plasma	1 M NaCl	130
Neurofilament protein	Bovine brain	5 M urea, pH 6.0 and 2.5, using rabbit anti-chicken IgG	124
Mouse F_c receptors	Mouse macrophage cell line J774	Phosphate-buffered saline containing 0.5% deoxycholate and 0.1 M triethylamine, pH 11.5	123
NADP-specific glutamate dehydrogenase (GDH)	*Chlorella sorokiniana* cell homogenate	Isolation of ^{35}S-labelled protein using rabbit anti-GDH IgG	131
Alpha-foeto-protein (AFP)	Mouse amniotic fluid	Use of anti-AFP IgG entrapped in polyacrylamide gel matrix	132
Terminal deoxy-nucleotidyl tranferase	Calf thymus and human leukaemic cells	—	133
Phospholipase A_2	Venom of *Vipera ammodytes*	5 mM HCl plus 1 M NaCl	134
2′,3′-Cyclic nucleotide 3′-phospho-hydrolase	Bovine brain	—	135
Kallikreins	Rat sub-mandibular gland and human plasma	Guanidine hydrochloride containing 0.5% bovine serum albumin	136, 137
Thyrotropin and α-subunits of chorionic gonadotropin	Human serum	Linear gradient 0.1—4.0 M guanidine HCl, pH 3.2	137*a*

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propyl Sepharose 6B has been used for the rapid purification of thiol-protein disulphide oxidoreductases from bovine liver. Two enzymes, a protein disulphide isomerase and glutathione–insulin transhydrogenase, were separated by eluting the column with L-cysteine followed by dithiothreitol.[141] A novel method has been described for the specific isolation of thiol-containing peptides or proteins. The peptide or protein is allowed to react with a maleimido-group attached to agarose by a cleavable phenyl ester linkage. The peptide and protein can be recovered as the *S*-succinyl cysteine derivative following treatment with 1M hydroxylamine at pH 7.0 for 10 min. This matrix has been shown to react with thiol groups in glutathione, bovine serum mercaptalbumin, and haemoglobin and rabbit muscle and yeast glyceraldehyde-3-phosphate dehydrogenase.[142] Derivatives of MPE agarose should prove useful in affinity chromatography and immunoadsorption where it is difficult to elute material bound to covalent affinity supports.

Metal Chelate Chromatography.—The presence of some amino-acid side chains, particularly cysteine and histidine, in proteins can result in an affinity for metal chelates.[143] This affinity may be exploited for purification and may also serve as a probe into the surface topography of protein molecules such as interferon.[144] To form metal chelate matrices, epoxy-activated agarose can be treated with iminodiacetic acid, with the formation of a bis-carboxymethylamino-agarose, which has a high affinity for divalent metal ions. Due to the pH dependent formation of transition-metal ion–protein complexes, particularly those of zinc and copper, pre-saturation of derivatized agarose with zinc ions to form a zinc chelate complex has been successfully used to purify inter-α-trypsin inhibitor from human serum.[145] Both copper and zinc chelate gels have been used in combination for the purification of rat liver nucleosidediphosphatase.[146] Copper chelate chromatography has been used in purification procedures for human leucocyte interferon[147] and embryo hamster cell interferon.[148]

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Phase Partition and Partition Chromatography.—Phase partition, although not a widely used technique, has been used for the rapid separation of rat α-foetoprotein from serum albumin, two proteins of very similar physicochemical properties.[149] Partition chromatography is a technique that is more suited to the purification of small peptides rather than proteins, although preliminary experiments have shown that the technique is applicable to some hydrophobic proteins, such as β-liprotropin (91 residues) and human growth hormone (191 residues).[150]

3 Isolation of Specific Classes of Protein

Membrane Proteins.—Triton X-100 and sodium cholate continue to be the most frequently used detergents for the solubilization of membrane proteins. The majority of membrane protein purifications involved an affinity chromatography step. Notable exceptions were the description of a new method for the purification of pyruvate and 2-oxoglutarate dehydrogenases from ox heart that did not require the initial preparation of isolated mitochondria. The enzymes were solubilized using Triton X-100 and purified by fractionation with poly(ethylene glycol) followed by gel chromatography on Sepharose CL-2B.[151] A protein kinase from human erythrocytes was purified to homogeneity by a two-step procedure involving two successive chromatographies on Sephadex G-200 under conditions of low and high salt concentration, which caused aggregation and disaggregation respectively of the enzyme.[152] Table 4 lists preparations of membrane proteins described during 1980.

Table 4 *Purification of membrane proteins*

Protein	*Source*	*Ligand*	*Eluant*	*Ref.*
Bacterial D-glucose dehydrogenase	*Pseudomonas* sp.	Phenyl	Triton X-100 gradient	153
Penicillin-binding proteins 1a, 1b, and 3	*E. coli*	Ampicillin or cephalexin	1 M NH_2OH, pH 8.7	138
Penicillin-binding proteins 5 and 6	*E. coli*	6-Aminopenicillanic acid	0.8 M NH_2OH, pH 7.0	140
Penicillin-binding proteins 1, 3, 4, and 5	*Bacillus megaterium*	Ampicillin	1 M NH_2OH, pH 8.8	139
Cytochrome P450	Rabbit liver and lung	Octyl	Emulgen 911	154

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Table 4 *(cont.)*

Protein	*Source*	*Ligand*	*Eluant*	*Ref.*
Cytochrome P450	Rabbit liver	ω-Amino-n-octyl	Emulgen 913	155
Cytochrome P450	Rabbit liver	Cytochrome b_5	0.5 M KCl	156
Retinol dehydrogenase	Bovine rods	Retinal	0.7 M KCl	157
Nucleotide pyrophosphatase	Rat liver	2-(6-Aminohexyl)-amino-5′-AMP	0.25% Tris–Sarkosyl	158
Dipeptidyl peptidase IV	Rat liver	Wheat germ agglutinin	0.5 M *N*-acetyl glucosamine	158
Translation protein	Dog pancreas	Amino-pentyl	0.05% Nikkol detergent	159
$Na^+ + K^+$ activated ATPase	Brine shrimp	Membranes isolated by sucrose density gradient centrifugation and enzyme purified by treatment with Lubrol WX and SDS in the presence of ATP		160
2′,3′-Cyclic nucleotide 3′-phosphodiesterase	Bovine brain	8-(6-Aminohexyl)-amino-2′-AMP	1 mM 2′-AMP	161
Androgen acceptor	Rat prostate	Prostatic cell DNA	0.6 M NaCl	162
Insulin receptor	Rat adipocytes	Protein A used to bind insulin anti-insulin–receptor complex	3 M KSCN	163
Insulin	Human placenta	Wheat germ lectin and receptor antibody	0.3 M *N*-acetyl glucosamine, 2.5 M $MgCl_2$	164
Acetylcholine receptor	*Torpedo californica*	*Naja naja siamensis* toxin III followed by concanavalin A	2 mM benzo-quinonium chloride	165
Acetylcholin-esterase	Rat brain	Concanavalin A	α-Methyl-D-mannoside	166

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Table 4 *(cont.)*

F_c-Receptors	Human B lymphocytes	Heat aggregated human IgG	6 M urea. Pre-column of Protein A Sepharose used to remove intrinsic IgG	167
Protein kinase	Human erythrocytes	Enzyme purified 190 × by two successive gel chromatographies on G100 Sephadex in low salt and high salt buffers		152
2-Oxoacid dehydrogenases (pyruvate and 2-oxoglutarate dehydrogenases)	Bovine heart	Solubilize with Triton X-100. Enzyme purified by fractionation with polyethyleneglycol and gel chromatography		151

Plasma Proteins.—Plasma has continued to be a rich source of proteins for study, and examples of the application of nearly all the isolation methods reviewed in this chapter can be found within the literature. The challenge of an increasing number of affinity chromatography procedures for the separation of specific plasma proteins has given an added impetus to the more traditional protein isolation methods and this in turn has led to the further characterization of plasma proteins, often available in homogeneous form for the first time. Characterization still mainly relies on SDS-PAGE and suitable biological assays, *e.g.* haemolytic or coagulation activity.

This section highlights some of the more active areas of plasma protein research and summarizes, with particular reference to affinity techniques, isolation procedures for a wide range of plasma proteins (Table 5).

Proteins Involved with Coagulation and Fibrinolysis. Proteins involved with the formation and dispersal of clots have continued to be of major interest. The purification procedures shown in Table 5 may soon extend beyond isolation from plasma, as a number of these proteins are major candidates for gene cloning strategies aimed at production in *E. coli* and other suitable microbial hosts.

Factor V has been purified from human plasma as a stable, single chain molecule with an apparent molecular weight of 330 000.[168] Activation with thrombin led to a 10- to 15-fold increase in activity with formation of two closely spaced doublets, one at 72 000 and one at 110 000 by SDS-PAGE. However, the inclusion of 2 mM di-isopropylfluorophosphate (DFP) resulted in a high molecular weight factor V (800 000—1 000 000) from both human and bovine blood.[169] Failure to include DFP led to recovery of a lower molecular weight factor V (500 000). Factor VII has been purified 100 000-fold with a yield of 30% from fresh or frozen human plasma, as a single chain polypeptide with a molecular weight of 48 000 by SDS-PAGE.[170] It is converted into a two chain form (factor VIIa) by the action of factor XII alone and factor Xa in the presence of phospholipids and

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Ca^{2+}. This conversion led to a 20- to 25-fold increase in coagulation assay activity. In the absence of heparin neither factor VII nor VIIa was inhibited by antithrombin III. Factor VII was shown to have an NH_2-terminal sequence Ala-Asn-Ala-Phe-Leu-(Gla)-(Gla)-Leu-(Arg)-Pro.

Affinity chromatography of factor VIII with insolubilized haemophilic antibody[171] showed that of 45—81% factor VIII:C the low molecular weight component of factor VIII and 0—33% of factor VIII were attached to the column. Cruikshank and Rock[172] visualized factor VIII:C as a 140 000 mol. wt. band on polyacrylamide gels when purified in the presence of a protease inhibitor (1 mM benzamidine). Following reduction of VIII:C with 2-mercaptoethanol a single band of mol. wt. 48 000 was detected. Peptide map analysis of plasma and platelet factor VIII[173] showed a high degree of correlation, but seven peptide fragments in plasma factor VIII were not detected in platelet factor VIII. Martin *et al.*[174] isolated a unique tryptic fragment of factor VIII-von Willebrand protein of mol. wt. 116 000 with ristocetin cofactor activity. Vehar and Davie[175] have purified bovine plasma factor VIII approximately 300 000-fold and detected a triplet on SDS-PAGE with apparent mol. wts. 93 000, 88 000, and 85 000. The purified factor VIII contained no platelet-aggregating activity. It was required for activation of factor X in the presence of factor IX, calcium, and phospholipid and was activated 30-fold by thrombin or factor Xa plus calcium and phospholipid, accompanied by a change in SDS-PAGE protein pattern.

The synthesis and use of Sephadex, sulphated by anhydrous reaction with chlorosulphonic acid, have been described for separation of factors II, IX, and X.[176] Recoveries of 70, 40, and 50%, respectively, of these proteins were obtained, homogeneous by SDS-PAGE, and 99% free of contaminating coagulation activities. Sulphated dextran compared favourably with heparin-Sepharose. Butyl-Sepharose has also been used to separate human blood clotting factors from fibrinogen and other plasma proteins.[177]

An improved method for the isolation of human fibrinogen by differential polyethylene glycol precipitation[178] yielded protein which was 95% clottable and contained no detectable prothrombin, thrombin, plasminogen, or plasmin. Two types of fibrinogen with differences in the γ-chain have been separated by DEAE-cellulose gradient elution chromatography,[179] and the mechanism of fibrinogen–fibrin conversion in solution was discussed.[180] A rapid and simple method has also been developed for isolating soluble fibrin complexes from fibrinogen by treatment with thrombin.[181]

Antithrombin III has been purified from rat plasma in 70% yield by a procedure including heparin-Sepharose 4B.[182] The preparation was homogeneous by SDS-

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PAGE and analytical ultracentrifugation, and was composed of a single polypeptide chain of 64 000 mol. wt. Antithrombin III is a glycoprotein containing 3.6% glucosamine, 0.2% fucose, 2.5% mannose, 1.6% galactose, and 3.9% sialic acid. Isoelectric focusing revealed four bands in the range pH 4.7—4.9. Human plasminogen activator has been partially purified using fibrin-Sepharose and hydrophobic chromatography on phenyl-Sepharose.[183] The plasminogen activator described had an apparent mol. wt. of about 60 000, which on reduction yielded two bands of 30 000 and 31 000 mol. wt. Isoelectric focusing of the activator yielded four major bands at pH 6.9, 7.4, 8.0, and 8.6. Human a_2-antiplasmin has been 80—90% purified in 50% yield by absorbtion on a high-affinity lysine binding site matrix (LBS1-Sepharose) followed by elution with 6-aminohexanoic acid.[184] The major impurity is fibrinogen, which can be readily removed by gel filtration. Lijnen *et al.*[185] also describe the isolation of a_2-antiplasmin and another protein using LBS1-Sepharose. The second protein was further purified by ion-exchange chromatography on CM-cellulose followed by chromatography on LBS1-Sepharose to yield 40 mg l^{-1} of plasma. The protein interacted with the high-affinity lysine binding site of plasmin with an apparent dissociation constant of 0.9 μM, resulting in a marked reduction in the reaction rate between plasmin and a_2-antiplasmin. The purified protein was a single-chain glycoprotein of 60 000 mol. wt. with an NH_2-terminal sequence Val-Ser-Pro-. It is apparently identical to a previously described plasma protein of unknown biological activity called histidine-rich glycoprotein.[186] An apparently new, fast-acting plasmin inhibitor has been isolated from human platelets.[187]

Plasma Fibronectin. The activities of various types of a glycoprotein, fibronectin, have been of increasing interest, particularly the comparison of fibronectin isolated from plasma (cold insoluble globulin, Cig) and from fibroblasts. Although they share the ability to mediate cell adhesion, there are some fundamental differences in the preparations. Pena *et al.*[188] isolated fibronectin from hamster plasma by affinity chromatography on gelatin coupled to Sepharose beads. Fibronectin was eluted with 8 M urea and SDS-PAGE showed two prominent polypeptide sub-units of mol. wt. 215 000 and 200 000. Variable amounts of lower molecular weight material in preparations of hamster and bovine fibronectin appear to be artefacts generated during isolation and storage. Antibodies raised against the major sub-units of hamster plasma fibronectin were coupled to Sepharose and used to isolate fibronectin extracted with urea from baby-hamster kidney cells. Cell fibronectin from this source was similar to plasma fibronectin in amino-acid and carbohydrate composition and produced very close peptide maps. A similar conclusion was reached after comparison of human fibronectin from amniotic fluid and plasma[189, 190] and fibronectin from bovine aortic endothelial

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cells and bovine plasma.[191] In both cases, however, some differences have been observed in polypeptide sizes after reduction. Bovine cell fibronectin (mol. wt. 440 000) yields a single band at 220 000 mol. wt., whereas bovine plasma fibronectin yields two bands, at 220 000 and 215 000.[191] A similar result was obtained for human plasma fibronectin.[192]

Klebe *et al.*[193] have also used gelatin-Sepharose for bovine plasma factionation and describe the use of lithium di-iodosalicyclic acid as a more effective chaotropic agent for eluting fibronectin from collagen. Hayashi *et al.*[194] have identified a heparin-binding site on chick cellular fibronectin following pronase digestion of material purified on heparin-Sepharose.

Complement and Associated Proteins. The C1q sub-fraction of complement protein C1 from several animal sources has been isolated and studied and considerable homology found.[195–198] All the proteins have molecular weights in the region of 400 000 and under non-reducing conditions SDS-PAGE yields sub-units around 50 000 and 45 000. Upon reduction, bands between 20 000 and 26 000 appear giving strong evidence of a covalently and non-covalently bound sub-unit structure.

An improved three-step method for the purification of C3[199] uses isoelectrofocusing in dextran gel with a 20% yield of protein homogeneous by SDS-PAGE and immunochemical criteria. Kunkel *et al.*[87] isolated C3 and C5 from serum absorbed on a hydrophobic resin, ω-aminohexyl-agarose. The C3 and C5 can be eluted with 0.2 M NaCl at pH 7.5 and if required C2, C4, C8, and C9 eluted with 0.7 M NaCl at pH 7.5. The C3 and C5 components were further purified using hydroxyapatite and salt-mediated hydrophobic chromatography to 34% and 46% yield respectively. Both gave bands at 115 000 and 75 000 mol. wt. on SDS-PAGE after reduction. This result was very similar to porcine C3[200] (116 000 and 74 000), again showing the high degree of homology among these proteins. The preparation of crystalline human C3a anaphylatoxin has also been described.[201]

C4 has been purified 157-fold with respect to haemolytic activity with 3% yield.[202] The protein was homogeneous, mol. wt. 200 000 on SDS-PAGE, and on reduction and alkylation yielded bands at 93 000, 75 000, and 33 000 plus a small amount of residual 200 000, which might represent a single-chain precursor polypeptide. C5 was also purified 570- to 710-fold by chromatography on monospecific goat anti-human C5 linked to Sepharose.[203] Conditions were critical to obtain yields of 20—50% C5 protein, which could be eluted from the affinity matrix with 2 M KBr following a 1 M NaCl wash.

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C8 has been isolated from Cohn fraction III in high yield.[204] The protein of mol. wt. 151 000 was shown to consist of α (64 000), β (64 000), and γ (22 000) chains. The α and γ chains were linked covalently with non-covalent attachment of the β chain. Neither the α and γ chains nor the β chain showed haemolytic activity on their own. C9 was shown to be a glycoprotein with a single polypeptide chain of mol. wt. 71 000.[205] A high titre rabbit antiserum to C9 could be used to deplete C9 from the serum, which was measured at $58 \pm 8\,\mu g\,ml^{-1}$ in human serum.

Activation of the classical complement pathway leads to the formation of the bimolecular complex C4b2a, which cleaves C3. This complex is intrinsically unstable owing to the decay of the catalytic site bearing protein C2a to yield C2i, which has no capacity to recombine. Burge *et al.*[206] report the isolation from guinea-pig plasma of a protein which acts as a C4b2a decay accelerating factor. The protein shows a single band on SDS-PAGE of mol. wt. 550 000 and after reduction and alkylation yields a single band of mol. wt. 72 000. The protein seems to represent an equivalent to the human C4 binding protein.

Regulation of C3b and C4b following proteolytic cleavage of C3 and C4 is achieved by C3b inactivator (C3b INA). Kai *et al.*[207] have isolated mouse C3b INA, which in solution cleaves the α' chain of mouse C4b, in the presence of mouse or human C4 binding protein, into three fragments. It also cleaves the α' chain of human C3b into two fragments in the presence of human β 1H. It would seem that mouse C3b/C4b inactivators are the same and that inactivators from different animals are very similar. C3b INA has also been isolated in high yield from human plasma[208] and further information on its properties and the mechanism of C3b cleavage obtained. A cofactor of C3b INA has been isolated from human plasma.[209] The protein has a mol. wt. of 450 000 and on reduction yields a single band of 75 000 on SDS-PAGE (hexamer). It is a glycoprotein and appears to have a similar function to C4 binding protein.

Harrison and Lachman[210] describe two previously uncharacterized proteolytic cleavages of human C3. In the first the intact a-chain is split in a C3b/C4b- and β 1H-dependent fashion to yield products of mol. wt. 78 000 and 43 000. In the second an unidentified proteinase yields a new a-chain of mol. wt. 107 000. Spitzer *et al.*[211] isolated a C3/C3b regulatory protein in human serum, which at normal concentrations fixes to cell bound C3b and prevents inactivation by C3b INA and β 1H. At high concentrations it is also capable of blocking the inactivation of C3 by both classical and alternative pathways. One of the major DNA binding proteins in plasma (DBP-1) was shown to be identical in amino-acid composition and molecular weight to β 1H of the alternative complement pathway.[212]

Other Plasma Proteins. Plasma lipoproteins have received considerable attention and a review of their structure and function, with particular regard to hyper-

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lipoproteinaemias and atherosclerosis, has appeared.[213] Two volumes of the CRC series 'Handbook of Electrophoresis' deal with the principles and concepts of lipoproteins and their role in disease.[214] Solution properties of plasma apolipoprotein have been described [215] and a comparison of high density lipoprotein and its major apoprotein from human, canine, bovine, and chicken plasma made.[216] It is not possible in the limits of this volume to discuss in detail other plasma proteins described in Table 5. The emphasis has been placed on purifications based on affinity chromatography or related techniques. On this basis Table 5 also includes some erythrocyte, leucocyte, and platelet proteins.

Heparin-Sepharose is a frequently used matrix and its application has been the subject of a review.[217] There is an increasing number of purifications based on dye-affinity chromatography [35] for proteins as diverse as human leucocyte interferon,[218] α-foetoprotein,[219] and human serum albumin.[220] However, affinity chromatography should not be looked at in isolation from other purification techniques. Hydrophobic chromatography can be used for a wide range of serum proteins [116] and the application of chromatographic methods in general to plasma fractionation has been reviewed.[221]

4 Protein Characterization

Protein Determination.—The dye-binding assay for protein based on Coomassie Brilliant Blue G-250 has found increasing use, and comparisons with the biuret and Lowry methods have been made.[249] The effects of pH and inorganic ions [250] and metrizamide [251] on the assay have been studied. Only extremely basic pH affected the assay significantly. Slight interference by ferrous and ammonium ions and metrizamide can be corrected. There has been a re-examination of the fluorescamine reaction for primary amines for use in a micromethod for protein determination.[252]

A dye-binding assay for protein solubilized in the presence or absence of SDS has been described [253] using Serva Blue G and Xylene Brilliant Cyanin G as alternatives to Coomassie Blue. Some of the problems associated with these dyes have been overcome.

The Lowry and modified biuret method have been compared for use in an automated and quantitative assay system for serum proteins along with a third method based on hydrolysis of the protein and measurement of the proline content.[254] Whitaker and Granum [255] have proposed the use of a method based on difference in u.v. absorbance at 235 and 280 nm. The method is less sensitive than measurement at 235 nm alone, but has certain advantages for use in the food industry. The proposed method has a sensitivity of 45% compared with the Lowry method.

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Table 5 *Purification of plasma proteins*

Protein	*Source*	*Major procedures*				*Ref.*
		Affinity chromatography	*Ion exchange*	*Gel filtration*	*Other*	
Proteins involved with coagulation and fibrinolysis						
Factor V	Human		DEAE-Sepharose	Ultrogel AcA22		168
Factor VII	Human		QAE-Sepharose	Sephadex G-100		170
Factor VIII	Human	Immobilized factor VIII antibody				171
Factor VIII	Bovine	Factor X-Sepharose	DEAE-Sephadex	Sephadex G-200	Sulphated-Sepharose	175
Factor IX	Human		DEAE-Sephadex		Sulphated-Sepharose	176
Factor X	Human		DEAE-Sephadex		Sulphated-Sepharose	176
Factor II (prothrombin)	Human		DEAE-Sephadex		Sulphated-Sepharose	176
Fibrinogen	Human				Polyethylene glycol pptn.	178
Antithrombin III	Rat	Heparin-Sepharose	DEAE-cellulose			182
Plasminogen activator	Human	Fibrin-Sepharose			Phenyl-Sepharose	183
Plasminogen activator	Human	Glass absorbed Kallikrein	QAE-Sephadex	Sephadex G-25		222
a_2-Antiplasmin	Human	LBS1-Sepharose				184
'New' plasmin inhibitor	Human	Plasminogen-Sepharose				187
Fibronectin						
Fibronectin	Hamster	Gelatin-Sepharose				188
Fibronectin	Human	Gelatin-Sepharose	DEAE-cellulose			190
Fibronectin	Bovine	Gelatin-Sepharose				193

Complement and associated proteins						
Clq	Human	Rabbit IgG-Sepharose Rabbit antihuman IgG-Sepharose			Euglobulin pptn.	195
C1q	Mouse	Human IgG-Latex beads			Exponential-gradient SDS gels	196
C1q	Rabbit	IgG-Sepharose	CM-cellulose			197
C1q	Bovine		DEAE-Sephadex, CM-cellulose	Sepharose 6B	EGTA pptn.	198
C3	Human		DEAE-cellulose		PEG pptn. isofocusing in dextran gel	199
C3	Human			Biogel P300	Aminohexyl-agarose, hydroxyapatite	88
C4	Guinea-pig	Lysine-Sepharose	DEAE-Sephacel, SP-Sephadex	Sepharose CL6B	PEG pptn.	202
C5	Human				Aminohexyl-agarose, hydroxyapatite	88
C5	Human	Goat anti-C5-Sepharose	QAE-Sephadex			203
C6	Human	Immobilized rabbit anti-C6 antibodies				223
C8	Human		CM-Sephadex, QAE-Sephadex	Sephacryl S200		204
C9	Human	Lysine-Sepharose	DEAE-Sephadex		Hydroxyapatite	205
C4 binding protein	Guinea-pig	Heparin-Sepharose, $C4^{gp}$-Sepharose	DEAE-cellulose	Sepharose 6B	Polyethylene glycol pptn.	206

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Table 5 *(cont.)*

Protein	*Source*	*Major procedures*				*Ref.*
		Affinity chromatography	*Ion exchange*	*Gel filtration*	*Other*	
C3b/C4b inactivator	Mouse		DEAE-Sephacel	Sephadex G-200, BioRex 70		207
C3b inactivator	Human	Wheat-germ agglutinin-Sepharose	QAE-Sephadex	Sephacryl S200	Hydroxyapatite	208
C3b inactivator-cofactor	Human	Gelatin-Sepharose, heparin-Sepharose, anti-IgM-Sepharose		Biogel A15m		209
Other plasma proteins						
Lipoproteins	Rat	Heparin-Sepharose				224
Lipoproteins	Human	Heparin-Sepharose				225
Lipoproteins	Human	Heparin-Sepharose				226
Lipoprotein 'transfer' proteins	Human	Concanavalin A-Sepharose	CM-cellulose		Phenyl-Sepharose	227
Lipoprotein 'transfer' proteins	Rabbit	Concanavalin A-Sepharose	DEAE-Sephadex	Sephadex G-200	Phenyl-Sepharose	228
Lipoprotein lipase	Human	Heparin-Sepharose		Biogel A5m		229
Pre-Kallikrein	Guinea-pig		DEAE-Sephadex, CM-Sephadex	Sephadex G-150		230
Pre-Kallikrein	Rabbit	Lectin chromatography				231
Kallikrein	Human	Soya bean trypsin inhibitor-Sepharose		Ultrogel ACA44		232
Kallikrein	Human	Immunoaffinity chromatography				137

Kallikrein	Human (colon)	Trasylol-Sepharose		Sephacryl S200		233
Kallikrein	Rat (stomach)	*p*-Aminobenzamidine-Sepharose	DEAE-Sephadex	Sephadex G-150		234
Angiotensin I-converting enzyme	Human	Angiotensin antibody-Sepharose		Sephadex G-200	Hydroxyapatite	127
Galactosyltransferase	Human	α-Lactalbumin-Sepharose				235
Lecithin-cholesterol acyltransferase	Human	HDL-Sepharose, Wheat-germ agglutinin-Sepharose	DEAE-Sephacel			236
α-Foetoprotein	Human	Affi-Gel Blue anti-albumin-Sepharose	DEAE-cellulose	Sephacryl S200		219
Enkephalin-degrading enzyme	Human	Sepharose-Blue Dextran	DEAE-Sephadex	Sephadex G-100		237
Lipid-binding protein	Human	Heparin-Sepharose				238

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Table 5 *(cont.)*

Protein	*Source*	*Major procedures*				*Ref.*
		Affinity chromatography	*Ion exchange*	*Gel filtration*	*Other*	
Sex steroid-binding protein	Human	5-Dihydrotestosterone-agarose	DEAE-cellulose			239
IgM	Human	Immobilized Protein A				240
Serum albumin	Human	Cholic acid-amino-hexylamino-Sepharose				241
Serum albumin	Human	Haematin and haematoporphyrin-agarose				242
Inter-α-trypsin inhibitor	Human	Zinc chelate-Sepharose	DEAE-Sephacel	Sephacryl S300	Phenyl-Sepharose	89
Endoglycosidase	Human platelet	Heparin-Sepharose				243
Platelet basic protein	Human platelet	Heparin-Sepharose				244

Fibrinolytic proteases	Human leukocyte	Fibrinogen-Sepharose, elastin-Sepharose, phenylbutylamine-Sepharose		245
Myeloperoxidase	Human leukocyte	Concanavalin A-Sepharose	Sephadex G-200	246
Insulin-degrading enzyme	Human erythrocyte	Organomercuri-Sepharose		247
Hexokinase	Rabbit	Sepharose-*N*-amino-hexanosylglucos-amine	DEAE Sephadex	248

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Molecular Weight Determination.—A new range of matrices for 'high-speed' gel filtration of proteins has been described by Kato *et al.*[256-259] Designated TSK-GEL types SW and PW, they may be used in the presence of sodium dodecyl sulphate (SDS) and denaturing agents such as 6 M guanidine hydrochloride. Separation ranges are in general 5000 to 7 000 000 daltons for globular proteins, 1000 to 500 000 daltons for dextrans, and from 500 to 250 000 daltons for polyethyleneglycols. For proteins, the highest separation efficiency for three SW type gels described was for mol. wts. $<30\,000$, 30 000—500 000, and $>500\,000$ daltons. In the presence of SDS the separation ranges were 15 000—25 000, 10 000—100 000, and 15 000—300 000 daltons. A combination of TSK-GEL columns has been used for the separation of serum lipoproteins from normal and pathological subjects.[260] Rumeloitis and Unger[261] also describe a 'high-performance size-exclusion' chromatography on Lichrosorb Diol for proteins in the mol. wt. range 10 000—100 000 daltons.

Two electrophoretic methods for molecular weight determination have been described; one is an SDS-pore gradient system suitable for multicomponent protein systems, and permits estimation in the range 1000—1 000 000 daltons.[262] The other uses linear polyacrylamide gradient gels and is suitable for molecular weight determination of native proteins.[263]

Some caution on the use of gel chromatography using Sepharose and Sephacryl matrices for molecular weight determination is advised.[264] Non-linearity in plots of Stokes radius *versus* $\mathrm{erf}^{-1}(1-K_D)$ was observed, probably reflecting the presence of two classes of pore sizes of the gel.

Electrophoretic Techniques.—Electrophoretic techniques are amongst the most important methods available for the characterization of proteins. Perhaps as a reflection of this, 1980 saw the emergence of a new international journal, *Electrophoresis*, which is devoted to the publication of reviews and research reports covering advances in electrophoretic techniques.

A significant polyacrylate contaminant to otherwise pure proteins introduced during preparative polyacrylamide gel electrophoresis was removed by ion-exchange chromatography.[265] A simple preparative apparatus has been designed that combines the beneficial features of high-resolution electrophoresis and gel filtration methods in sequential steps.[266] The procedure has been used to isolate

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two structurally similar subunit types of ferritin. High-voltage paper electrophoresis in borate has been used for the resolution of seven glycopeptides from ovalbumin and seven glycopeptides from human IgG.[267] The kinetics of unfolding and refolding of several proteins have been examined by rapid electrophoresis in polyacrylamide gels containing a linear gradient of urea perpendicular to the direction of migration.[268] The preparation of natural pH gradients on polyacrylamide gels using either two (glutamic acid and lysine) or three (glutamic acid, histidine, and lysine) amino-acids as the sole carrier ampholyte species has been described.[269]

A slab gel system containing phenol–acetic acid–urea (PAU) was successfully used for the solubilization and separation of the hydrophobic protein composing the cell vacuoles of a variety of prokaryotic micro-organisms.[270] Gels produced using the PAU system can be successfully fluorographed.

Lithium dodecyl sulphate-polyacrylamide gel electrophoresis at 4 °C provided a convenient and rapid procedure for detection and isolation of pigment–protein complexes present in the chromatophase membrane of *Rhodopseudomonas sphaeroides*.[271] Enzymes such as amylases, dehydrogenases, and proteases were renatured after SDS-polyacrylamide electrophoresis by incubation of the gels in a solution containing substrate.[272] This technique allowed the identification of these enzymes following SDS-PAGE.

The dependence of electrophoretic mobility on agarose concentration was measured by embedding several agarose gels (running gels), each of a different agarose concentration, within a single 1.5% agarose slab.[273] Multiple forms of glutathione S-transferase in rat and human liver were separated by electrophoresis in starch gel and localized on the gel surface by a specific stain, 1-chloro-2,4-dinitrobenzene, in the presence of reduced glutathione as substrate.[274]

Polyacrylamide gels cross-linked by disulphide bonds have been prepared over a concentration range of 3.5—12.5% polyacrylamide.[275] These can be solubilized by thiols, yielding high molecular weight polymers. The most attractive feature of these gels is that they permit a very convenient method of recovering samples from gels.

A flat-bed electrophoretic elution in Sephadex by isoelectric focusing for the removal of proteins from polyacrylamide gels following electrophoresis has been developed.[276] A narrow slice of the polyacrylamide gel, containing the protein to be eluted, is finely ground and applied to a horizontal bed of Sephadex G-200 superfine containing carrier ampholytes. The method ensures the recovery of proteins in concentrated form with a high yield. Efficient transfer of proteins and nucleic acids from either polyacrylamide or agarose slab gels to nitro-cellulose

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membrane and diazobenzyloxymethyl cellulose has been developed,[277] as an alternative method to blotting.

Isoelectrofocusing. Preparative IEF is now widely used for the purification of proteins. The method is convenient and affords high resolution, but is somewhat limited by the amount of protein, and in particular by the small volume of sample, that can be applied. Larger volume samples containing more protein have been electrofocused by a continuous-flow system.[278] A haemoglobin mixture, containing HbA2 and HbA, was separated at a sample loading of 4 g of haemoglobin per day by this method. A large column containing Ampholines in a density gradient was used to resolve sperm whale myoglobins in a time of 4 h.[279] This was achieved by a rapid preliminary focusing over the width of the column (20 cm) followed by fine-focusing over the length (112 cm). IEF in 60 cm long gels was claimed to improve resolution in multiple-component systems by providing a very shallow ($0.1\,\mathrm{pH\,cm^{-1}}$), linear pH gradient with consistently reproducible results.[280] An apparatus for the large scale fractionation of proteins in IEF was developed using a column of 46 closed compartments (total volume 7.6 l).[281] Internal stirring and cooling were effected by slow rotation of the whole apparatus in a tank of cold water.

A rapid IEF technique with very high resolution has been developed using a voltage gradient of up to $460\,\mathrm{V\,cm^{-1}}$, employing the Peltier cooling method on thin (0.25 mm) gels backed with polyester film.[282] As many as 150—200 protein bands were resolved over a distance of 5.4 cm. Although Pharmalyte carrier Ampholines from Pharmacia had better continuity and conductance than Ampholines from LKB, over the pH range 3—10, they required much higher field strengths to achieve the same degree of separation resulting in higher gel temperatures. The effect of temperature on the measured pI in Pharmalyte-containing gels has now been documented, so accurate pI determinations are now possible.[283] Improved resolution on horizontal slab gel IEF has been obtained by overlaying ampholyte-containing gel strips at specific locations on the base gel, creating areas of increased pH gradients.[284]

Ultrathin gels (0.05—0.1 mm) were prepared by the 'flap technique' in which polyacrylamide was covalently bound to glass plates or to polyester films pretreated with methacryloxypropyltrimethoxy silane.[285] IEF of proteins of high molecular weight cannot be successfully performed in polyacrylamide gels owing to sieving effects and the subsequent trailing of protein bands. This was overcome using Sephadex G-15 as the anticonvectant.[286] The main advantage of this new technique is its rapidity over the classical focusing method based on sucrose gradients.

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A convenient method for IEF of intact polymeric IgA and IgM was described employing composite gels of 1% acrylamide and 0.75% agarose.[287] Separation of proteins by IEF in gels of acrylamide–ethyl acrylate co-polymers in dimethyl sulphoxide–water mixtures at subzero temperatures was reported.[288] Details of the rate of formation and stability of the pH gradient under these conditions were given.

Segmental voltages along IEF gels made of polyacrylamide were measured without disturbing the ongoing electrofocusing by embedding platinum wires in the walls of the glass tubes at regular intervals.[289]

Isotachophoresis. ITF offers distinct advantages in resolving substances of very similar structure. The technique is sufficiently sensitive to detect picomole quantities in sample volumes of 1—10 μl. Preparative ITF has been used with Ampholine carrier ampholytes as spacers of the purification of pepsinogen 1 from human urine.[290] Many substances such as nucleotides,[291, 292] oxalates,[293] co-enzyme M (2-mercaptoethanesulphonic acid) derivatives,[294] and sulphur-containing amino-acids[295] were successfully measured by ITF.

Selective trapping of isotachophoretic zones by using a bifurcation has been developed.[296] An electronic device was introduced for automatic isotachophoretic analysis using the coupled column system, which allows high sample loads, large concentration differences between sample constituents, and the use of different electrolyte systems.

Two-dimensional Polyacrylamide Gel Electrophoresis. A detailed analysis of mammalian cell surface proteins was described using a new two-dimensional PAGE technique.[297] The first-dimension gel contained 2% acrylamide, 0.1% SDS, 0.3% Triton CF10, and 9 M urea, while the second dimension was a conventional SDS-PAGE system. Complex protein mixtures were separated by an improved high-resolution two-dimensional gel system, which allowed a three-fold increase in the number of proteins detected.[298]

A modification of the two-dimensional electrophoresis system for protein separation was introduced to include the use of flat-bed agarose IEF in the first dimension followed by horizontal SDS-electrophoresis in an exponential acrylamide gradient in the second dimension, with molecular weight standards and radiolabelled proteins as internal standards.[299]

Two-dimensional gel electrophoresis of eukaryotic ribosomal proteins under conditions in which thiol groups are not maintained in a reduced state has been

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found to cause marked changes in the migration of certain proteins, owing to the formation of intra-molecular disulphide bridges.[300]

Micro-slab gels were utilized to separate proteins in homogeneous or gradient gels, as well as by IEF or combination of these techniques. Different samples of biological material were separated by micro-slab ($3 \times 3.5 \times 0.03$ cm) gel electrophoresis to demonstrate the high resolving power of this method.[301]

Detection of Proteins in Gels. Several methods have been described for the detection of proteins in polyacrylamide gels. Formaldehyde has been used to retain certain basic and low molecular weight proteins, which are not retained in gels by standard acid fixation.[302] For visualization of polypeptides in gels a rapid and sensitive silver stain has been described.[303] The staining procedure utilizes only three solutions and allows protein patterns to be visualized in less than one hour, with a sensitivity 100-times that of the Coomassie Blue stain. A highly sensitive radioactive method makes use of [^{125}I]-*N*-succinimidyl-3-(4-hydroxyphenyl) propionate (Bolton–Hunter reagent) and requires 1000-times less protein for detection compared with Coomassie Blue.[304]

Two staining methods have been described for use with isoelectric focusing gels. The first uses Fast Green in 10% acetic acid.[305] Fast Green has the advantage of not binding to ampholytes and maximum staining is achieved in 5 min with bands visible after 3—6 h of destaining. In the second method, described for the detection of peptides, gels are exposed to iodine vapour for a short period to yield white peptide zones against a brown background. The reaction is fully reversible and can be used for small-scale preparative procedures.[305]

Nitroblue tetrazolium and phenazine methosulphate were shown to give purple formazan bands with proteins following electrophoresis on polyacrylamide.[307] Sulphydryl blockers in the incubation medium inhibited this colour development.

Trypan Blue (0.01%) in methanol (25%) and acetic acid (7%) was shown to stain proteins following separation by electrophoresis.[296] Proteins prefixed in the gel with TCA (12.5%) showed sharper resolutions and required less time for destaining. It was found that the subunits of the RNA polymerase were stained differently when they were separated by PAGE and exposed to a carbocyanine dye (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine bromide) in 50% formamide in the dark.[308] Second staining was carried out by the same dye or by Coomassie Blue to give more brilliant coloration. The channels were cut out and scanned at 600 nm and 535 nm to register the blue and red bands, respectively. Protein sulphydryl groups were stained in SDS-PAGE slab gels by 2,2′-dihydroxy-6,6′-dinaphthyl disulphide (DDD).[309] The method was reported to be sensitive enough to detect 0.25 μg of lysozyme and 1 μg of most other proteins.

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A diazo-print method was presented for reproducing most of the isoenzyme patterns obtained with electrophoresis or isoelectrofocusing on polyacrylamide slab gels.[310] Positive transparencies were produced in only a few minutes. Detection of proteolytic enzyme zones on electrophoretograms by making contact print zymograms was described.[311] The technique was claimed to be applicable to electrophoretograms prepared on a variety of support media, immunoelectrophoretograms or IEF patterns on various media. The contact print zymograms were prepared by placing unfixed, unstained electrophoretograms in contact with a thin film of casein which had diffused into a layer of agarose supported by a hydrophilic polyester film. After staining the casein film with Coomassie Blue, the proteolytic zones were detected as clear zones against a blue background.

Complete recovery of ^{3}H-labelled material within polyacrylamide gel slices was efficiently obtained by the use of Soluene-350 with Permablend III (0.55%) in toluene.[312] This particular solubilizer cocktail system eliminates almost all chemiluminescence, making it a suitable alternative to combustion.

A method for the quantitative measurement of proteins in samples separated by PAGE was described, based on the solubility in periodic acid of polyacrylamide gels crosslinked with *NN*-diallyltartardiamide (DATD).[313] Proteins were first stained with Coomassie Brilliant Blue R-250, and the quantity of the dye in the solubilized gel sections was measured spectrophotometrically at 560 nm. Colour stability was maximal at pH 3.0 and 25 °C.

Ca^{2+}-binding proteins were identified after electrophoresis on polyacrylamide gels by equilibrating the gel with ^{45}Ca either during or after electrophoresis, followed by visualization of the ^{45}Ca-binding proteins by autoradiography.[314] Videodensitometry based on a television technique has also been shown to be suitable for recording gel electrophoretic patterns.[315]

Peptide Mapping.—A number of analytical and preparative methods are used to map proteins following limited proteolytic or chemical digestion. The separation of peptides obtained by a combination of paper electrophoresis, t.l.c., ion exchange chromatography, and reversed-phase h.p.l.c. still remains as the most widely used technique.[316–319] However, recently the trend has been towards improving the resolving power and sensitivity of some of these techniques such that separation can be achieved at the nanomole [320, 321] or even picomole level [322] for subsequent sequencing and quantitative analysis.

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PART IB: Chemical Modification of Proteins *By A. J. Garman and R. A. G. Smith*

1 Introduction

A comprehensive list of protein modifications is given in the Table. Modifications for sequencing purposes, naturally occurring and enzymic modifications, simple radio-labelling, modifications of prosthetic groups, and non-covalent modifications have, in general, been excluded.

Table *Chemical modification of proteins*

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Acetate kinase	*E. coli*	*N*-ethylmaleimide	Cys	inactivation; ATP, ADP, AMP protect	1
Acetylcholine receptor	*Electrophorus electricus*	5- and 7-azidodimethisoquin, 5-azido[^{3}H]trimethisoquin		photo-affinity labelling	2, 3
Acetylcholine receptor	rat brain	various disulphide and thiol reagents	$(Cys)_2$, Cys	involved in affinity state transitions	4
Acetylcholine receptor	rat muscle	ethyl *N*-5-azido-2-nitrobenzoylamino-acetimidate derivative of α-bungaro-toxin		affinity labelling of *in situ* receptor	5
Acetylcholine receptor	bovine brain	*N*-ethylmaleimide	Cys	differential effect on agonist binding	6
Acetylcholine receptor	*Torpedo californica*	*N*-carboxy-D,L-alanine anhydride	Lys	binding and myasthenic properties dissociated	7
Acetylcholine receptor	*Torpedo californica*	bromoacetylcholine, maleimidobenzyl-trimethylammonium		affinity alkylation	8
Acetylcholine receptor	*Torpedo marmorata*	5-azido[^{3}H]trimethisoquin		66 K chain selectively labelled	9
Acetylcholine receptor	*Torpedo marmorata*	dithiothreitol, 4-(*N*-maleimido)phenyl trimethylammonium	Cys	essential and non-essential thiols	10
Acetylcholine receptor	*Torpedo marmorata*, cat muscle	bromoacetylcholine		affinity alkylation, stoicheiometry determined	11

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Acetylcholinesterase	human erythrocyte membrane	glutaraldehyde		cross-linking in vesicles	12
Acetylcholinesterase	*Electrophorus electricus*	*p*-dimethylaminobenzene diazonium fluoroborate		photo-affinity labelling by Trp energy transfer	13
Acetylcholinesterase	*Electrophorus electricus*	u.v. irradiation	Trp	inactivation, Trp fluorescence lost	14
Acetylcholinesterase	*Torpedo californica*	butane-2,3-dione, phenylglyoxal	Arg	essential Arg	15
ACTH receptor	porcine	2-nitro-4-azidophenylsulphenyl and 2-nitro-5-azidophenyl sulphenyl derivatives of ACTH		photo-affinity labelling	16
ACTH receptor	rat	[(2-nitro-5-azidophenylsulphenyl)-Trp^9]ACTH		photo-affinity labelling	17
Actin	rabbit muscle	*N*-(1-pyrene)maleimide	Cys	pulse-fluorometry study	18
Actin	rabbit muscle	*N*-iodoacetyl-*N'*-(sulpho-1-naphthyl)-ethylenediamine	Cys	fluorescent probe	19
Actin	rabbit muscle	*o*-iodosobenzoic acid	Tyr, Trp	cleavage at Tyr and Trp	20
Actin	rabbit muscle	*p*-phenylene *NN'*-bis(maleimide)	Cys	cross-linking to myosin	21
Acyl-CoA dehydrogenase	porcine liver	EDC, [^{14}C]taurine, [^{14}C]Gly(OMe)	carboxyl	inactivation	22
Adenosine transport protein	rat adipocyte	8-azido-2-[^{3}H]adenosine		labelling of intrinsic protein	23
Adenylate cyclase	bovine brain, rat liver	2',3'-dialdehyde ATP, sodium cyanoborohydride	Lys	inhibition	24
Adenylate cyclase	bovine corpus luteum	3,3'-dithiobispropionimidate	Lys	activation, stabilization	25
Adenylate cyclase	rat brain	methyl acetimidate, dimethyl suberimidate	Lys	effects on activity and regulation	26
Adenylate cyclase	rat brain	phenylglyoxal, butane-2,3-dione, cyclohexane-1,2-dione	Arg	essential Arg	27
Adenylate cyclase	rat brain	formaldehyde, glutaraldehyde	Lys	effect on regulation	28
Adenylate cyclase	various	3-chloromercuri-2-methoxypropyl urea, sodium tetrathionate	Cys	inactivation *via* GTP regulatory proteins	29
ADP, ATP carrier	bovine heart mitochondria	phenylglyoxal	Arg	inhibitor binding studies	30

β-Adrenergic receptor	rat reticulocyte	acebutolol azide		photo-affinity labelling	31
Adrenodoxin	bovine	methylmercury acetate, methylmercury thioglycollate	Cys	sulphur extrusion	32
Agglutinin	wheat germ	dithiothreitol, [^{14}C]iodoacetic acid	$(Cys)_2$	all-or-none reduction	33
Agglutinin	wheat germ	2-(1-thio-β-glucopyranosyl)-ethanoyl-L-leucyl daunorubicin, sodium periodate	Lys	properties of anti-tumour conjugate	34
Agglutinin	soybean	4′-azidoazobenzene-4-oxysuccinimide ester, *N*-[4-(*p*-azidophenylazo)-benzoyl]-3-amino-alicyl-*N*′-oxysuccinimide ester		heterobifunctional photo-cross-linker	35
Alanine aminotransferase	porcine heart	β-cyano-L-alanine		suicide inhibitor	36

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Albumin	bovine serum	ozone-oxidized alkenyl glycosides, sodium cyanoborohydride	Lys	synthesis of model glycoproteins	37
Albumin	bovine serum	*O*-alkoxypolyethyleneglycoxy *S*-carboxamidomethyldithiocarbonates	Lys	reduction of antigenicity	38
Albumin	bovine serum	(i) [^{125}I]NaI, chloramine T (ii) formaldehyde, [^{3}H]KBH_4	(i) Tyr (ii) Lys	iodinated proteins degraded more rapidly after endocytosis	39
Albumin	bovine serum	methylglyoxal	Lys, Arg	u.v. spectroscopic study	40
Albumin	bovine serum	3-imino-2-methoxyethyl 1-thioglycosides	Lys	synthesis of neo-glycoproteins	41
Albumin	bovine serum	isothiocyanatophenyl 6-phospho-α-D-mannofuranoside	Lys	lysosomal enzyme recognition model	42
Albumin	bovine serum	[^{14}C]formaldehyde, sodium cyanoborohydride, Ni^{2+}	Lys	labelling efficiency optimized	43
Albumin	bovine serum	various disulphides	Cys	thiol reactivity assay	44
Albumin	bovine, human serum	2,2′-dithiopyridine	Cys	reactivity at various pH's	45
Albumin	bovine serum	isothiocyanate activated β-(*p*-aminophenyl)ethylamine derivatives of sialyloligosaccharides		antibodies raised	46
Albumin	human serum	citraconic anhydride	Lys	changes in physical properties	47
Albumin	human serum	tetranitromethane	Tyr-41	one very reactive Tyr	48
Albumin	rabbit serum	glutaraldehyde	Lys	neo-antigens from pronase digest	49
Albumin	various	*N*-bromosuccinimide	Trp	fluorescent quenching studied	50
Alcohol dehydrogenase	horse liver	iodoacetic acid, bromo-imidazoylpropionic acid	Cys	phosphate binding demonstrated	51
Aldolase	rabbit muscle	various α-dicarbonyl compounds	Arg	essential Arg reactive due to reduced pK_a	52

Aliphatic	*Pseudomonas aeruginosa*	chloroacetone		active-site directed inhibition	53
Alkaline phosphatase	calf intestine	(i) phenylglyoxal (ii) iodoacetamide	(i) Arg	inactivation	54
Alkaline protease inhibitor	*Streptomyces griseoincarnatus*	iodoacetic acid, pH 3.0	Met	essential Met-Ile bond	55
Amino-acid transport system	sheep erythrocytes	various thiol reagents	Cys	3 classes of essential thiols	56
Amino-acyl tRNA synthetases	*E. coli*	γ-(*p*-azidoanilide)-ATP		photo-affinity labelling of ATP site	57
Aminobutyrate aminotransferase	porcine brain	DTNB	Cys	reactivity of 1 essential Cys	58
Aminobutyrate aminotransferase	porcine brain	4-amino-5-halopentanoic acids		suicide inhibitors	59

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Aminopeptidase	*Aspergillus oryzae*	various thiol reagents	Cys	role in binding metal	60
Aminopeptidase	porcine microvillus	[^{125}I]iodonaphthazide		membrane insertion study	61
cAMP receptor	*E. coli*	8-azido-[^{32}P]cAMP		photo-affinity labelling	62
α-Amylase	*Bacillus amyloliquefaciens*	citraconic anhydride	Lys	tryptic digestion limited to Arg	63
α-Amylase	various	(i) *N*-acetylimidazole (ii) ethoxyformic anhydride	(i) Tyr (ii) His	(i) essential Tyr	64, 65
β-Amylase	soybean	DTNB, iodoacetamide, iodoacetic acid	Cys	essential Cys	66
Amyloglucosidase	*Aspergillus niger*	acryloyl chloride	Lys	immobilized by polymerization	67
Amylo-1,6-glucosidase–4α-glucanotransferase	rabbit muscle	1-*S*-dimethylarsino-1-thio-β-D-glucopyranoside		active-site irreversible inhibitor	68
Androgen binding protein	rat epididymis	17β-hydroxy-[1,2-^{3}H]4,6-androstadien-3-one, irradiation, dimethyl suberimidate		characterization using gel electrophoresis	69
Androgen binding protein	rat epididymis	[^{3}H]17β-hydroxy-4,6-androstadien-3-one		photo-affinity labelling	70
Antigen E	ragweed	*O*-alkoxypolyethyleneglycoxy *S*-carboxamidomethyldithiocarbonates	Lys	reduction in antigenicity	71
Antithrombin III	bovine plasma	dimethyl(2-hydroxy-5-nitrobenzyl) sulphonium bromide	Trp	1 Trp essential for heparin binding	72
Antithrombin III	human plasma	dimethyl(2-hydroxy-5-nitrobenzyl) sulphonium bromide	Trp	heparin binding site labelled	73
Antithrombin III	human plasma	dithiothreitol, [^{14}C]iodoacetamide	$(Cys)_2$	essential for heparin acceleration	74
Antithrombin III	human plasma	dithiothreitol, iodoacetamide	$(Cys)_2$	inactivation, conformation little changed	75
Antithrombin III–thrombin complex	human plasma	[^{14}C]methoxamine	Arg	covalent bonding in complex	76

Arginine kinase	lobster	[8-^{14}C]ATP-γ-*p*-azidoanilide		photo-affinity labelling	77
Arginine kinase	lobster	DTNB	Cys	inhibition	78
Asparaginase	*E. coli*	activated polyethylene glycol	Lys	antigenicity reduced	79
Aspartate aminotransferase	porcine heart	pyridoxal 5'-phosphate, sodium borohydride	Lys	stereospecificity of Schiff-base formation	80
Aspartate aminotransferase	porcine heart mitochondria	*N*-ethyl [1-^{14}C]maleimide, DTNB	Cys	crystal-state thiol reactivity	81
Aspartate aminotransferase	rat mitochondria	dimethyl adipimidate	Lys	membrane translocation inhibited	82
Aspartate-β-semi-aldehyde dehydrogenase	*E. coli*	L-2-amino-4-oxo-5-chloropentanoic acid, 3-chloroacetylpyridine ADP		affinity labelling	83, 84

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Aspartate transcarbamylase	*E. coli*	tetranitromethane	Tyr	inactivation; Tyr-160 essential	85
ATPase	bovine heart	[^{35}S]diazobenzenesulphonic acid	Tyr	sub-unit distribution	86
ATPase	bovine heart mitochondria	4-azido-2-nitrophenyl phosphate		photoreactive P_i analogue	87
ATPase	rabbit muscle	1,5-difluoro-2,4-dinitrobenzene		inhibition, intramolecular cross-links	88
ATPase	rabbit muscle	succinic anhydride	Lys	tryptic cleavage at Arg	89
ATPase	rat ascites cells	4-diazobenenesulphonic acid		cell-surface localization	90
ATPase	rabbit muscle	FDNB	Cys, Tyr	Ca^{2+}-dependent conformations dissociated	91
ATPase	yeast	[^{14}C]*NN'*-dicyclohexylcarbodi-imide	Glu	single Glu in proteolipid sub-unit	92
ATPase F_1	bovine heart mitochondria	(i) FDNB (ii) 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (iii) 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline	(i) Lys (ii) Tyr (iii) carboxyl	identification of active-site functional groups	93
ATPase F_1	ATCC 398	pyridoxal 5'-phosphate, $NaBH_4$	Lys	6 residues modified	94
ATPase F_1	*Micrococcus luteus*	3'-*O*-{3-[*N*-(4-azido-2-nitrophenyl)-amino]propionyl}8-azido ATP		photo-cross-linking	95
ATPase F_1	*Micrococcus luteus*	8-azido-1,N^6-etheno-ATP		fluorescent photo-label	96
ATPase [Ca^{2+}]	rabbit sarcoplasmic reticulum	disulphides of thioinosine triphosphates	Cys	2 different reactive thiols	97
ATPase [Ca^{2+}]	rabbit sarcoplasmic reticulum	2-(2-nitro-4-azidophenyl)amino-ethyl 16-doxyl stearate		spin label, photo-affinity label	98
ATPase [Ca^{2+}, Mg^{2+}]		*N*-ethylmaleimide	Cys	functionally distinct thiols modified	99
ATPase [Ca^{2+}, Mg^{2+}]	*E. coli*	various cleavable cross-linking agents	Lys	sub-unit structure	100
ATPase [Ca^{2+}, Mg^{2+}]	*E. coli*	2',3'-ATP dialdehyde	Lys	different strains labelled	101

ATPase [K^+]	porcine gastric mucosa	(i) 2-methoxy-2,4-diphenyl-3-dihydrofuranone (ii) butane-2,3-dione, cyclohexane-dione	(i) Lys (ii) Arg	inhibition, ATP protects	102
ATPase [K^+, Na^+]	electric eel	thiol-specific cross-linking reagents	Cys	spatial organization of thiols	103
ATPase [K^+, Na^+]	dog kidney medulla	Cu^{2+}, *o*-phenanthroline	Cys	conformational probe	104
ATPase [K^+, Na^+]	porcine gastric mucosa	butane-2,3-dione	Arg	essential Arg	105
ATPase [K^+, Na^+]	dog kidney	[^{3}H]adamantane diazirine		membrane-embedded regions identified	106
ATPase [K^+, Na^+]	*Electrophorus electricus*	3‴- and 4‴-diazomalonyldigitoxin		photo-affinity labelling	107

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
ATPase [K^+, Na^+]	porcine kidney	*N*-[*p*-(2-benzimidazolyl)phenyl]-maleimide		ATP dependent, conformational change	108
ATPase [K^+, Na^+]	rabbit kidney	*N*-ethylmaleimide, DTNB		effect of cations on inhibition	109
ATPase inhibitor IF_1	bovine heart mitochondria	phenyl [^{14}C]isothiocyanate *N*-(ethoxycarbonyl)-2-ethoxy-dihydroquinoline		PTH-IF_1 cross-linked	110
ATP synthetase complex	bovine heart mitochondria	[^{14}C]dicyclohexylcarbodi-imide		covalent binding to complex	111
Bacteriorhodopsin	*Halobacterium halobium*	various reagents	Lys, Trp Asp, Glu, Tyr, Arg	isotope effects and activation parameters	112
Bacteriorhodopsin	*Halobacterium halobium*	*p*-azidophenylisothiocyanate	Lys	hydrophobic cross-linking	113
Benzodiazepine receptor	rat CNS	kenazepine (bromoacetamidoethyl benzodiazepine derivative)		different types of receptor identified	114
Benzodiazepine receptor		[^{3}H]flunitrazepam, u.v. irradiation		photo-affinity labelling	115
α-Bungarotoxin	*Bungarus multicinctus*	[^{125}I]NaI, chloramine T	Tyr	label is on Tyr-54	116
Carbamate kinase	*Streptococcus faecalis*	butane-2,3-dione	Arg	essential Arg	117
Carbamyl phosphate synthetase	*E. coli*	5′-*p*-fluorosulphonylbenzoyladenosine		two functionally different ATP-binding sites	118
Carboxypeptidase A	bovine	(i) *N*-ethyl-5-phenylisoxazolium-3′-sulphonate	(i) Glu-270	prevents binding of *Ascaris* inhibitor	119
		(ii) tetranitromethane, diazotized arsanilic acid	(ii) Tyr-248		
Carboxypeptidase A	bovine	tetranitromethane	Tyr-248	pH dependence of peptidase activity	120
β-Casein (guanidinated)		various photolabile transglutaminase substrate cross-linking agents	Gln	cross-linking	121
Cathepsin B	porcine liver	[^{14}C]iodoacetamide	Cys	essential thiol	122
Cathepsin D	human	*N*-(2,4-dinitrophenyl)diaminoalkane derivatives of pepstatin		haptenic inhibitor-antibody localization technique	123

Cell proteins	*E. coli*	α-dehydrobiotin		reaction with biotin-utilizing proteins	124
β-Chorionic gonadotropin	human	6-maleimidocaproic acid *N*-oxy-succinimide ester and *N*-acteyl homocysteine thiolactone	Lys Cys	conjugation to flagellin and tetanus toxoid carriers	125
Chromatin	sea urchin	formaldehyde, $[^3H]NaBH_4$	Lys	aid to 2-D electrophoresis	126
Chymopapains A and B	*Carica papaya*	2,2'-dipyridyl disulphide, DTNB	Cys	study of active-centre pK_a's	127
Chymotrypsin	bovine	numerous α-bromo-amides	Met, Ser	stereospecificity of alkylation	128, 129
α-Chymotrypsin	bovine	$^{13}CH_3I$	Met-192	n.m.r. probe	130
α-Chymotrypsin		palmitic chloro-anhydride	Lys	incorporation into liposome membranes	131

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Chymotrypsin	bovine	*cis*-cinnamoyl imidazole	Ser	photodeacylation gives enzyme photographic process	132
α-Chymotrypsin	bovine	*o*, *m*, and *p*-(trifluoromethyl)bromo-acetanilides	Met-192	^{19}F n.m.r. study	133
α-Chymotrypsin		*p*-trifluoromethylbenzenesulphonyl fluoride	Ser	^{19}F n.m.r. study	134
α-Chymotrypsin		CF_3CO-Ala-PheCHO	Ser	^{1}H n.m.r. study	135
Collagen peptides	bovine achilles tendon	KB^3H_4 galactose oxidase		location of carbohydrate	136
Colipase		*N*-acetylimidazole	Tyr	important in phospholipid triglyceride system	137
Collagenase	*Achromobacter iophagus*	various reagents	carboxyl, Tyr, Trp, Arg, Lys	essential carboxyl, Trp, and Tyr	138
Collagenase	human leucocyte	*N*-ethylmaleimide *p*-aminophenyl-mercuric acetate	Cys	activation	139
Complement C2	human	I_2–KI	Tyr	increased affinity of C2a for C4b	140
Complement C3	human	[^{14}C]methylamine, [^{14}C]iodoacetamide	Glu, Cys	internal thioester bond	141
Complement C3	human	[^{14}C]methylamine	Glu	single Glu in 135 000 mol. wt. sub-unit	142
Complement C3	human, guinea-pig	hydroxylamine, [^{14}C]methylamine		internal thioester bond reacts	143
Calcium-activated neutral protease	chicken muscle	*d,l-trans*-epoxysuccinate derivatives		inhibition	144
Complement C3		formaldehyde, sodium [^3H]boro-hydride	Lys	labelling method	145
Complement C4	human	[^{14}C]methylamine		inhibition	146
Complement system Factor D	human	di-[1,3-^{14}C]isopropylphospho-fluoridate	Ser	location of active centre	147
Coproporphyrinogen oxidase	bovine liver	tetranitromethane	Tyr	essential Tyr	148

Coupling factor	spinach chloroplast thylakoid	(i) *o*-iodosobenzoic acid (ii) 2,2′-dithiobis(5-nitropyridine)	Cys	(i) uncoupling (ii) uncoupling or energy-transfer inhibition	149
Coupling Factor 1	*E. coli*, bovine heart	[^{14}C]dicyclohexylcarbodi-imide	carboxyl	F_1 labelled at acidic pH	150
Coupling Factor 1	spinach chloroplast	Woodward's reagent K	carboxyl	essential carboxyl	151
Coupling Factor 1	spinach chloroplast	dicyclohexylcarbodi-imide		inactivation of ATPase	152
Coupling Factor CF_0	spinach chloroplast	1-(ethoxycarbonyl)-2-ethoxy-1,2,-dihydroquinoline, 1-(isobutoxy-carbonyl)-2-isobutoxy-1,2-dihydro-quinoline	carboxyl	inhibition of photo-phosphorylation	153
Creatine kinase	rabbit muscle	[8-^{14}C]ATP-γ-*p*-azidoanilide		photo-affinity labelling	154

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Creatine kinase	rabbit muscle	4-(*N*-2-chloroethyl-*N*-methylamino)-benzyl-γ-amide of ATP		affinity labelling	155
Creatine kinase	rabbit muscle	2,4-dinitrophenylthiocyanate	Cys	sub-unit selective modification	156
α-Crystallin	bovine lens	various bis-imide esters	Lys	sub-unit structure	157
Cyclitol permease	*Klebsiella aerogenes*	(i) *N*-ethyl [2-^{3}H]maleimide (ii) diazotized [^{35}S]sulphanilic acid	(i) Cys (ii) Tyr	identification of protein components	158
Cytochrome *c*	bovine heart	[^{3}H]- and [^{14}C]-acetic anhydride	Lys	differential chemical modification	159
Cytochrome *c*	bovine heart mitochondria	methyl-3-imidate-2,2,5,5-tetramethyl-pyrroline-1-oxyl·HCl	Lys	spin-label study of membrane interactions	160
Cytochrome *c*	horse heart	maleic anhydride	Lys	chemical reactivity of fully maleylated protein	161
Cytochrome *c*	horse heart	4-chloro-3,5-dinitrobenzoic acid	Lys	activity of five singly substituted derivatives; C_1 binding domain mapped	162, 163
Cytochrome *c*	horse heart	butane-2,3-dione	Arg	Arg-38 in ascorbate binding site	164
Cytochrome *c*	horse heart	dithiobis(succinimidylpropionate) and yeast peroxidase	Lys	complex catalyses oxidation at cytochrome c_1 by H_2O_2	165
Cytochrome *c*	horse heart	semi-synthetic analogues prepared: Met-80 replaced by (i) ethionine or (ii) *S*-methyl cysteine		(i) 96% active (ii) inactive	166
Cytochrome *c*	horse heart	4-chloro-3,5-dinitrobenzoic acid, TNBS	Lys	carbonate binding site located	167
Cytochrome *c*	tuna heart	EDC		Spin state and reduction potential changes	168
Cytochrome *c*	yeast	[^{3}H]-*p*-azidophenacylbromide(methyl-4-mercaptobutyrimidate)		photo-labelling of cytochrome *c* oxidase	169
Cytochrome *c* oxidase	bovine heart	*N*-(1-anilino-4-naphthyl)-maleimide	Cys	conformation vesicle system	170

Cytochrome *c* oxidase	bovine heart	[^{14}C]dicyclohexylcarbodi-imide	H^+ translocating activity reduced	171
Cytochrome *c* oxidase	bovine heart	various bis-imidates	cross-linking to mitochondria and cytochrome *c*	172
Cytochrome *c* oxidase	bovine heart	4-azido-2-nitrophenyl (Lys-13 and Lys-22) cytochrome *c*	photo cross-linking	173
Cytochrome *c* oxidase	bovine heart	1,5-difluoro-2,4-dinitrobenzene	sub-units III and IV cross-linked	174
Cytochrome P450	rabbit liver	Cu^{2+}–1,10-phenanthroline	cross-linking reaction	175
Cytochrome P450	rabbit liver	Cu^{2+}–1,10-phenanthroline	evidence for molecular aggregates	176
Cytochrome P450	rat liver	4-(3-iodo-2-oxopropylidene)-2,2,3,5,5-pentamethylimidosolydene-1-oxyl Cys	affinity labelling; e.p.r. spectroscopy	177

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Cytochrome P450	rat liver microsomes	dimethylsuberimidate, dimethyl-3,3′-dithiobis-(propionimidate)	Lys	cross-linking studies	178
NADPH-cytochrome P450 reductase		various cross-linking agents	Lys	purified proteins cross-linked	179
Cytokinin binding protein	wheat germ	methylene-[^{14}C]2-azido-6-benzylamino-purine		photo-affinity labelling	180
D-Amino-acid oxidase	porcine kidney	[^{36}Cl]*N*-chloro-D-leucine	Tyr	active-site Tyr located	181
D-Amino-acid oxidase	porcine kidney	*N*-chloro-D-leucine	Tyr	chlorination of active-site Tyr	182
D-Amino-acid oxidase	porcine kidney	(i) dansyl chloride, ethoxyformic anhydride	His	essential His	183
		(ii) butane-2,3-dione	Arg	essential Arg	
		(iii) dinitrofluorobenzene	Tyr	essential Tyr	
Deoxyribonuclease	bovine pancrease	*N*-bromosuccinimide	Trp	reactivities of Trp residues compared	184
Digitalis receptor	*Electrophorus electricus*, crab axon chick heart	*p*-nitrophenyltriazene-ouabain		affinity labelling	185
Dihydrofolate reductase	bovine liver, chicken liver	methylmercuric hydroxide, *p*-hydroxy-mercuribenzoate	Cys	activation	186
Dihydrofolate reductase	chicken liver	sodium [^{35}S]tetrathionate	Cys	activity increased	187
Dihydrofolate reductase	*Lactobacillus casei*	*N*-bromosuccinimide	Trp-21	binding studies	188
Dihydrofolate reductase	*Lactobacillus casei*	EDC	carboxyl	essential carboxy-group	189
Dioldehydrase (B_{12} dependent)	*Klebsiella pneumoniae*	butane-2,3-dione, phenylglyoxal	Arg	essential Arg	190
Diphtheria toxin		ATP, NAD, u.v. irradiation		ATP and NAD cross-linked	191
Diphtheria toxin		mixed anhydride derivative of chlor-ambucil, anti-(human lymphocyte) globulin		conjugate is specifically cytotoxic	192
Diphtheria toxin, A chain		dithiothreitol, SPDP-derivatized epidermal growth factor	Cys	conjugate is non-toxic (*cf.* Ricin A)	193
Diphtheria toxin, A chain		2-mercaptoethanol and SPDP derivatized monoclonal antibody		selectively toxic to carcinoma cells	194

Diphtheria toxin, A chain, cholera toxin A chain		EDC	Lys	hybrid toxin prepared	195
Diphtheria toxin, B chain		periodate-oxidized [γ-^{32}P]ATP, sodium cyanoborohydride		affinity labelling	196
Disulphide isomerase	bovine liver	thiopropyl-Sepharose 6B	Cys	covalent chromatography	197
DNA polymerase	various sources	phenylglyoxal	Arg	inhibition; template binding affected	198
DNA polymerase I	*E. coli*	α-ATP γ-4-(*N*-2-chloroethyl-*N*-methylamino)benzylamidate		affinity labelling	199
Dopa decarboxylase	pig kidney	2-(fluoromethyl)-3-(3,4-dihydroxylphenyl)alanine		suicide substrate	200

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Dopamine β-hydroxylase	bovine adrenal	*p*-hydroxybenzylcyanide		suicide inactivation	201
Dopamine receptor	bovine	various thiol reagents	Cys	essential thiols	202
Dopamine receptor	rat muscle	(−)*N*-(chloroethyl)norapomorphine		alkylation and receptor blockade	203
DNA polymerase	dogfish	*N*-ethylmaleimide	Cys	α and β form inhibited	204
Elastase	human pancreas	peptide chloromethyl ketones		kinetics of inhibition	205
Elongation factor G	*E. coli*	*N*-ethylmaleimide, iodoacetamide, iodoacetyl *p*-azidobenzylamine	Cys	study of interactions with ribosome	206
Elongation factor G	*E. coli*	tetranitromethane, [^{125}I]KI–H_2O_2–lactoperoxidase	Tyr	essential Tyr	207
Elongation factors T and Tu	rabbit reticulocyte	N^{ε}-bromoacetyl-Lys-tRNA		affinity labelling	208
Elongation factor Tu	*E. coli*	ethoxyformic anhydride, photo-oxidation	His	essential His residues	209
Elongation factor Tu	*E. coli*	butane-2,3-dione	Arg	5 reactive arginines	210
Elongation factor 2	rat liver	periodate-oxidized guanine nucleotides		Schiff-base formation?	211
Enoyl-CoA reductase	goose	pyridoxal-5′-phosphate, $NaBH_4$	Lys	essential Lys	212
Erythrocytes	sheep	2,4-dinitrophenylalanylglycylglycine, EDC, and *N*-hydroxysuccinimide		preparation of dinitrophenylated cells	213
Factor Xa	human	[^{3}H]DFP, [^{3}H]-di-isopropyl phosphofluoridate	Ser	localization of active centre	214
Factor Xa	bovine	[^{3}H]*m*-[*o*-(2-chloro-*S*-fluorosulphonylphenyl ureido)phenoxybutoxy]-benzamidine		affinity labelling	215
Fatty acid synthetase	rat mammary glands	(i) phenylglyoxal	(i) Arg	(i) inhibition of ketoacyl and energy reductase	216
		(ii) pyridoxal 5′-phosphate	(ii) Lys	(ii) inhibition of enoyl reductase	
Fatty acid synthetase	yeast	1,3-dibromopropan-2-one	Cys	sub-units cross-linked	217
Ferricytochrome *c*	horse	succinic anhydride	Lys, α-NH_2	method for integral NH_2 determination	218

Ferritin	horse spleen	bis-hydrazides, carbodi-imides	carboxyl	electron-dense probe for glycoconjugates	219
α-Foetoprotein	rat	16-diazoestrone, 4-azidoestradiol		photo-affinity labelling	220
Fibrin monomer	human	H_2O_2	Trp	D-domain Trps essential for association	221
Fibrinogen	bovine	diazomethane, dimethyl sulphate	carboxyl	methylation and polymerization	222, 223
Ficin	*Ficus glabrata*	chromogenic thiol reagents	Cys	titrants used as reactivity probes	224
Flavocytochrome b_2	yeast	[2-^{14}C]bromopyruvate	Cys	affinity labelling	225

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Follicle-stimulating hormone	human	azidobenzoyl derivative of a fetuin glycopeptide		activity increased by conjugation	226
Formate dehydrogenase	methylotrophic bacteria	DTNB	Cys	inactivation	227
Formate dehydrogenase	methylotrophic bacteria	butane-2,3-dione	Arg	essential Arg	228
Formyl peptide chemotactic receptor	human neutrophil	(i) formyl-Nle-Leu-Phe-Nle-[^{125}I]-Tyr-Lys, dimethyl suberimidate (ii) formyl-Nle-Leu-Phe-Nle-[^{125}I]-Tyr-Lys-N^{ε}-4-azido-2-nitrophenyl, irradiation (iii) formyl-Nle-Leu-Phe-Me-[^{125}I]-Tyr-Lys-N^{ε}-bromoacetyl		affinity labelling, receptor identified	229
Formyltetrafolate synthetase	*Clostridium cyclindrosporum*	dimethyl suberimidate	Lys	activity and 4° structure stabilized	230
Fructose diphosphatase	spinach chloroplast	H_2O_2, pH 8.0	Cys	inactivation released by dithiothreitol	231
Fructose-1, 6-diphosphatase	rabbit liver	(i) DTNB, (ii) ethoxyformic anhydride	(i) Cys (ii) His	essential groups identified	232
Fumarate reductase	*Vibrio succinogenes*	various sulphydryl reagents	Cys	essential Cys	233
GABA–benzodiazepine–Cl^- ionophore complex	rat	avermectin B_{1a}		irreversible stimulation of receptor	234
Galactose oxidase	*Dactylium dendroides*	(i) iodoacetamide (ii) *N*-bromosuccinimide	(i) His (ii) Trp	co-ordination chemistry study	235
Galactose-transporting membrane vesicle protein	*E. coli*	[^{14}C]*N*-ethylmaleimide		inactivation; glucose protects	236
Galactosyltransferase	bovine milk	[^{125}I]ICl		inactivation	237
Galactosyltransferase	bovine	*S*-mercuric *N*-dansyl cysteine	Cys	fluorescence mapping	238
Galactosyltransferase		KI–H_2O_2–lactoperoxidase, *N*-acetylimidazole	Tyr	inactivation	239

Gelatin		4-chloro-7-nitrobenzofurazan	Lys	cross-linking observed; two-step reaction	240
Gelonin	*Gelonium multiflorum*	SPDP, concanavalin A derivatized with SPDP	Lys	conjugate is cytotoxic	241
Glucagon	porcine	various alkyl imidates	Lys, α-NH_2	binding and activity studied	242
Glucocorticoid receptor	rat liver	*N*-ethylmaleimide, iodoacetamide	Cys	effect on activation	243
Glucocorticoid receptor	rat	pyridoxal-5′-phosphate, $NaBH_4$	Lys	metabolizable receptor forms identified	244
Glucocorticoid receptor		various	Arg, His, and Lys	identification of DNA binding-site residues	245
Glucosephosphate isomerase	human placenta	*N*-bromoacetylethanolamine	His	active-site His located	246
Glucose-6-phosphatase	rat liver	*p*-chloromercuribenzenesulphonic acid	Cys	inhibition	247

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Glucose-6-phosphate dehydrogenase	yeast	dichlorotriazine dyes		reaction at NAD^+, $NADP^+$, and ATP sites	248
Glucose-6-phosphate dehydrogenase		derivatized gentamycin	Cys	homogeneous enzyme immunoassay	249
Glucose transporter	human erythrocyte	maltosyl isothiocyanate		affinity labelling	250
Glutamate dehydrogenase	yeast	ethoxyformic anhydride	His	important for conformation	251
γ-Glutamylcysteine synthetase	rat kidney	3-amino-1-chloropentan-2-one	Cys	irreversible inactivation	252
γ-Glutamyl transferase	rat kidney	PMSF, *N*-acetylimidazole, iodoacetamide	Ser	study of active-centre functionalities	253
γ-Glutamyl trans-peptidase	rat kidney	AT-125[L-(*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid]		potent inhibition	254, 255
γ-Glutamyl trans-peptidase	rat	L-(α*S*, 5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid	Cys(?)	enzyme inactivation	256
Glutathione reductase	human erythrocyte	1,5-difluoro-2,4-dinitrobenzene	Cys, His	cross-linking of sub-units	257
Glutathione reductase	human, rat, calf	TNBS		oxidase activity induced	258
α-Glutathione-*S*-transferase	bovine liver	*S*-(*p*-azidophenacyl)glutathione		photo-affinity label	259
Glyceraldehyde-3-phosphate dehydrogenase	*B. stearothermophilus*	iodoacetic acid, NAD, u.v. irradiation		energy transfer with Trp residues	260
Glyceraldehyde-3-phosphate dehydrogenase	human erythrocyte membrane	glutaraldehyde	Lys	cross-linking and inhibition	261
Glyceraldehyde 3-phosphate dehydrogenase	rabbit muscle	*p*-nitrophenoxy carbonylmethyl disulphide	Cys	new methanethiolating reagent	262
Glyceraldehyde 3-phosphate dehydrogenase	rabbit muscle	iodoacetic acid, NAD, u.v. irradiation		fluorescent derivative crystallized	263
Glycogen phosphorylase	rabbit muscle	8-azido AMP		photo-affinity labelling	264
Glycogen phosphorylase	rabbit muscle	various bis-imidoesters	Lys	probe of structural change	265
Glycolytic enzymes	various sources	methylglyoxal, phenylglyoxal	Arg	inactivation rates compared	266
Glyoxalase I	yeast	*S*-(*p*-azidophenacyl)-glutathione		photo-affinity labelling	267
Glyoxalase II	bovine liver	*S*-(*p*-azidophenacyl)-glutathione		photo-affinity labelling	268
Gonadotrophin receptor	rat ovary	u.v. irradiation		inactivation, uncoupling	269

Growth hormone	bovine	chloramine T	Met	reactivity order established	270
Haemoglobin	human	dextran treated with cyanogen bromide, diaminoethane, and bromoacetyl bromide		enhanced stability, reduced haptoglobin affinity	271
Haemoglobin	human	FDNB	α-NH_2	chloride binding study	272
Haemoglobin	human	[^{14}C]glyceraldehyde	Val (α-NH_2)	ketoamine adduct	273
Haemoglobin	human	various bimanes	Cys	β-93 fluorescence-labelled	274, 275

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Haemoglobin	human	various aspirin analogues	Lys	acylation properties compared	276
Haemoglobin	human	polyethylene glycol derivatives		blood substitute	277
Haemoglobin		various cross-linking agents		random collisional cross-links insignificant	278
Haemoglobin α	human	3-bromo-3-methyl-2-(2-nitrophenyl-thio)-(3*H*)indole, *N*-bromo-succinimide	Trp, Tyr, His	cleavage reaction	279
Haemoglobin β	human	DTNB, 2,2′-dithiopyridine	Cys (β-93)	pH dependence of modification	280
Haemoglobin S	human	bis(3,5-dibromosalicyl)fumarate	Lys	cross-linking of DPG binding site	281
Haemoglobin S (CO)	human	[^{14}C]glyceraldehyde	α-NH_2, Lys	labelled residues located	282
Haemocyamin	keyhole limpet	isothiocyanate-activated β-(*p*-amino-phenyl)-ethylamine derivatives of sialyloligo-saccharides		antibodies raised	283
Hexokinase	yeast	dichlorotriazine dyes		reaction at NAD^+, $NADP^+$, and ATP site	284
Hexon protein	adenovirus	[^{14}C]iodoacetic acid	Cys	reactive Cys located	285
Histidine decarboxylase	rat hypothalamus	α-fluoromethylhistidine		irreversible histidine neurotransmitter analogue	286
Histones	calf thymus	1-fluoro-2,4-dinitro[3,5-^{3}H]benzene	Cys, α-NH_2	competitive labelling	287
Histones	calf thymus	EDC	carboxyl, NH_2	H1-histone octamer contacts	288
Histone H3	calf thymus	various sulphydryl reagents	Cys	Cys-110 structurally important	289
Histones H3	rat liver, mouse thymocyte	*N*-3-pyrene maleimide	Cys (Lys)	fluorescent labelling of H3 in chromatin	290
Histones H3, H4	calf thymus	acetyl adenylate	Lys	role in SV40 transformation	291, 292
Histones H3, H4	chicken erythrocyte	bisulphite	Cys	reversal of SH-mediated ageing	293
L-α-Hydroxy-acid oxidase	rat kidney	ethoxyformic anhydride	His	essential His	294

p-Hydroxybenzoate hydroxylase	*Pseudomonas desmolytica*	phenylglyoxal	Arg	essential Arg	295
D-3-Hydroxybutyrate dehydrogenase	bovine heart mitochondria	*N*-ethyl maleimide, methylmercury	Cys	essential Cys	296
D-3-Hydroxybutyrate dehydrogenase	rat mitochondria	phenylglyoxal, butane-2,3-dione, cyclohexane-1,2-dione	Arg	essential Arg	297
D-3-Hydroxybutyrate dehydrogenase		methyl methane thiosulphonate	Lys	inactivation, Cys specificity questioned	298
3α,20β-Hydroxysteroid dehydrogenase	*Streptomyces hydrogenans*	17β-(1-oxo-2-propynyl)androst-4-en-3-one		enzyme-generated affinity alkylator	299
3α,20β-Hydroxysteroid dehydrogenase	*Streptomyces hydrogenans*	17-(bromoacetoxy)steroids		affinity alkylation	300

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
α-L-Iduronidase	human fibroblasts	butane-2,3-dione	Arg	cellular uptake inhibited	301
Immune complexes	rabbit	toluene-2,4-di-isocyanate		cross-linking of complex	302
Immunoglobulins	mouse	[^{3}H] and [^{14}C]FDNB	His, Lys, Cys, NH_2	comparative reactivity study	303
Immunoglobulins	sheep	*S*-acetylmercapto succinic anhydride *NN'-o*-phenylenedimaleimide, *NN'-p*-phenylenedimaleimide, *NN'*-(oxydimethylene) dimaleimide	Lys	conjugates for enzyme immunoassay	304
Immunoglobulin	mouse (monoclonal)	*NN*-bis(2,4-dinitro-6-fluorophenyl)-pimelic acid amide		immune complex models	305
Immunoglobins (anti-Thy-1)	mouse	fluorescein isothiocyanate		fluorochrome labelling	306
Immunoglobulin G	rabbit	dithiothreitol, iodoacetamide	$(Cys)_2$	effect on dimensions with and without hapten	307
Immunoglobulin G	rabbit	dithiothreitol, iodoacetamide, 1-anilinonaphthalene-8-sulphonic acid	$(Cys)_2$	fluorescence probe for Ig flexibility	308
Immunoglobulin G	rabbit	D-2-*N*-acetylhomocysteinethiolactone, 2-pyridinealdoxime triaziquinone	Met	drug–antibody conjugate	309
Immunoglobulin G	rabbit	activated peroxidase	Lys	conjugation method	310
Immunoglobulin G	rabbit	dithiobis(succinimidylpropionate)	Lys	antigen–antibody cross-linking	311
Immunoglobulin G	rat	methyl-3,5-di[^{125}I]iodohydroxybenzimidate	Lys	high specific activity iodination	312
Initiation factor eIF-2	rabbit reticulocyte	sodium selenite	Cys	indirect inactivation	313
Initiation factor eIF-2	rat liver	methyl-*p*-azidobenzoylaminoacetimidate, methyl-5-(*p*-azidophenyl)-4,5-dithiapentanimidate, diepoxybutane		α and γ sub-units cross-linked to 18S rRNA	314

Initiation factor eIF-2	rat liver	methyl *p*-azidobenzoylaminoacetimidate, 5-(*p*-azidophenyl)-4,5-dithiapentanimidate		β-sub-unit close to Met-$tRNA_f^{Met}$	315
Initiation factor IF-3	*E. coli*	$^{125}I^-$—lactoperoxidase, –chloramine T		ribosomal binding studies	316
Inorganic pyrophosphatase	yeast	methyl phosphate	carboxyl	affinity labelling	317
Inorganic pyrophosphatase	yeast	[^{14}C]phenylglyoxal	Arg	identification of PP_i binding site	318
Inosine S^1 monophosphate dehydrogenase	*E. coli*	6-chloro-9-β-D-ribofuranosyl S^1 phosphate	Cys	active-site modification	319
Insulin	bovine	tetranitromethane	Tyr	4 Tyrs not essential	320
Insulin	bovine	dithiothreitol	$(Cys)_2$	calorimetric study	321
Insulin	porcine	I^-—lactoperoxidase	Tyr	properties of monoiodoinsulins	322

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Insulin B chain	bovine	pyromellitic di-anhydride, *o*-sulphobenzoic anhydride	Lys	new acylating agents	323
Insulin receptor	porcine liver	4-azido-2-nitrophenylacetyl-[^{125}I]insulin		identification by gel electrophoresis	324
Insulin receptor	rat adipocyte	B2-(2-nitro-4-azidophenylacetyl)-des-Phe-insulin		gives prolonged lipogenesis signal	325
Insulin receptor	rat adipocyte	4-azido Phe B1 insulin, 4-azido-phenylacetyl-B29 insulin-4-azidophenylacetyl-D,α,β-diaminobutyryl A1 insulin		photo-affinity labelling	326
Insulin receptor	rat liver	$N^{\varepsilon B1}$-(*p*-azidobenzoyl)insulin		photo-affinity labelling	327
Insulin receptor	rat	$N^{\varepsilon B29}$-mono-(azidobenzoyl)insulin		photo-labelling of receptor proteins	328
Insulin receptor	rat, guinea-pig, mouse	$N^{\varepsilon B29}$-(azidobenzoyl)insulin, $N^{\alpha A1}N^{\varepsilon B29}$-di-(azidobenzoyl)insulin		photo-affinity labelling in membranes	329
Isocitrate dehydrogenase		adenosine 5′-(2-bromoethyl)-phosphate		new affinity label	330
Kallikrein	rat urine	peptidyl chloromethyl ketones	(His)	subsite specificity study	331
α-Ketoglutarate dehydrogenase		butane-2,3-dione	Arg	Arg in active centre	332
Δ^5-3-Ketosteroid isomerase	*Pseudomonas putida*	1,4,6-androstatrien-3-one-17β-ol, u.v. irradiation		active-site-directed photo-inactivation	333
Δ^5-3-Ketosteroid isomerase	*Pseudomonas testosteroni*	spiro-17β-oxiranyl steroids		irreversible inhibition	334
lac Carrier protein	*E. coli*	[^{3}H]4-nitrophenyl-α-D-galacto-pyranoside		photo-affinity labelling	335
α-Lactalbumin	goat	*N*-acetylimidazole	Tyr, Lys	effect on galactosyl transferase interaction	336
β-Lactamase	*Bacillus cereus*	toluene-2,4-di-isocyanate		cross-linking study	337
β-Lactamase	*Bacillus cereus*	6β-bromopenicillanic acid	Ser	active-site-directed inhibition	338
β-Lactamase	*Bacillus cereus*	6β-bromopenicillanic acid		suicide substrate	339, 340

β-Lactamase	*Bacillus cereus*	6β-bromopenicillanic acid	Ser	binds *via* ester linkage as dihydrothiazine	341
β-Lactamase	*Bacillus cereus, Staphylococcus aureus, E. coli*	phenylpropynal		irreversible inhibition	342
β-Lactamase	*Bacillus licheniformis*	Compound PS-5, *N*-acetyl thienamycin		active-site-directed irreversible inactivation	343
β-Lactamase	*E. coli*	[^{14}C]cefoxitin		isolation of acyl-enzyme intermediate	344
β-Lactamase	*E. coli*	penicillanic acid sulphone		irreversible inhibition	345

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Lactate dehydrogenase	*Megasphaera elspenii*	bromopyruvic acid, DTNB	Cys	comparison of Cys reactivities	346
Lactate dehydrogenase	mouse	pyridoxal 5′-phosphate, $NaBH_4$, or cysteine	Lys	inactivation	347
Lactate dehydrogenase	porcine heart	dichlorothiazine dyes		reaction at NAD^+, $NADP^+$, and ATP sites	348
Lactate dehydrogenase	potato	dimethyl suberimidate	Lys	sub-unit arrangement determined	349
Lactate dehydrogenase	rabbit muscle	3-(3*H*-diazirino)pyridine adenine dinucleotide (DAD^+), irradiation		labelling of NAD^+ binding site	350
Lactate transporter	human erythrocyte	isobutylcarbonyl lactylanhydride		inhibition of transport by acylation	351
Lactose synthase	bovine	[^{14}C]acetic anhydride, [^{3}H]acetic anhydride	Lys	differential kinetic labelling	352
Laticauda semifasciata III	*Laticauda semifasciata*	acetic anhydride	α-NH_2, Lys	Lys-23 and Lys-35 essential	353
Lipophilin	human myelin	DTNB, [l-^{14}C]iodoacetamide	Cys	reactivity studied	354
Low-density lipoprotein	human	glutaraldehyde, malondialdehyde		effect on cholesteryl ester accumulation in monocyte-macrophages	355
Low-density lipoprotein	human	dimethyl suberimidate	Lys	low yield of apo B dimer	356
Luciferase	*Beneckea harveyi*	3-(maleimidomethyl)-2,2,5,5-tetramethyl 1-pyrolidinyloxyl, 3-(3-maleimidopropylcarbamoyl)-2,2,5,5-tetramethyl-1-pyrollinyoxyl	Cys	spin-label attachment	357
Lutrophin receptor	porcine ovary	^{125}I-labelled 4-azidobenzoyl-glycylglycyl choriogonadotrophin		photo-affinity labelling	358
Lysozyme	ovine	biotin, EDC	carboxyl	avidin determination	359
Lysozyme	ovine	*N*-bromosuccinimide	Trp-62	oxidation to oxindolealanine	360
Lysozyme	ovine	1-[^{14}C]iodoacetic acid, dithiothreitol	$(Cys)_2$	gives active derivative	361
Lysozyme	various	*N*-bromosuccinimide	Trp	fluorescence quenching studied	362

α_2-Macroglobulin	human	alkylamines	pGlu	internal pyroglutamyl bond proposed	363, 364
α-Macroglobulin	human	dimethyl suberimidate	Lys	comparison with pregnancy-associated protein	365
Malic enzyme	pigeon liver	*SS'*-dithiobis(2-nitrobenzoic acid) bromopyruvate and other thiol reagents	Cys	location of non-functional SH groups	366
Malic enzyme	pigeon liver	*N*-acetylimidazole, tetranitromethane	Tyr	essential for substrate binding	367
Membrane proteins	Change liver cells	[^{14}C]*N*-ethylmaleimide	Cys	effect of amino-acids on reactivity	368
Membrane proteins	Ehrlich ascites cells	1-isothiocyanate-4-benzene sulphonic acid		inhibition of anion transport	369

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Membrane proteins	human erythrocyte	1-azido-4-[^{125}I]iodobenzene		photo-affinity labelling of hydrophobic domain	370
Membrane proteins	human erythrocyte	phenylisothiocyanate	Lys	band 3 modified; phosphate transport inhibited	371
Membrane proteins	human erythrocyte	eosin 5-maleimide		band 3 rotational diffusion	372
Membrane proteins	human erythrocyte	(i) ethyl acetimidate (ii) light, Methylene Blue	Lys	(i) did not inhibit cross-linking by (ii)	373
Membrane proteins	human erythrocyte	eosin isothiocyanate	Lys	lateral mobility of band 3 studied	374
Membrane proteins	human erythrocyte	1-[^{3}H]spiro[adamantane 4,4′-diazirine], irradiation		labelling of lipid contact areas	375
Membrane proteins	human erythrocyte	4-methylazidobenzoimidate		globoside cross-linked to protein	376
Membrane proteins	human, mouse, and rat lymphocytes	hexanoyl di-iodo-*N*-(4-azido-2-nitrophenyl)tyramine, irradiation		photo-labelling of membrane associated proteins	377
Membrane proteins	mouse leukaemia cells	bis{2-[succinimido-oxy-carbony(oxy)ethyl]} sulphone	Lys	topographical study	378
Membrane proteins	porcine kidney	3,*S*-di-[^{125}I]iodo-4-azido-benzene sulphonate		photo-affinity labelling	379
Membrane proteins	sarcoplasmic reticulum	FDNB, TNBS, methyl acetimidate	Lys	differential labelling	380
Membrane proteins	rabbit sarcoplasmic reticulum	TNBS, fluorodinitrobenzene		interaction of probes studied	381
Membrane proteins	rat mitochondria	dimethyl suberimidate		dynamic changes studied	382
Methionyl-tRNA transformylase	*E. coli*	periodate-oxidized[^{14}C]tRNA$_f^{Met}$, sodium cyanoborohydride	Lys	affinity labelling	383
Methotrexate transport system	L1210 cells	EDC-activated methotrexate and other folate compounds		irreversible	384
Mitochondrial genome proteins	*Drosophila melanogaster*	4,5′,8-trimethylpsoralan, u.v. irradiation		DNA-protein cross-linking	385
Modeccin toxin	*Adenia digitata*	*N*-ethyl[^{14}C]maleimide	Cys	toxicity inhibition	386
Monoamine oxidase	bovine liver	*trans*-phenylcyclopropylamine	Cys	suicide inactivation	387

Monoclonal antibody		liposomes, SPDP	Lys	cell targetting	388
α-MSH receptor	*Xenopus laevis*	[*p*-azidophenylanine[13]]-α-MSH		irreversible stimulation melanophores	389
Muscarinic antagonist binding sites	rat brain	*p*-azidophenylacetate esters of tropine		photoattachment study	390
Myelin	bovine brain	TNBS	Lys	Lys accessibility study	391
Myelin basic protein	human CNS	3-(2-iodoacetamido)-2,2,5,5,-tetramethyl-1-pyrrolininyloxyl and other spin labels	Cys	effect of lipid environment	392
Myosin	porcine heart	DTNB	Cys	actin-induced conformational changes	393, 394

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Myosin	porcine heart	*N*-methyl-2-anilino-6-naphthalene-sulphonyl chloride	Lys	accessibility study; effect of divalent cations	395
Myosin	rabbit muscle	*N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl)iodoacetamide	Cys	oxidation of spin label	396
Myosin	rabbit muscle	*N*-(7-dimethylamino-4-methylcoumarinyl)maleimide	Cys	fluorometric studies on light chain	397
Myosin		TNBS	Lys	reactive Lys in ATPase located	398
Myosin ATPase		[^{14}C]*p*-*NN*'-phenylenedimaleimide	Cys	—SH_1 and — SH_2 cross-linked	399
Myosin subfragment 1	rabbit muscle	various cross-linking agents	Cys, Lys	thiol cross-linkers trap nucleotides	400
Myosin subfragments 1	rabbit muscle	DTNB	Cys	inactivation and $(Cys)_2$ formation	401
NADH-CoQ reductase	ox heart	arylazido-β-[3-^{3}H]alanyl NADP^{+}		affinity labelling	402
NADH-cytochrome P450 reductase	liver	*N*-ethyl 2,3-[^{14}C]maleimide cyclohexane-1,2-dione	Cys	difference labelling	403
NADH dehydrogenase	bovine heart mitochondria	diazotized [^{35}S]sulphanilic acid, ^{125}I–lactoperoxidase	Tyr	membrane impermeable probes	404
NADH dehydrogenase	bovine heart mitochondria	*S*-[^{125}I]iodonaphth-1-yl azide, irradiation		phospholipid bilayer contacts	405
Neocarzinostatin	*Streptomyces carzinostaticus*	(i) cyclohexane-1,2-dione (ii) trypsin	(i) Arg (ii) Lys	active 89-residue fragment isolated	406
Nerve growth factor	mouse submandibular gland	[^{3}H]DFP	Ser	active-site labelling	407
α-Neurotoxin	*Naja naja siamensis*	2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid *N*-hydroxysuccinimide ester		interaction of spin-labelled toxin with acetylcholine receptor	408
Neurotoxins A and E	*Clostridium botulinum*	cyclohexane-1,2-dione	Arg	nicking site identified	409
Neurotoxic esterase	hen brain	various organophosphates	Ser	study of reactivation and ageing	410
Neutral proteinase	human uterine cervix	DFP, TLCK	Ser	trypsin-like specificity	411
Nitrate reductase	*E. coli*	diazotized [^{125}I]di-iodosulphanilic acid, diazobenzene [^{35}S]sulphonate		β sub-unit located on cytoplasmic surface of membrane	412

Nuclear envelope nucleoside triphosphatase	sheep liver	*N*-bromosuccinimide, chloramine T	His	essential His	413
Nucleoprotein complex	yeast	citraconic anhydride	Lys	reversible modification to dissociate complex	414
Oestradiol dehydrogenase	human placenta	3-(arylazido-β-alanine)oestrone, 17β-(arylazido-β-alanine)-oestradiol, 3-methyl ether		affinity labelling	415
Oestrogen receptor	rat mammary tumour	photo-oxidation (Rose Bengal)		DNA binding lost	416
Oestrogen receptor	rat uterus	pyridoxal 5'-phosphate, $NaBH_4$	Lys	8S → 4S species	417
Opiate receptor	bovine caudate nucleus	azido enkephalin analogues		affinity labelling	418

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Opiate receptor	rat brain	*N*-ethylmaleimide	Cys	evidence for two receptor classes	419
Ornithine trans-carbamylase	bovine liver	butane-2,3-dione, phenylglyoxal	Arg	Arg at carbamyl phosphate binding site	420
Ornithine trans-carbamylase	bovine liver, *Streptococcus faecalis*	various aromatic disulphides	Cys	essential Cys	421
Ornithine trans-carbamylase	bovine liver, *Streptococcus faecalis*	2-chloromercuri-4-nitrophenol	Cys	pH dependence of spectrum	422
Pantoate dehydrogenase	*Pseudomonas fluorescens*	(i) DTNB, iodoacetic acid, *p*-chloromercuribenzoic acid (ii) phenylglyoxal	(i) Cys (ii) Arg	inactivation; NAD, D-pantoate protect	423
Papain	*Carica papaya*	chloromercurinitrophenols	Cys	reporter groups	424
Papain	*Carica papaya*	2,2′-dipyridyldisulphide, *N*-propyl-2-pyridyl disulphide, 4-(*N*-aminoethyl)-2′-pyridyl disulphide, 7-nitrobenzo-2-oxa-1,3-diazole	Cys	comparison of active-site reactivity with ficin	425
Penicillinase	*Staphylococcus*	dimethyl suberimidate	Lys	enhancement of substrate, induced deactivation	426
Pepsin	porcine	α-bromo-4-amino-3-nitroacetophenone	Met, His, carboxyl	chromphores generated; inactivation	427
Peptidyl transferase	*E. coli*	pyridoxal 5′-phosphate, photo-irradiation	Lys	adjacent His in L16 modified	428
Peroxidase	horseradish	fluorodinitrobenzene, $NaIO_4$, $NaBH_4$		attachment to liposomes	429
Peroxidase	horseradish	monosulphuric anhydride of mesohaem	Lys-174	isolation of haem crevice peptide	430
Phenylalanyl-tRNA synthetase	*E. coli*	pentane-2,4-dione	Arg	essential Arg residues	431
Phenylalanyl-tRNA synthetase	*E. coli*	azidonitrophenyl derivatives of $tRNA^{Phe}$		photo-affinity labelling of the β sub-units	432

Phenylalanyl-tRNA synthetase	*E. coli*	β-(*p*-azidoanilide)-GDP, *N*-methyl, *N*-(*p*-azidobenzyl)-γ-amide-ATP		affinity modification blocks nucleotide enhancement	433
Phosphoenolpyruvate carboxykinase	hog liver	3-[^{14}C]bromopyruvate	Cys	difference labelling inactivation	434
Phosphofructokinase	rabbit muscle	[^{3}H]cAMP		photo-affinity labelling in the frozen state	435
Phosphofructokinase	rabbit muscle	2-nitro-5-thio-[^{14}C]cyanobenzoic acid	Cys	cleavage; fragments give Cys positions	436
Phosphofructokinase	rabbit muscle	5′-(*p*-fluorosulphonylbenzoyl)-2-aza-1, N^6-ethenoadenosin, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide	Cys	mapping of cAMP binding site	437
Phosphofructokinase	sheep heart	*p*-fluorosulphonyl[^{14}C]benzoyl-5′-adenosine		affinity labelling	438
Phosphofructokinase	yeast	DTNB	Cys	differential chemical labelling	439

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
D-3-Phosphoglycerate	chicken	*N*-alkylmaleimides	Cys	labelling of NAD^+ binding site	440
3-Phosphoglycerate kinase	yeast	1-cyclohexyl-3-(2-morpholinoethyl) carbodi-imide, nitrotyrosine ethyl ester	Glu	essential Glu identified	441
Phospholipase A_2	cobra venom	*p*-bromophenacyl bromide	His	activity and toxicity lost	442
Phospholipase A_2	cobra venom	various	various	essential *N*-terminal Asn	443
Phospholipase A_2	porcine pancreas	phenylglyoxal, butane-2,3-dione, cyclohexane-1,2-dione	Arg	essential Arg	444
Phosphomevalonate kinase	porcine	(i) DTNB (ii) pyridoxal 5′-phosphate, $NaBH_4$	Cys Lys	essential functional groups	445
Phosphorylase *b*		(i) 4-(2,4-dinitro-5-fluorophenoxy)-2,2,6,6-tetramethyl-1-piperidinyl-oxyl	(i) Lys	spin-labelling study	446
		(ii) DTNB	(ii) Cys	changes e.s.r. spectrum	
Photosystem II herbicide binding protein	spinach	4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol		photo-affinity labelling	447
Phytohaemagglutinin	kidney beans	various reagents		essential carboxyls, Tyrs	448
Pinguinain	*Bromelia pinguin*	β-mercaptoethanol, iodoacetamide	$(Cys)_2$	essential for conformation	449
Plasminogen	human	(i) photo-oxidation, ethoxyformic anhydride	(i) His	(i) essential His	450
		(ii) MeOH–HCl	(ii) carboxyl	(ii) essential carboxyl	
Plasminogen	human	(i) EDC, GlyOMe (ii) cyclohexane-1,2-dione	(i) carboxyl (ii) Arg	essential Arg and carboxyl	451
Platelet factor 4	human	dansyl chloride	Lys	fluorescence anisotropy reporter group	452
Poliovirus		4-vinyl pyridine	Cys, Met	side reaction during acid hydrolysis	453
Prekallikrein	human plasma	DFP	Ser	inactivation of zymogen	454
Progesterone receptor	chick oviduct	17α,21-dimethyl-19-norpregna-4-diene-3,20-dione (R S020), irradiation		photo-affinity labelling with unsaturated keto-steroid	455

Proline reductase	*Clostridium sticklandii*	$LiBH_4$, NH_2OH		evidence for Ser-Glu ester linkage	456
Prorenin	human amniotic sac	ethyl diazoacetyl glycinate–Cu^{2+}	Asp	labellin of zymogen	457
Protein A	*Staphylococcus aureus*	liposomes, SPDP	Lys	cell targetting	458
Protein A	*Staphylococcus aureus*	fluorescein isothiocyanate		labels porcine lymphocytes	459
Proteinase inhibitor I	potato	dithiothreitol, [^{14}C]iodoacetamide	$(Cys)_2$	single disulphide not important	460
Protein inhibitor	potato	tetranitromethane	Tyr	decreased activity	461
Protein kinase	rabbit muscle	EDC, [^{14}C]GlyOMe	Glu	arginine recognition site	462
Protein kinase (cAMP dependent)	bovine brain	Cibacron Blue F36A		affinity labelling of Mg^{2+} binding site	463

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Protein kinase II (cAMP dependent)	porcine heart	8-azido-AMP	Tyr	single Tyr identified	464
Prothrombin	bovine	methyl acetimidate	Lys	activation kinetics and fluorescence study	465
Prothrombin	bovine	^2H_2O	Gla	identification of Gla	466
Protochlorophyllide oxidoreductase	oat	[^{3}H]-*N*-phenylmaleimide	Cys	identification of labelled peptides	467
Putrescine oxidase	*Micrococcus rubens*	EDC	carboxyl	inactivation	468
Pyruvate dehydrogenase	*E. coli*	*N*-ethylmaleimide, *N*-(*p*-benzimidazol-2-yl)phenyl maleimide	Cys	kinetic studies of inactivation	469
Pyruvate kinase	yeast	5′-*p*-fluorosulphonylbenzoyl adenosine		affinity labelling	470
Pyruvate phosphate dikinase	*Bacteroides symbions*	2′,3′-dialdehyde of adenosine 5′-phosphate		ATP binding site labelled	471
Quinolinate phosphoribosytransferase	porcine kidney	various reagents	Cys, Lys, His	essential His, Cys	472
Reverse transcriptase	avian myeloblastosis virus	$tRNA^{Trp}$, deoxythymidine 5′-triphosphate		photochemical cross-linking	473
Rhodanese	bovine liver	2-nitro-5-thiocyanobenzoic acid	Cys	essential SH and SSH cyanylated	474
Rhodopsin	bovine rods	*N*-(4-azido-2-nitrophenyl)-2-aminoethanesulphonate		membrane impermeable photolabelling	475
Rhodopsin		2-(methylsulphonyl)-ethyl acetimidate isothionyl acetimidate	Lys	rhodopsin is a transmembrane protein	476
Riboflavin binding protein	chicken egg	various	various	essential Trp	477
Ribonuclease	*Aspergillus satoi*	[^{14}C]iodoacetic acid, [^{14}C]iodoacetamide	carboxyl, His	essential His	478
Ribonuclease	bovine pancreas	6-chloropurine 9-β-D-ribofuranosyl 5′-monophosphate	α-NH_2	affinity labelling	479
Ribonuclease	bovine pancreas	L-3α-hydoxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid	Cys	gives 2-thioester derivatives of Trp	480

Ribonuclease	bovine pancreas	D-gluconyl glycine azide	Lys	glycoprotein analogue	481
Ribonuclease	bovine pancreas	6-chloropurine riboside 5′-monophosphate	Lys, NH_2	n.m.r. study	482
Ribonuclease	bovine semen	*N*-ethylmaleimide, *N*-(3-dimethylaminopropyl)maleimide	Cys	reduced/denatured forms labelled	483
Ribonuclease		*o*-benzoquinone	Met	conformationally determined fast reaction	484, 485
Ribonuclease	*Rhizopus sp.*	*N*-bromosuccinimide, H_2O_2–dioxan, *o*-nitrophenylsulphenyl chloride	Trp	1—2 essential Trps	486
Ribonuclease A	bovine	succinic anhydride	Lys αNH_2	method for integral NH_2 determination	487
Ribonuclease A		tetranitromethane	Tyr	kinetics of unfolding	488

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Ribonuclease A		iodoacetic acid	Cys	3-disulphide intermediate isolated in refolding study	489
Ribonuclease A		(i) iodoacetic acid	(i) His-12, His-119	(i) increased binding to placental inhibitor	490
		(ii) iodoacetic acid	(ii) Lys-41	(ii) decreased binding	
		(iii) sodium cyanate, methyl acetimidate	(iii) Lys	(iii) decreased binding	
Ribonuclease A		α-methylene-γ-butyrolactones	Cys	models for allergic contact dermatitis	491
Ribosomal proteins	*Bacillus subtilis*	sodium [^{3}H]borohydride, [^{14}C]-formaldehyde	Lys	conformation study	492
Ribosomal proteins	*E. coli*	*N*-acetyl-*N'*-(*p*-glyoxylbenzoyl) cystamine, *M*-maleimidomethyl-ω-(2-methoxy-4-nitrophenoxy)-carboxamidopropane		new RNA-protein cross-linking procedure	493
Ribosomal proteins	*E. coli*	[^{3}H]puromycin		affinity labelling, immuno-electron microscopy	494
Ribosomal proteins	*E. coli*	[^{3}H]chloroamphenicol, u.v. irradiation		affinity labelling	495
Ribosomal proteins	*E. coli*	phenylglyoxal, butane-2,3-dione	Arg	essential for mRNA binding	496
Ribosomal proteins	*E. coli*	[^{3}H]tetracycline		labelled proteins identified	497
Ribosomal proteins	*E. coli*	β-azidophenylacetyl imidoesters and others		RNA-protein photocross-linking	498
Ribosomal proteins	*E. coli*	methyl *p*-nitrobenzene sulphonic acid	His	essential for peptidyl transferase activity	499
Ribosomal proteins	*E. coli*	EDC	carboxyl	protein–RNA cross-linking	500
Ribosomal proteins	*E. coli*	4-azidophenylglyoxal		RNA protein cross-linking	501
Ribosomal proteins	*E. coli*	active esters of 3-(4-bromo-3-oxo-butane-1-sulphonyl)propionic acid	Lys	nucleic acid–protein cross-linking	502
Ribosomal proteins	*E. coli*	photoirradiation		S7—16S tRNA	503
Ribosomal proteins	*E. coli*	ethyl 4-azidobenzoylaminoacetimidate	Lys	RNA-protein cross-linking	504
Ribosomal proteins	hamster fibroblasts	O_2 exposure	Cys	anomalous migration on 2-D gel electrophoresis	505

Ribosomal proteins	L-cells	u.v. irradiation		cross-linking to mRNA	506
Ribosomal proteins	rat liver	u.v. irradiation		L5–5S RNA cross-link	507
Ribosomal proteins	rat liver	[^{14}C]iodoacetic acid	Cys	L6–L2a interaction demonstrated	508
Ribosomal proteins	rat liver	dimethyl suberimidate, dimethyl 3,3′-dithiobispropionimidate	Lys	60S sub-unit protein pairs identified	509
Ribosomal proteins	rat liver	u.v. irradiation		80S RNA–protein cross-linking	510
Ribosomal proteins	rat liver	dimethyl suberimidate	Lys	protein pairs in 40S identified	511
Ribosomal protein S1	*E. coli*	2-nitro-5-thiocyanobenzoic acid, [^{14}C]*N*-ethylmaleimide	Cys	thiols located	512

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Ribulose-1,5-diphosphate carboxylase	spinach	ethoxyformic anhydride	His	active-site residue	513
Ricin toxin	*Ricinus communis*	*m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester and partially reduced anti-Thy 1.2 monoclonal antibody		conjugate selectivity toxic	514
Ricin toxin A chain	*Ricinus communis*	dithiothreitol, SPDP-derivatized epidermal growth factor	Cys	conjugate is a potent toxin	515
Ricin toxin A chain	*Ricinus communis*	dithiothreitol, *Wistaria floribunda* lectin sub-unit	Cys	hybrid toxin of high toxicity	516
RNA polymerase	*E. coli*	9-β-D-arabinofuranosyl-6-thiopurine	Cys	affinity labelling	517
RNA polymerase	*E. coli*	periodate-oxidized UTP		2 types of binding site	518
RNA polymerase	*E. coli*	γ-(azidobenzyl)amide of GTP, γ-azido GTP		photo-affinity labelling	519
RNA polymerase	*E. coli*	5-bromo-UTP, 5-iodo-UTP		affinity labelling	520
RNA polymerase	*E. coli*	polynucleotides containing 8-azido-adenosine or 8-azidoinosine residues		photo-affinity labelling	521
RNA polymerase	*E. coli*	phenylglyoxal	Arg	inhibition template binding affected	522
RNA polymerase	*E. coli*	[^{14}C]methyl acetimidate	Lys	DNA protects *ca.* 17 lysines	523
Saccharopine dehydrogenase	yeast	pyridoxal, pyridoxal 5′-phosphate	Lys	inactivation	524
Saccharopine dehydrogenase	yeast	ethoxyformic anhydride	His	inactivation	525
Secretagogue receptor	guinea-pig pancreatic acini	2-nitro-5-azidobenzoyl-Gly-Asp-Tyr-(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2		irreversible photo-inactivation	526
Serine proteases		[^{3}H]DFP	Ser	interactions with α_2-macroglobulin	527

Serine proteases		*N*-acylsaccharins and *N*-acylbenzo-isothiozolinones	Ser	active-site acylation	528
Serine proteases		(*p*-amidinophenyl)methane sulphonyl fluoride	Ser	affinity version of DMSF	529
Serine proteases		*N*-dansyl peptide chloromethyl ketones		affinity alkylation with fluorescent labels for enzyme localization	530
Sodium channels	frog nerve	trimethyloxonium fluoroborate	carboxyl	role in ion conductance	531
Sodium channels	frog muscle	*N*-bromosuccinimide, TNBS, 4-acetamido-4-isothiocyanato stilbene 2,2′-disulphonic acid, isethionylacetamidate HCl, glyoxal	various	prevention inactivation phase	532
Sodium channels	N18 neuroblastoma cells, rat brain synaptosomes	5-azido-2-nitrobenzoyl mono-[^{125}I]iodoscorpion toxin		photo-affinity labelling	533

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Sodium channels		3-azido-2,4-dinitrophenyl scorpion neurotoxin M_{10}		photo-affinity labelling	534
Spectrin	human erythrocyte	various spin-labelled maleimides		12 Å cavities indicated	535
Spectrin	human erythrocyte	methyl 3-(4-azidophenyldithio)-propionimidate, methyl 4-azidobenzimidate, 4-azidobenzene acid *N*-hydroxysuccinimide ester	Lys	sub-unit structure study	536
Subtilisin-like serine protease	*Thermactinomyces vulgaris*	(i) TPCK (ii) mercuric nitrate (iii) H_2O_2	(i) Ser (ii) Cys (iii) Met	functional group identification and classification	537
Succinyl-CoA synthetase	rat liver	[^{3}H]GDP 3′,5′-dialdehyde		affinity labelling	538
Superoxide dismutase	human erythrocyte	6,6′-dithionicotinic acid organomercurial agarose	Cys	SH-containing peptides	539
Superoxide dismutase	yeast	4-hydroxy-3-nitrophenyl glyoxal	Arg	Arg-143 essential	540
Testosterone S-reductase	rat	(5α20-*R*)-4-diazo-21-hydroxy-20-methypregnan-3-one		enzyme-activated alkylating agent	541
Thioredoxin	*E. coli*	dithiothreitol and various thiol reagents	$(Cys)_2$	differential reactivity of thiols	542
Thymidylate synthetase	*Lactobacillus casei*	various disulphides	Cys	kinetics of inhibition	543
Thymidylate synthetase	*Lactobacillus casei*	5(*E*)-(3-azidostyryl)-2′-deoxyuridine 5′-phosphate		also light-dependent inhibitor of tumour growth	544
Thymidylate synthetase	*Lactobacillus casei*	5-fluorodeoxyuridylate	Cys	^{19}F n.m.r. of covalent adduct	545
Thymidylate synthetase	*Lactobacillus casei*	2-[^{14}C]phenylglyoxal	Arg	essential Arg	546
Thyroid hormone receptor	rat liver	*N*-bromoacetyl derivatives of L-thyroxine and 3,3′,5-tri-iodo-L-thyronine		affinity labelling	547
Toxin B-IV	*Cerebratulus lacteus*	(i) tetranitromethane (ii) 2-hydroxy-5-nitrobenzylbromide	(i) Tyr-9 (ii) Trp (5, 30)	essential residues	548, 549
Transcortin	human	various N-spin-labelled maleimides	Cys	cortisol binding site topography	550

Transcriptase	reovirus	pyridoxal-5′-phosphate–[^{3}H]boro-hydride	Lys	active-site identification	551
Transferrin, ovotransferrin	human, chicken	sodium periodate	Tyr	inactivation	552
Transglutaminase	rat kidney	*N*-carbobenzoxy-diazonorvaline *p*-nitrophenylester		irreversible inhibition of α_2-macroglobulin binding	553
Transhydroxymethylase	rabbit liver	[^{14}C]iodoacetic acid	Cys	active-site thiol	554
Transketolase		phenylglyoxal, butane-2,3-dione	Arg	essential Arg	555
α-Tropomyosin	rabbit muscle	dimethyl adipimidate	Lys	α-sub-unit dimer major product	556

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Troponin, troponin C	bovine	2-(4′-iodoacetamidoanilino)-naphthalene 6-sulphonic acid	Cys	fluorescence study of Ca^{2+} binding	557
Troponin	rabbit muscle	various cross-linking reagents	Lys	Ca^{2+}-induced conformation changes studied	558
Troponin		methyl 4-azidobenzimidate	Lys	photo-cross-linking	559
Troponin C	rabbit muscle	1,3-difluoro-4,6-dinitrobenzene	Lys, Cys	intramolecular cross-link	560
Troponin C	rabbit muscle	dansylaziridine		Ca^{2+}-induced conformational change studied	561
Troponin C	rabbit muscle	*N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide		fluorescence stopped-flow study	562
Troponin I		methyl 4-azidobenzimidate, irradiation	Lys	photo-cross-linking	563
Trypsin	bovine pancreas	*p*-amidinophenyl *trans*-*N*-(1-dimethyl-aminonaphthalene *S*-sulphonyl)aminomethylcyclo-hexanecarboxylate	Ser	fluorescent acyl-enzyme formed	564
Trypsin	bovine pancreas	3-carboxy-2,2,5,5-tetramethyl-l-pyrrolidinyloxyl and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinyloxy-*p*-amidinophenyl esters		spin-labelled inverse substrate	565
Trypsin	bovine pancreas	PMSF–0.05M-KOH	Ser	formation of anhydrotrypsin	566
Trypsin		various inverse substrates	Ser	induced activation of deacylation	567
Trypsin		D- and L-*N*-(2,4-dinitrophenyl) alanine *p*-amidinophenyl esters	Ser	c.d. probe of active site	568
Trypsin		(i) dithiothreitol (ii) mersalyl	(i) $(Cys)_2$ (ii) Cys	(i) inactivates (ii) reactivates	569
Trypsin		(i) dithiothreitol, thiol inhibitor (ii) mersalyl, 4-amino-phenylmercuric acetate	Cys	(i) inactivation (ii) reactivation studied	570
Trypsin		PMSF	Ser	calorimetry of soybean inhibitor binding	571

Trypsin inhibitor	soybean	camphorquinone-10-sulphonic acid, camphorquinonesulphonyl norleucine	Arg	new reversible Arg reagents	572
Trypsin inhibitor	lima bean	pyromellitic di-anhydride, *o*-sulphobenzoic anhydride	Lys	new acylating agents	573
Tryptophan synthetase	*E. coli*	*N*-ethylmaleimide, 2-nitro-5-thiocyanobenzoic acid, bromoacetylpyridoxamine phosphate	Cys	essential thiols located	574
Tryptophan synthetase	*E. coli*	(i) [^{14}C]bromoacetylpyridoxamine (ii) pyridoxal phosphate and [^{3}H]$NaBH_4$	(i) Cys-61 (ii) Lys-86	active-site residues identified	575
Tubulin	porcine	acetaldehyde, [^{3}H]$NaBH_4$	Lys	1 mole/dimer	576
Tubulin		ethyl acetylacrylate	Cys	antibiotic analogues; assembly inhibited	577

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Table (*cont.*)

Protein	*Source*	*Reagent**	*Residue*	*Comments*	*Ref.*
Tumour cell proteins	Ehrlich ascites cells	u.v. irradiation		protein–mRNA cross-linking in intact cells	578
Ubiquinol-cytochrome *c* reductase		*N*-(4-azido-2-nitrophenyl)-*p*-alanyl ubiquinone		photo-affinity label	579
UDP-glucose-4-epimerase	*Saccharomyces fragilis*	diazene dicarboxylic acid bis-*NN*-dimethylamide (or diamide)	Cys	two vicinal thiols in active site	580
Uricase	*Aspergillus flavus*	cyanide ion		enzyme, urate, O_2, and CN^- complex	581
Uridine diphospho-glycose dehydrogenase	bovine	various fluorescent thiol-specific reagents	Cys	fluorescence probes	582
Urocanase	*Pseudomonas putida*	4′(5′)-imidazozone 5′(4′)-propionic acid, O_2	Cys	product-induced inactivation	583
Uroporphyrinogen I synthetase	wheat germ	various sulphydryl reagents	Cys	1 or more Cys at active site	584
Various		*O*-methylisourea	Lys	guanidinated proteins are more stable	585
Various		*p*-iodoethyltrifluoracetamide	Cys	gives 2-aminoethyl cysteine	586

Various		*p*-hydroxyphenyl glyoxal	Arg	chromophoric Arg modified	587
Various		*p*-aminophenyl-*p*-isothiocyanatophenyl derivatives of muramyl dipeptide		protein–peptide conjugates	588
Viral proteins	cowpea chlorotic mottle virus	pyridoxal 5'-phosphate	Lys	fluorescence study	589
Viral proteins	mouse mammary tumour	dithiobis-(succinimidyl propionate), methyl 4-mercaptobutyrimidate		cleavable cross-linking of glycoproteins	590
Viral proteins	turnip yellow mosaic	RNA, u.v. irradiation		*in situ* cross-linking	591
Vitamin D binding protein	human plasma	TNBS	Lys	vitamin inhibits modification	592

* Some reagents have been abbreviated as follows: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TLCK, tosyl-lysine chloromethylketone; DFP, di-isopropyl fluorophosphate; TPCK, tosylphenylalanine chloromethylketone; PMSF, phenylmethylsulphonylfluoride; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)-propionate.

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2 Reinvestigation of Known Reagents and Reactions

Methyl Methanethiosulphonate.—The specificity of this reagent for thiol groups has been questioned.[593] In the case of D-3-hydroxybutyrate dehydrogenase the inactivation obtained with the reagent is due to amino-group modification.

4-Vinylpyridine.—Conversion of methionine residues into *S*-β-(4-pyridylethyl)-L-homocysteine has been reported as a side reaction when proteins are thiol-protected with this reagent and then hydrolysed in 6M-HCl.[594] This may be prevented by trapping any remaining 4-vinylpyridine with mercaptoethanol and repeatedly freeze-drying.

***o*-Iodosobenzoic Acid.**—This reagent is believed specifically to cleave peptide bonds at tryptophan residues. Under normal conditions extensive cleavage at four or five tyrosine residues was observed with carboxymethylated actin.[595] In the absence of any simple explanation for tyrosine susceptibility from consideration of neighbouring sequences, a broader specificity for the reagent is suggested.*

α-Dicarbonyl Compounds.—An explanation for the observed selectivity of these compounds for active-site arginine residues of enzymes acting on anionic substrates or co-enzymes has been given.[596] It is suggested that the positively charged nature of the anionic binding sites causes a reduction in the pK_a of the active-site arginine, resulting in hyper-reactivity.

Iodination.—Protein iodination by the chloramine T or similar methods is a common labelling procedure. Studies on the degradation of labelled proteins by *Xenopus laevis* oocytes following endocytosis have shown that proteins iodinated using chloramine T are apparently degraded abnormally quickly; proteins tritiated by reductive methylation were degraded more slowly.[597] Caution is clearly in order in similar situations.

Reductive Methylation.—Specific activities approaching those obtained by radio-iodination have been obtained with the recently available high specific activity sodium borohydride.[598] Compared with iodination, the advantages are increased safety, minimal change in protein properties, and longer shelf-life.

Sodium Cyanoborohydride.—The efficiency of the labelling procedure using sodium cyanoborohydride and [^{14}C]formaldehyde can be improved by the

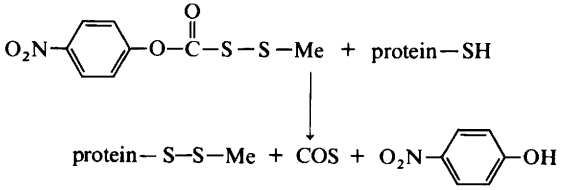
* Lack of specificity is believed to be due to contaminating *o*-iodoxybenzoic acid (from W. C. Mahoney, P. K. Smith, and M. A. Hermodson, *Biochemistry*, 1981, **20**, 443).

[593] R. Kluger and W.-C. Tsui, *Can. J. Biochem.*, 1980, **58**, 629.
[594] J. Heukeshoven, *Anal. Biochem.*, 1980, **109**, 421.
[595] P. Johnson and V. B. Stockmal, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 697.
[596] L. Patthy and J. Thész, *Eur. J. Biochem.*, 1980, **105**, 387.
[597] L. Opresto, H. S. Wiley, and R. A. Wallace, *Proc. Natl. Acad. Sci. U.S.A.*, 1980, **77**, 1556.
[598] N. Jentoft and D. G. Dearborn, *Anal. Biochem.*, 1980, **106**, 186.

addition of Ni^{2+} ions.[599] These form a complex with the cyanide produced by the reaction and therefore prevent cyanohydrin formation with formaldehyde.

3 New Reagents and Techniques

Aminoethylation of cysteine residues has usually necessitated the use of ethylenimine, a carcinogen with a strong tendency towards polymerization; it is therefore pleasing to be able to report an alternative reagent, *N*-(β-iodoethyl)trifluoroacetamide.[600] Specific aminoethylation of cysteines and deacylation is achieved in a single step; separate removal of the trifluoroacetyl group with piperidine is not required. An interesting reagent for the methanethiolation of cysteines has been prepared;[601] *p*-nitrophenyloxycarbonyl methyl disulphide reacts according to the Scheme and the *p*-nitrophenol released readily enables the extent of modification to be determined.



Scheme

S-Mercuric *N*-dansyl cysteine has been used to map the metal binding sites of bovine galactosyl transferase.[602] Reaction is specific for the thiol in or near the UDP-galactose binding site. Resonance energy transfer measurements between dansyl and Co^{2+} in binding site I gave an inter-site spacing of 19 Å, consistent with earlier findings.

A method for staining thiol-containing proteins after polyacrylamide gel electrophoresis has been described.[603] The gel is treated with 2,2′-dihydroxy-6,6′-dinapthyl disulphide, which is then coupled with Fast Black K. Approximately 1 μg of most proteins can be detected, sensitivity depending on thiol content.

Two new reversible arginine-modifying reagents, camphorquinone-10-sulphonic acid and camphorquinone sulphonyl norleucine, have been reported; the extent of modification by the latter can be achieved by acid hydrolysis and norleucine estimation.[604] Reversal of modification by both reagents is achieved by 0·2M *o*-phenylenediamine at pH 8—9. Another chromophoric analogue of phenylglyoxal, *p*-hydroxyphenylglyoxal, has been described for mild specific arginine modification; quantitation, however, requires removal of excess reagent.[605] It is suggested that, because of the titratable phenolic proton, the modified arginine may be of use as a reporter group.

[599] B. F. Tack, J. Dean, D. Eilat, P. E. Lorenz, and A. N. Schechter, *J. Biol. Chem.*, 1980, **255**, 8842.
[600] W. E. Schwarz, P. K. Smith, and G. R. Royer, *Anal. Biochem.*, 1980, **106**, 43.
[601] R. V. Nair and D. J. Smith, *Anal. Biochem.*, 1980, **101**, 316.
[602] E. T. O'Keefe, R. L. Hill, and J. E. Dell, *Biochemistry*, 1980, **19**, 4954.
[603] A. Telser and B. Rovin, *Biochim. Biophys. Acta*, 1980, **624**, 363.
[604] C. S. Pande, M. Pelzig, and J. D. Glass, *Proc. Natl. Acad. Sci. U.S.A.*, 1980, **77**, 895.
[605] R. B. Yamasaki, A. Vega, and R. E. Feeney, *Anal. Biochem.*, 1980, **109**, 32.

A method for obtaining integral values of the amino-group content of proteins has been developed.[606] The protein is treated with increasing amounts of succinic anhydride and a sample of the reaction mixture analysed after each addition by polyacrylamide gel electrophoresis at pH 3.6. By counting the bands obtained, the number of modified lysines in the maximally modified species can be easily determined. Two new reversible lysine acylating agents have been prepared, pyromellitic dianhydride and *o*-sulphobenzoic anhydride.[607] Deacylation is achieved at acid pH.

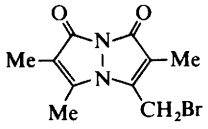
High specific activity labelling of immunoglobins has been reported using methyl 3,5-di[^{125}I]iodohydroxybenzimidate.[608] The reagent, similar to the Bolton and Hunter reagent, has the added advantage of preserving the charge of the lysine upon modification.

2-Imino-2-methoxyethyl 1-thioglycosides provide a means of studying the role of carbohydrate groups in glycoproteins.[609] Many such compounds have been synthesized and allowed to react with the amino-groups of bovine serum albumin. The stereospecificity of the rabbit hepatic carbohydrate-binding system was thereby investigated.

The oxidation of tryptophan residues with dimethyl sulphoxide and HCl has been described.[610] The strongly acidic conditions required, however, restrict application to stable peptides. An activated tryptophan derivative, L-3a-hydroxy-1,2,3,3a, 8, 8a-hexahydropyrrolo[2,3-*b*]-indole-2-carboxylic acid, may be obtained by mild peracetic acid oxidation of tryptophan.[611] This is able to react with protein thiols under acidic conditions to give 2-thioether derivatives of tryptophan. The reagent provides a route to 2-[L-3-alanyl(thiol)]-L-tryptophan, the double amino-acid found in the toxic cyclopeptides of *Amanita phalloides*.

Conversion of γ-carboxyglutamic acid residues to ($\gamma\gamma$-$^2H_2$) glutamic acid has been described.[612] This is achieved by isotopic exchange in ^2H_2O followed by decarboxylation in the same medium. The doubly deuteriated glutamic acid thus formed can then be analysed by mass spectrometry of the derivatized peptide fragment. The use of azidofluoroescein diacetate has been suggested for the labelling of intracellular proteins.[613] The reagent is able to enter the cell where rapid hydrolysis of the ester groups ensues, trapping the reagent inside the cell; upon irradiation proteins are labelled. The technique enabled the microviscosity changes of platelets before and after thrombin activation to be studied. A new class of fluorescent labels based on *syn*-9,10-dioxabimanes, *e.g.* monobromobimane (1), have been used to label haemoglobin in intact and lysed erythrocytes.[614, 615]

[606] M. Hollecker and T. E. Creighton, *FEBS Lett.*, 1980, **119**, 187.
[607] A. Bagree, I. K. Sharma, K. C. Gupta, C. K. Narang, A. K. Saund, and N. K. Mathur, *FEBS Lett.*, 1980, **120**, 275.
[608] G. P. Der-Balian, *Anal. Biochem.*, 1980, **106**, 411.
[609] C. P. Stowell and Y. C. Lee, *Biochemistry*, 1980, **19**, 4899.
[610] W. E. Savige and A. Fontana, *Int. J. Pept. Protein Res.*, 1980, **15**, 285.
[611] W. E. Savige and A. Fontana, *Int. J. Pept. Protein Res.*, 1980, **15**, 102.
[612] K. Rose, J. D. Priddle, R. E. Offord, and M. P. Esnouf, *Biochem. J.*, 1980, **187**, 239.
[613] A. Rotman and J. Heldman, *FEBS Lett.*, 1980, **122**, 215.
[614] N. S. Kosower, G. L. Newton, E. M. Kosower, and H. M. Ranney, *Biochim. Biophys. Acta*, 1980, **622**, 201.
[615] N. S. Kosower, E. M. Kosower, G. L. Newton, and H. M. Ranney, *Proc. Natl. Acad. Sci. U.S.A.*, 1979, **76**, 3382.



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Finally, an interesting technique for the localization of contact areas in protein complexes has been described.[616, 617] Briefly, differential chemical modification involves modifying the complex with trace amounts of radio-labelled reagent, *e.g.* [^{3}H]acetic anhydride, followed by treatment with excess unlabelled reagent under denaturing conditions. Protein without ligand is treated likewise. After protein digestion, the activity of each peptide is determined and the protective effect of the ligand assessed. To obviate difficulties of assessing peptide yields, completely [^{14}C]acetylated protein is added after the tracer labelling step and ^{3}H : ^{14}C ratios are measured. For cytochrome *c* complexed with cytochrome *c* oxidase, cytochrome *c* peroxidase, or cytochrome *bc*, the results are in excellent agreement with those obtained by kinetic analysis of singly substituted lysine derivatives.

4 Cross-linking

Conjugates of two proteins, one cytotoxic, the other providing specificity, are being explored as an approach to cancer therapy; a review of current research effort has recently appeared.[618] Monoclonal antibody and protein A have been coupled to liposomes for cell targeting purposes.[619] *N*-Hydroxysuccinimyl 3-(2-pyridyldithio)propionate (SPDP) was first treated with phosphatidylethanolamine and the liposomes were prepared; the thiolated protein was then coupled.

The base-reversible 13 Å homo-bifunctional cross-linker bis[2-(succinimido-oxycarbonyloxy)ethyl]sulphone has been used to map the membrane surface of transformed mouse leukaemia cells.[620] Cross-linked, radio-iodinated proteins were analysed by two-dimensional SDS polyacrylamide gel electrophoresis, treating the gel with base (pH 11.6) between dimensions. A new hetero-bifunctional reagent, 6-maleimidocaproic acid *N*-hydroxysuccinimide ester, has been described and used to couple chorionic gonadotrophin peptides to carrier proteins for the raising of antibodies.[621]

The possibility of using transglutaminase and specially designed substrates for cross-linking has been investigated.[622] The substrates contain an amino-group that is enzymatically condensed with protein glutamine residues; the second reactive group is either arylazido or a further amine. Cross-linking with guanidated casein was demonstrated but it is uncertain to what extent other proteins will be good substrates.

[616] M. R. Bosshard, *Methods Biochem. Anal.*, 1979, **25**, 273.
[617] R. Reider and M. R. Bosshard, *J. Biol. Chem.*, 1980, **255**, 4732.
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[621] A. C. J. Lee, J. E. Powell, G. W. Tregear, H. D. Niall, and V. C. Stevens, *Mol. Immunol.*, 1980, **17**, 749.
[622] J. J. Gorman and J. E. Folk, *J. Biol. Chem.*, 1980, **255**, 1175.

Several new photo-activatable cross-linking agents have been used to study ribosome structure. These include methyl- and ethyl-4-azidobenzoylaminoacetimidate,[623, 624] methyl-5-(4-azidophenyl)-4,5-dithiopentanimidate,[623] and *p*-azidophenylacetyl imidoesters.[625] Similar reagents including 3-(4-azidophenyldithio)propionimidate have been employed to study membrane-associated spectrin sub-unit structure.[626] Also for intramembrane studies, the hydrophobic azidophenylisothiocyanate has been used to cross-link bacteriorhodopsin.[627]

5 Photoaffinity Labelling

Affinity labelling using photoactivatable low molecular weight ligands or photolabile protein derivatives has continued to be a very popular approach to the characterization of receptor sites in complex biological systems. A review of the applications of this method to the study of antigen–antibody combining sites has appeared.[628] Aromatic or heteroaromatic azides which act as chemically stable precursors of non-rearranging nitrenes are still the most widely used reagents, although stabilized diazoketones and diazirines have also found some applications. There have been increasing numbers of reports on 'direct' photoaffinity labelling experiments using unmodified ligands that do not undergo fragmentation to high-energy intermediates, and on photoactivated substitution reactions.

Nucleotide Analogues.—A γ-azidoanilide derivative of ATP has been used to label the site of amino-acid activation in an aminoacyl tRNA synthetase.[629] Polynucleotide phosphorylase was utilized to prepare azidopolynucleotides based on 8-azidoadenosine and 8-azidoinosine for use in a study of the sub-unit topology of RNA polymerase.[630] A photoreactive derivative of $tRNA^{Phe}$ has also been shown to label the β subunit of phenylalanyl tRNA synthetase.[631] A bifunctional ATP analogue with azide functions in the purine ring and in a side chain attached to the ribose moiety has been used for cross-linking studies on F_1 ATPase.[632] A carbene precursor analogue of NAD^+, 3-(3-*H*-diazirino)pyridine adenine dinucleotide, has been shown to photolabel the nucleotide binding site of rabbit muscle lactate dehydrogenase.[633]

Peptide Analogues.—An interesting series of arylazide derivatives of insulin in which the photoreactive centres are located at specific points in the polypeptide

[623] P. Westermann, O. Nygård, and H. Bielka, *Nucleic Acid Res.*, 1980, **8**, 3065.
[624] R. Millon, M. Olomucki, J.-J. Le Gall, B. Golinska, J.-P. Ebel, and B. Ehresmann, *Eur. J. Biochem.*, 1980, **110**, 485.
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[626] C. R. Middaugh and T. H. Ji, *Eur. J. Biochem.*, 1980, **110**, 587.
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[628] F. F. Richards and J. Lifter, *Ann. N.Y. Acad. Sci.*, 1980, **346**, 78.
[629] N. Abulichev, O. I. Lavrik, and G. A. Nevinsky, *Mol. Biol.*, 1980, **14**, 558.
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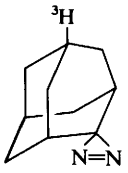
[632] H.-J. Schafer, P. Scheurich, G. Rathgeber, K. Dose, A. Mayer, and M. Klingenberg, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 582.
[633] D. N. Standring and J. A. Knowles, *Biochemistry*, 1980, **19**, 2811.

have been reported.[634-638] These derivatives have been used to locate insulin receptors in various tissues; in one case adipocyte lipogenesis was reported to have been irreversibly 'switched on' by irradiation in the presence of the analogue.[639] Azido-enkephalin analogues have been used in a study of opiate receptors[640] and azidophenyl sulphenyl derivatives of ACTH in which the modification has been restricted to tryptophan have been used as probes of the ACTH receptor.[641, 642] A *p*-azidophenylalanine derivative of α-MSH has been shown to produce irreversible stimulation of melanopores in *Xenopus laevis* and two studies have reported photoaffinity labelling of neuroblastoma sodium channels using azidophenyl and azidobenzoyl derivatives of scorption neurotoxin.[643, 644]

Lipophilic Probes.—There has been increasing interest in both the photoaffinity labelling of intrinsic membrane proteins and in the development of reagents which can react with lipid components of membranes. The high reactivity required of the latter substances has restricted their photoprecursors to diazirines and certain azides. For example, 1-[^{3}H]spiro-[adamantane 4,4′-diazirine], (2), shows promise as a lipophilic precursor of non-rearranging adamantylidene.[645, 646] 3-Trifluoromethyl 3-phenyldiazirine has also been proposed as a (moderately) lipophilic carbene precursor.[647] A combination of spin and photoaffinity labels has been reported in the lipophilic [2-(2-nitro-4-azidophenyl)aminoethyl]-16-doxyl stearate, a reagent which has been used for modifying sarcoplasmic APTase.[648] A lipophilic azide derivative of tyramine has also been reported to photolabel plasma membrane proteins in lymphocytes.[649]



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[649] M. J. Owen, J. C. A. Knott, and M. J. Crumpton, *Biochemistry*, 1980, **19**, 3092.

Direct Photoaffinity Labelling.—The nature of the excited states of reactive intermediates involved in photoaffinity labelling processes in which the protein is irradiated in the presence of a 'natural' ligand is often obscure. However, there is evidence that in photoaffinity labelling with α,β unsaturated ketones or benzophenones, a triplet state is involved in abstraction of hydrogen from the labelled substrate. Recent examples of this type of photoaffinity labelling have utilized an androstatrienone derivative to label Δ^5 3-keto-steroid isomerase[650] and a norpregnadiene dione to label progesterone receptor.[652] [^{3}H]Flunitrazepam has been shown to label benzodiazepine receptors irreversibly upon irradiation.[653] The mechanism of this labelling process is not clear at present although it is known to be dependent on tight non-covalent binding to the receptor. Photoattachment of ribosomal subunits to [^{3}H]puromycin also belongs to this category of labelling and has been studied by immunofluorescent methods using antibodies directed against a puromycin analogue.[654]

Other Reagents.—4-Nitrophenyl α-D-galactopyranoside has been used to photolabel the *lac* carrier protein.[655] Photolabelling with nitrophenyl ethers is known to be mediated *via* nucleophilic aromatic photosubstitution reactions and the process has also been utilized in a maleimido nitrophenoxypropane derivative which can act as a photoactivated heterobifunctional reagent.[656] Diazomalonyldiogoxin derivatives have been used to label *Electrophorus* ATPase[657] and diazosteroids have been employed to label rat α-fetoprotein.[658] A photoarylation reaction, which can be sensitized by energy transfer from excited tryptophan as well as by direct irradiation, is involved in the labelling of *Electrophorus* acetylcholinesterase by *p*-(*NN*-dimethyl)aminobenzene diazonium fluoroborate.[659]

6 Affinity Labelling

Classical affinity labelling is distinguished from most types of photoaffinity labelling by the relatively limited number of functional groups in proteins that can react with the equally limited number of ligand functionalities that are stable to physiological conditions. Affinity ligands have therefore been reviewed by reaction mechanism rather than by structural type. In the case of mechanism-based or 'suicide' inhibitors, where a reactive centre is usually generated by enzyme-mediated elimination or isomerization reactions, the precise reaction mechanism is often unclear.

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Alkylation and Arylation.—Several affinity labels for nucleotide binding sites have been described, based on a chloroalkylaminobenzyl γ-amide of ATP,[660, 661] the 3-chloroacetylpyridine analogue of ADP,[662, 663] and on the dichlorotriazine dyes which are known to bind to the 'dinucleotide fold ' of phosphokinases. The latter reagents have been used to label binding sites in hexokinase, lactate dehydrogenase,[664] and protein kinase.[665] Other affinity labels based on nucleophilic displacements from heterocyclic systems have included 6-chloro-9-β-ribofuranoside (inosine monophosphase dehydrogenase[666]) and a 3-chlorodihydroisoxazoleacetic derivative which has been used to label γ-glutamyl transpeptidases.[667–669] Alkylating derivatives of drugs have not been widely used for probing receptor sites in unpurified systems because of the problem of non-specific labelling of protein sulphydryl groups. However, a bromoacetaminobenzodiazepine (kenazepine) has been used to label benzodiazepine receptors in the rate CNS.[670] *N*-ε-(bromoacetyl)lysine $tRNA^{lys}$ has been used to label tRNA binding sites in elongation factors T and Tu of rabbit reticulocytes,[671] and bromacetyl thyroxine analogues have been utilized in studies of the thyroid hormone receptor.[672] An unusual methylation reaction involving transesterification from methyl phosphate to an essential carboxyl-group in yeast inorganic pyrophosphatase has been reported.[673] Epoxide ring opening has been utilized in the labelling of Δ^5-3-keto-steroid isomerase by spiro-oxiranyl steroids.[674] Recently available information about the sub-site specificity of endopeptidases has allowed the design of more specific chloromethyl ketone derivatives of peptides for the inhibition of pancreatic elastase[675] and urinary kallikrein.[676]

Acylation.—Extensive use continues to be made of the relatively stable sulphonyl fluoride functionality in affinity labels that function by acylation of, for example, active site serine residues. *p*-Amidinophenylmethyl sulphonyl fluoride has been described as an irreversible inhibitor of trypsin-like enzymes.[677] These enzymes also undergo reversible active site acylation by *p*-amidinophenyl esters and this 'inverse' acylation has been used for the synthesis of fluorogenic[678] and spin-

[660] Z. S. Mkrtchyan, L. S. Nersesova, Ah. I. Akopyan, G. T. Babkina, V. N. Buneva, and D. G. Knorre, *Biokhimiya*, 1980, **45**, 616.
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labelled [679] acyl-enzymes. 5'-(*p*-Fluorosulphonyl benzoyl)adenosine has been used to label carbamyl phosphate synthetase [680] and pyruvate kinase;[681] the corresponding 2-aza-N^6-ethenoadenosine derivative has been used as a fluorescent label for phosphofructokinase.[682] (An 'exo' affinity label, in which the fluorosulphonylphenyl moiety is separated from an active-site specific benzamidine function, has been used to map the active centre of bovine coagulation Factor Xa.)[683] Acylation of β-lactamase by the antibiotic cefoxitin has been reported.[684] An unusual example of the use of a mixed anhydride has been reported in the labelling of the erythrocyte lactate transporter by isobutylcarbonyl lactyl anhydride.[685] A similar study of the glucose transporter in human erythrocytes was reported using maltosyl isothiocyanate.[686]

Schiff-base Formation.—Periodate-oxidized (dialdehyde) nucleoside di- and triphosphates have found many applications in labelling of nucleotide binding sites, either alone or in combination with borohydride or cyanoborohydride as reductant. Dialdehyde-ATP has been used to label ATPase,[687] adenylate cyclase,[688] diphtheria toxin,[689] and pyruvate phosphate dikinase.[690] Dialdehyde derivatives of UTP and GPD have also been used in labelling studies on RNA-polymerase and succinyl-CoA reductase, respectively,[691, 692] and periodate-oxidized $tRNA^{met}$ in combination with sodium borohydride was an effective affinity label for metRNA transformylase.[693] The dialdehyde derivatives appear to be fairly specific for lysine residues.

'Suicide' Substrates.—Several suicide substrates of β-lactamase have been studied. In the case of 6-β-bromopenicillanic acid, acylation of a serine residue in the enzyme during opening of the β-lactam ring is followed by rearrangement to give a dihydrothiazine.[694-697] The mechanisms of β-lactamase inactivation by penicillanic acid sulphone[698] and compound PS-5 and *N*-acetyl thienamycin[699] are not clear at present.

trans-Cyclopropylamine has been shown to inactivate monoamine oxidase by formation of an intermediate cyclopropanone or cyclopropanone imine followed

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by the formation of a stable adduct with a protein sulphydryl group.[700] Suicide reagents based on alkyne analogues have included 17β-(1-oxo-2-propynyl)androst-4-en-3-one for 3α,20β-hydroxysteroid dehydrogenase[701] and phenylpropynal for β-lactamase.[702] Also in the steroid field, a diazo-pregnan-3-one has been shown to act as an affinity alkylating agent, possibly by enzyme-catalysed protonation to give a diazonium cation.[703] Dopa-decarboxylase was inactivated by 2-(fluoromethyl)-3-(3,4-dihydroxy)phenylalanine,[704] in a process involving alkylation with loss of fluorine. A similar mechanism probably operates during the inactivation of histidine decarboxylase with α-fluoromethyl histidine.[705]

Dopamine β-hydroxylase is inactivated by *p*-hydroxybenzyl cyanide by a process that does not involve *p*-hydroxybenzaldehyde but may be mediated by an arginine-reactive agent such as *p*-hydroxyphenylglyoximine.[706] An interesting example of 'suicide' product formation occurs in the inactivation of urocanase by a combination of oxygen and the product: 4'-imidazolone 5-propionic acid.[707]

Other Reagents.—Despite the widespread use of chromogenic disulphides for estimation of protein thiols, the use of disulphide interchange reactions in the design of affinity labels has not been widely exploited. However, a dimer of thioinosine triphosphate has now been used to label ATPases in rabbit sarcoplasmic reticulum.[708] An interesting specific halogenation reaction has been used to label D-amino-acid oxidase with radioactive chlorine derived from *N*-chloro-D-leucine. A specific tyrosine was labelled.[709, 710]

PART II: *X*-Ray Studies *By W. D. Mercer*

1 Introduction

The results of *X*-ray crystallographic studies on amino-acids, peptides, and proteins published in 1980 suggest a year of 'tidying up' of structures with a concomitant decrease in the numbers of new structures reported.

Amino-acid structures both in the absence and presence of metal ions are presented in Tables 1 and 2, respectively. Table 3 details preliminary crystal data for proteins and Table 4 presents some of the results obtained by low-angle scattering methods on proteins and other biological structures.

The reports of crystallographic equipment and methods in 1980 show an increased ability to refine structures to obtain thermal parameters that give an

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Table 1 *Amino-acids and peptides*

Compound	*Space group*	*Cell dimensions*						*Z*	*R*	*Ref.*
		a/nm	*b*/nm	*c*/nm	α/°	β/°	γ/°			
α-Glycine[a]	$P2_{1/n}$	0.5084	1.1820	0.5458	90	111.95	90	4	0.046	*b*
Glycine γ form[c]	$P3_2$	0.7046	0.7046	0.5491	90	90	120	3	0.025	*d*
Glycine γ form[e]	$P3_2$	0.6975	0.6975	0.5473	90	90	120	3	0.024	*d*
N-Phthaloylglycine hydroxamic acid[f]	$P2_{1/C}$	0.8353	0.7774	1.5097	90	99.86	90	4	0.039	*g*
Benzoyloxycarbonyl-bis-(α-amino-isobutyryl)-L-alanyl methyl ester[h]	$P2_1$	0.8839	1.0818	1.1414	90	95.69	90	2	0.053	*i*
L-Serine-L-ascorbic acid	$P2_12_12_1$	0.5335	0.8769	2.5782	90	90	90	4	0.036	*j*
L-Glutamic acid[k]	$P2_12_12_1$	0.7068	1.0277	0.8755	90	90	90	4	0.034	*l*
L-Glutamic acid[m]	$P2_12_12_1$	1.0282	0.8779	0.7068	90	90	90	8	0.026	*n*
2,4-Methanoglutamic acid monohydrate	$P\bar{1}$	0.5863	0.8141	0.9310	72.32	74.87	75.22	2	0.089	*o*
L-Arginine-L-ascorbate	$P2_1$	0.5060	0.9977	1.5330	90	97.50	90	2	0.067	*p*
2-Methyl-4-nitroimidazole	$P2_{1/n}$	0.3770	1.5159	0.9769	90	95.58	90	4	0.043	*q*
1-(D-3-Mercapto-2-methylpropionyl)-L-proline[r]	$P2_12_12_1$	0.8811	1.7984	0.6837	90	90	90	4	0.071	*s*
t-Butoxycarbonyl-L-phenylalanine	$P2_12_12_1$	1.1462	2.4453	1.0752	90	90	90	8	0.066	*t*
D,L-Tryptophan	$P2_{1/c}$	1.8986	0.5768	0.9379	90	101.84	90	4	0.085	*u*
D,L-Tryptophan hydrogen oxalate	$P2_12_12_1$	0.5877	0.7722	2.8302	90	90	90	4	0.049	*u*
cyclo(-D-Ala-L-Ala-)	$P2_{1/c}$	0.8498	0.6148	0.7209	90	111.08	90	2	0.034	*v*
Pivaloyl-D-ala-*N*-isopropyl-D-prolinamide monohydrate	$P2_12_12_1$	1.1976	1.6978	1.8457	90	90	90	8	0.058	*w*
Isobutyryl-L-Ala-*N*'-isopropyl-L-prolinamide[x]	$P4_1$	0.9668	0.9668	1.8589	90	90	90	4	0.46	*y*
N-(t-Butoxycarbonyl)-L-Met-Gly-benzyl ester	$P2_1$	1.5884	0.5083	1.3296	90	94.54	90	2	0.055	*z*
cyclo(D-*N*-Methylvalyl-D-α-hydroxy-isovaleryl)	$P2_12_12_1$	1.0968	1.7007	0.6494	90	90	90	4	0.044	*aa*

Pivaloyl-L-Pro-*N*'-isopropyl glycinamide[bb]	$P2_1$	0.9392	0.8458	1.1543	90	100.59	90	2	0.055	*cc*
N-Acetyl-L-Pro-L-4-hydroxyprolyl-[dd]	$P2_12_12_1$	0.7204	0.8322	2.1240	90	90	90	4	0.067	*ee*
(L-Pro-L-4-hydroxyprolyl)mono-hydrate[dd]	$P2_1$	0.6264	0.8940	1.0336	90	101.50	90	2	0.059	*ee*
cyclo(-Pro-L-4-hydroxyprolyl)mono-hydrate[dd]	$P2_12_12_1$	1.0377	1.1777	1.7123	90	90	90	8	0.048	*ee*
Gly-L-4-hydroxyproline[dd]	$P2_12_12_1$	0.5894	0.7894	1.7713	90	90	90	4	0.086	*ee*
cyclo(-Di-L-Pro-D-Pro-)	$P2_12_12_1$	0.8742	1.5423	2.1987	90	90	90	8	0.107	*ff*
cyclo[-Di(benzylglycyl)-L-prolyl-]-monohydrate	$P2_12_12_1$	1.0348	0.8856	2.3235	90	90	90	4	0.104	*ff*
t-Butoxycarbonyl-L-prolylsarco-sinebenzyl ester	$P2_12_12_1$	1.1271	1.8751	0.9372	90	90	90	4	0.070	*gg*
L-Prolylsarcosine monohydrate	$P2_12_12_1$	1.1003	1.1916	0.07795	90	90	90	4	0.097	*gg*
(Glutamyl-α-lactam)histidinylpro-line tartrate monohydrate[hh]	$P2_1$	1.0522	1.6209	0.7472	90	98.88	90	2	0.048	*ii*
L-Leu-L-Pro-Gly monohydrate	$P2_1$	0.9422	0.6724	1.2105	90	100.18	90	2	0.055[jj]	*kk*
t-Butoxycarbonyl-L-Pro-L-Ile-Gly hydrate[ll]	$P2_12_12_1$	1.2909	1.7567	1.0055	90	90	90	4	0.109	*mm*
N-(t-Butoxycarbonyl)-L-Pro-L-Val-Gly hemihydrate[nn]	$P2_1$	1.5783	1.3428	0.9815	90	90.94	90	4	0.084	*oo*
cyclo[Bis-(L-methylvalyl-D-hydroxyisovaleryl)-][pp]	$P2_12_12_1$	1.2625	1.5635	1.2421	90	90	90	4	0.086	*qq*
cyclo[-(D-Ile-lactyl-Ile-D-hydroxyisovaleryl)$_2$-][rr]	$P2_12_12_1$	1.3390	1.6678	2.1349	90	90	90	4	0.105	*ss*
cyclo-Bis(D-lactyl-Ile-D-hydroxyisovaleryl) dihydrate	$P2_12_12_1$	1.1900	1.7090	2.2941	90	90	90	4	0.052	*tt*
cyclo(L-Val-L-Pro-Gly-L-Val-Gly)$_3$[uu]	$R3$	2.8474	2.8474	1.0044	90	90	120	3	0.109	*vv*
Prolinomycin rubidium picrate toluene–chloroform solvate[ww]	$P\bar{1}$	1.6139	1.6312	1.8270	106.70	86.95	106.70	2	0.130	*xx*
Valinomycin barium thiocyanate	$C2$	2.948	1.649	1.992	90	111.4	90	4	—	*yy*
Valinomycin barium perchlorate hydrate[zz]	$P2_12_12_1$	2.8304	1.6938	1.9543	90	90	90	4	0.130	*yy*

Table 1 (*cont.*)

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Table 2 *Metal complexes of amino-acids and peptides*

Compound	*Space group*	*Cell dimensions*						*Z*	*R*	*Ref.*
		a/nm	*b*/nm	*c*/nm	α/°	β/°	γ/°			
Chloroglycylglycinatoimidazole-cadmium	$P2_{1/c}$	0.7338	1.6136	1.1554	90	124.0	90	4	0.042	*a*
Tris(glycine)calcium(II) dibromide	$Pbc2_1$	0.915	1.484	2.031	90	90	90	8	0.095	*b*
[*N*-(2-Pyridylmethyl)-L-aspartato]-(L-phenylalaninato)cobalt(III) trihydrate	$P2_12_12_1$	0.9821	2.3069	0.9564	90	90	90	4	0.059	*c*
(−)5,8,9-Ammineglycinato(1,4,7-triazacyclononane)cobalt(III) di-iodide–0.84-water	$P2_12_12_1$	1.5075	1.7674	1.2625	90	90	90	8	0.0253	*d*
(+)5,8,9-β_2-[(*R*)-Alaninato]{1,7-bis[2(*S*)-pyrrolidyl]-2,6-diazaheptane}cobalt(III) perchlorate hydrate	$P4_12_12_1$	0.9951	0.9951	5.2537	90	90	90	8	0.056	*e*
Histamine : copper(II) chloride 1 : 1	$P\bar{1}$	0.8592	0.9045	0.5893	91.2	98.7	79.9	2	0.061	*f*
[Dicupric tetraglycinato]cuprous chloride complex	$P\bar{1}$	0.8267	0.8408	0.8535	84.89	100.54	114.97	1	0.052	*g*
Aqua-[(*R,S*)-*NN'*-ethylene-bis-(serinato)]copper(II)	$P2_{1/c}$	1.0516	1.2204	1.5631	90	143.88	90	4	0.05	*h*
Aqua-(pyridoxylidene-*O*-phospho-D,L-threoninato)copper(II) dimer monohydrate	$P\bar{1}$	0.9756	0.9866	1.0115	112.44	111.36	89.08	1	0.044	*i*
Diaqua-bis(*N*-acetyl-D,L-tryptophanato)-bis(pyridine)-copper(II)	$P2_{1/c}$	0.9377	1.9341	1.1615	90	123.2	90	2	0.071	*j*
catena-Diaqua-dichloro(μ-glycine)-manganese(II)	$P2_{1/c}$	0.8413	0.5613	1.6816	90	90.20	90	4	0.051	*k*
catena-Octakis-μ-(β-alanine)-trimanganese(II) hexaperchlorate dihydrate	*Pbca*	2.3455	2.1159	1.1187	90	90	90	4	0.082	*l*

Table 2 (*cont.*)

Compound	*Space group*	*Cell dimensions*						*Z*	*R*	*Ref.*
		a/nm	*b*/nm	*c*/nm	*α*/°	*β*/°	*γ*/°			
Bis(D,L-α-alanine)diaqua-manganese(II) dibromide dihydrate	$P2_{1/c}$	0.4790	1.9596	0.9289	90	102.26	90	2	0.065	*m*
Tetrakis(glycylglycine)dimolyb-denum(II) tetrachloride hexahydrate	$P\bar{1}$	0.9775	1.0886	0.9595	107.06	113.15	91.07	1	0.037	*n*
Tetrakis(L-leucine)dimolyb-denum(II) dichloride bis(*p*-toluene sulphonate) dihydrate	$P2_1$	1.2557	2.9938	1.4532	90	92.09	90	4	0.059	*o*
Bis[D-β-(2-pyridyl)-α-alaninato]-nickel(II) dihydrate	$P2_1$	1.4942	1.2091	1.0090	90	90.3	90	4	0.084	*p*
Triaqua(pyridoxylidene-*O*-phospho-D,L-threoninato)-nickel(II) dihydrate	$P\bar{1}$	0.9245	1.4243	0.9754	126.89	79.63	109.97	2	0.052	*i*
trans-Chloroglycinoglycinato-palladium(II)	*Pbca*	0.8443	1.0522	1.9356	90	90	90	8	0.060	*q*
Dichloro(*S*-methyl-L-cysteine methyl ester)palladium(II) monohydrate[r]	$P4_12_12$	0.8309	0.8309	3.386	90	90	90	8	0.062	*s*

Dichloro[(*S*-methyl-L-cysteine)-sulphoxide]palladium(II) monohydrate	$P2_12_12_1$	1.7858	0.8690	0.7008	90	90	90	4	0.061	*t*
cis-Bis(glycinato)platinum(II) oxalic acid dihydrate	$P2_{1/c}$	0.5551	1.7596	1.0169	90	106.88	90	2	0.037	*u*
cis-Amminechloro(glycinato)-platinum(II)	$P2_12_12_1$	0.8228	0.9691	0.7694	90	90	90	4	0.111	*v*
cis-Amminedichloroglycinato-platinum(II)	$P4_12_12$	0.8376	0.8376	2.1193	90	90	90	8	0.054	*v*
cis-Dichloropyridine-glycine-platinum(II)	$P\bar{1}$	0.6752	0.7043	1.2398	96.77	79.40	107.67	2	0.108	*w*
Glycinato-trimethyltin(IV)	$P4_1$	0.7839	0.7839	1.4659	90	90	90	4	0.069	*x*

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Table 3 *Preliminary protein crystallization reports*

Protein	*Source*	*Space group*	*Cell dimensions*			
			a/nm	*b*/nm	*c*/nm	*β*/deg
Mellitin	bee venom	$P6_122$ or $P6_522$	3.65	3.65	12.7	—
		$C222_1$	6.09	3.85	4.23	—
Gene 5 protein	fd bacteriophage	$P6_3$	10.7	10.7	20.6	—
		$C222_1$	11.0	18.0	11.7	—
		$R32$	20.0	20.0	20.5	—
		$P3_1$	14.3	14.3	8.3	—
Protein S	*Myxococcus xanthus*	$P2_12_12_1$	5.299	6.010	10.216	—
Troponin C	rabbit	$P4_2$	8.94	8.94	7.99	—
	chicken	$P3_221$ or $P3_121$	6.67	6.67	6.08	—
rec A Protein		$P6_1$ or $P6_5$	10.31	10.31	8.20	—
		$P4_12_12$ or $P4_32_12$	8.10	8.10	12.74	—
Calmodulin	bovine brain	$P2_1$	6.18	5.67	4.00	92.7
S100	bovine brain	$P4_1$	5.60	5.60	11.28	—
Sarcoplasmic calcium binding protein	crayfish	$P2_12_12_1$	5.89	6.85	11.61	—
Calmodulin	rat testis	$P1$	2.979	5.374	2.478	$\alpha = 93.46$ $\beta = 96.98$ $\gamma = 89.05$
Protocatechuate-3,4-dioxygenase	*Pseudomonas aeruginosa*	$I2$	20.4	12.9	13.7	97.5
Prothrombin fragment 1	bovine blood	$P4_12_12_1$ or $P4_32_12$	7.76	7.76	8.48	—
Deglycosylated fragment 1	bovine blood	$P4_12_12$ or $P4_32_12$	7.76	7.76	8.53	—
Cytochrome oxidase (nitrite reductase)	*Pseudomonas aeruginosa*	$P2_12_12$	9.2	11.5	7.6	—
Ribonuclease T_1	*Aspergillus oryzae*	$P2_12_12_1$	9.18	3.74	7.79	—
Aspartyl tRNA synthetase	yeast	$P4_12_12$	9.2	9.2	18.5	—
Aspartyl tRNA synthetase + tRNA	yeast	$P4_12_12$	8.91	8.91	48.0	—
Glutamate dehydrogenase	tuna liver	$I23$ or $I2_13$	22.84	22.84	22.84	—
Cyclic AMP receptor protein	*E. coli*	$P2_12_12_1$	4.56	9.71	10.54	—
β-Glucuronidase	rat preputial gland	$P4_12_12$ or $P4_32_12$	10.35	10.35	27.98	—
Erysimum latent virus	—	$P2_13$	41.4	41.4	41.4	—
		$B2$	44.2	42.2	38.7	$\gamma = 95°$
Desulphoredoxin	*Desulfovibrio gigas*	$P3_121$ or $P3_221$	4.228	4.228	7.246	—
Ribulose biphosphate carboxylase	*Alcaligenes eutrophus*	$P4_22_12$	11.27	11.27	20.14	—

Mol. wt. and no. of subunits	*Molecular weight of asymmetric units*	V_m nm^3/dalton $\times 10^3$	*Precipitant*	pH	*Ref.*
11 360 (4)	5680	2.1	ammonium sulphate	7.2	*a*
11 360 (4)	5680	2.3	ammonium sulphate	7.2	*a*
20 000 (2)	120 000	2.84	PEG[b]	7.5	*c*
20 000 (2)	120 000	2.42	PEG	7.5	*c*
20 000 (2)	120 000	3.3	PEG	7.5	*c*
20 000 (2)	120 000	2.2	PEG	7.5	*c*
23 000 (1)	46 000	1.77	2-methylpentane-2,4-diol	6.0	*d*
18 000 (1)	72 000	2.20	ammonium sulphate	5.1	*e*
18 000 (1)	18 000	2.17	ammonium sulphate	4.9	*e*
42 000 (1)	42 000	3.0	PEG	5—6	*f*
42 000 (1)	42 000	2.5	PEG + ADP	5—6	*f*
16 680 (1)	33 360	2.09	PEG	5.1—5.3	*g*
21 000 (2)	42 000	2.11	PEG	4.7—4.9	*g*
44 000 (2)	44 000	2.70	2-methylpentane-2,4-diol	6.5—7.0	*g*
16 700 (1)	16 700	2.35	2-methylpentane-2,4-diol	6.0	*h*
783 100 (8)	390 000	2.27	ammonium sulphate	8.0	*i*
20 000 (1)	20 000	3.15	PEG	7.0	*j*
20 000 (1)	20 000	3.19	PEG	7.0	*j*
125 000 (2)	63 000	3.27	ammonium sulphate	6.0	*k*
11 000 (1)	22 000? 33 000?	3.03? 2.02?	ammonium sulphate	7.2	*l*
114 000 (2)	57 000	3.44	ammonium sulphate	6.7	*m*
114 000 (2)	114 000	—	PEG	6.2	*m*
333 000 (6)	166 500	2.98	PEG	5.5	*n*
45 000 (2)	45 000	2.6	potassium phosphate	8.0	*o*
275 000 (4)	1 375 000	2.72	2-methylpentane-2,4-diol	7.5	*p*
—	1/3 virus	—	PEG	7.0	*q*
—	1 virus	—	PEG	7.0	*q*
7740 (2)	7740	2.42	ammonium sulphate	5.2	*r*
534 000 (8 + 8)	133 500	2.39	sodium sulphate	7.8	*s*

Table 3 (*cont.*)

Protein	*Source*	*Space group*	*Cell dimensions*			
			a/nm	*b*/nm	*c*/nm	*β*/deg
Ribulose bisphosphate carboxylase	*Nicotiana sylvestris*	$I422$	14.87	14.87	13.75	—
Neurophysin + dipeptide	pig	$P2_12_12$	15.278	6.908	3.630	—
Aldolase	rabbit	$P2_1$	16.3	6.1	8.2	103.00
Cucurbitin	cucumber	$F23$	13.6	13.6	13.6	—
Glycerol-3-phosphate dehydrogenase	chicken	$P1$	5.89	5.45	5.85	$\alpha = 91°$ $\beta = 95°$ $\gamma = 89°$
Mitochondrial coupling factor BF_1	beef heart	$C222_1$	15.0	13.2	18.0	—
Phytoagglutinin	*Abrus precatorius*	$P2_12_12_1$	13.8	14.2	17.3	—
		$P4_12_12$	13.6	13.6	17.6	—
Somatomammotropin	human chorion	$C222$ or $C222_1$	5.8	8.4	7.5	—
δ-Crystallin	turkey lens	$P2_12_12$	9.99	13.34	6.91	—
Anaphylatoxin C3a	human	$P4_12_12$ or $P4_32_12$	4.40	4.40	10.71	—

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Mol. wt. and no. of subunits	*Molecular weight of asymmetric units*	V_m nm^3/dalton $\times 10^3$	*Precipitant*	pH	*Ref.*
534 000 (8 + 8)	133 500	2.84	PEG	7.0	*t*
10 000 (1)	40 000	2.39	Manganese chloride	5.4	*u*
150 000 (4)	150 000	2.64	ammonium sulphate	6.3	*v*
325 000 (6)	325 000	1.93	sodium chloride	—	*w*
75 000 (2)	75 000	2.57	PEG	7.6	*x*
350 000 (8)	175 000	2.54	ammonium sulphate	7.8	*y*
260 000 (4)	260 000	3.26	ammonium sulphate	—	*z*
260 000 (4)	130 000	3.13	ammonium sulphate	—	*z*
22 300 (1)	22 300	2.05	citrate/pH	5.0	*aa*
200 000 (4)	100 000	2.30	PEG	4.5	*bb*
9100 (1)	9100	2.84	phosphate	4.5	*cc*

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Table 4 *Low-angle scattering results*

Protein	*Source*	*Mol. wt.*	R_G/nm	V/nm^3	*Comments*	*Ref.*
DNA-dependent RNA polymerase—subunit σ	*E. coli*	92 000	4.2	136	A Y-shaped model fits the data best	*a*
DNA-dependent RNA polymerase—subunit α_2	*E. coli*	36 500 × 2	4.4	146	A disc-like model with a deep crevice fits the data best	*b*
DNA-dependent RNA polymerase—core enzyme $\beta^1\beta\alpha_2$	*E. coli*	395 000	6.5	675	A model which fits other observations is presented	*c*
DNA-dependent RNA polymerase—subunit $\beta\alpha_2$	*E. coli*	228 000	6.86	410	—	*c*
DNA-dependent RNA polymerase—core enzyme $\beta^1\beta\alpha_2$	*E. coli*	395 000	—	—	A neutron scattering study which proposes a model for the holo-enzyme	*d*
lac Repressor	*E. coli*	154 000	5.30	329	Also presents data from the proteolytically derived core protein	*e*
lac Repressor	*E. coli*	154 000	4.25	196	A model is proposed which explains the difference between core protein and entire repressor (neutron scattering)	*f*
Ribosomal protein S4	*E. coli*	22 000	1.8	—	Data suggest that S4 is a compact structure, which is unaltered during 30S subunit assembly	*g*
13S Fragment of 16S RNA + protein S4	*E. coli*	200 000	5.0	—	Neutron scattering study	*h*
IgG3 Immunoglobulin	human	180 000	8.6	—	Models are presented for the three forms of the protein	*i*
Fch fragment	human	61 000	3.8	—		
Fc fragment	human	50 000	2.9	—		
IgG Antibodies	rabbit	—	5.17	303	The effects of hapten binding and chemical modifications are described	*j*
Myosin subfragment 1	rabbit	120 000	3.28	151	Models are compared with recent electron microscope work	*k*
Ribonuclease	beef pancreas	—	—	—	The aggregation of the four proteins during *X*-irradiation has been	*l*
Lactate dehydrogenase	pig heart	—	—	—		

Glyceraldehyde-3-phosphate dehydrogenase	yeast	—	—	—	compared to a previously studied protein	
Serum albumin	beef serum	—				
Malate dehydrogenase	yeast	—	—	—	Measurements on the *X*-ray induced aggregation of the enzyme. Conditions which slow this process have been examined	*m*
Malate synthase	yeast	—	—	—	The effects of substrates on *X*-ray induced aggregation and inactivation have been examined	*n*
Haemocyanin 245 component	crayfish	854 000	6.90	1440	Models have been compared with electron micrographs	*o*
Cytochrome oxidase—oxidized	*Pseudomonas*	—	4.05	177	—	*p*
Cytochrome oxidase—reduced	*aeruginosa*	—	3.70	150		
Transcortin	human	52 000	3.55	—	Neutron scattering study	*q*
Colipase–taurodeoxycholate micelles	pig	10 000	1.39	—	Neutron scattering study	*r*
Prothrombin—without Ca^{2+}	human	78 000	3.55	—	The effect of calcium on the proposed	*s*
with Ca^{2+}		78 000	3.95	—	structure is discussed	
Aspartate transcarbamylase	—	—	—	—	A stopped-flow *X*-ray scattering technique is described which allows protein dissocation to be followed	*t*

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insight into the dynamic structure of protein molecules. Other notable publications are a comparison of film scanning equipment and processing programs and a program for determining molecular co-ordinates from published stereo pictures. The use of graphics systems to display macromolecular structures and to model their interactions with other structures, large or small, seems to be increasing.

Sections 3 to 10 cover structure determinations of proteins and viruses. Of interest are: intact immunoglobulin Kol, cytochrome c^1, cytochrome *c* peroxidase (the first haem-containing enzyme to have its structure determined), aspartate transaminase, uteroglobin, ribosomal proteins L7/L12, levansucrase, southern bean mosaic virus, and satellite tobacco necrosis virus.

Sections 11 to 15 cover results from *X*-ray studies on other biologically interesting structures and Section 16 selectively reviews the analysis and prediction of protein conformations.

Hawkins has shown that about ten thousand crystallographic papers are published each year. Faced with such huge amounts of material the choice of material for inclusion in this review has been fairly arbitrary although it is hoped that the coverage of high-resolution protein structures is as complete as possible.

2 Methods and Equipment

Crystallographic Literature.—Hawkins [1] has performed a bibliometric analysis of the crystallographic literature and found that in the period from 1972 to 1976 the number of journal articles on crystallography remained fairly constant at just under 10000 per annum. He has shown that there are 22 core journals, which account for over half the papers published and these journals are ranked on the basis of crystallographic content.

Equipment and Data Collection.—A computer-controlled slit unit suitable for the Phillips PW1100 single-crystal diffractometer has been described,[2] and Hovmöller [3] has reported a fast and accurate way of aligning *X*-ray cameras. Methods for increasing the lifetime of the filament in rotating-anode machines[4] and for lengthening the focal spot in Elliott rotating-anode *X*-ray sets [5] have been published.

A double-stage cryorefrigerator capable of cooling to 10 K without the use of liquid nitrogen or helium has been described.[6] The unit can be mounted on the cradle of a neutron four-circle diffractometer and causes only minor interference with the operation of the diffractometer.

An absorption correction for Weissenberg diffractometers has been described [7] and de Meester [8] has shown how triclinic cell parameters can be obtained from one crystal setting on a Weissenberg camera. Lenhert [9] has reported a method for

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[8] P. de Meester, *Acta Crystallogr.*, 1980, **A36**, 732.
[9] P. G. Lenhert, *J. Appl. Crystallogr.*, 1980, **13**, 199.

testing the uniformity of an *X*-ray beam while Harkema and co-workers [10] have described a correction procedure which allows for such inhomogeneity in the primary *X*-ray beam.

Wilson [11] has examined the relationship between the observed and true intensity of an *X*-ray reflection for four different counting modes.

Direct Methods.—Several significant papers on the methods and computer programs of direct-method structure solution have been published.[12–16] Silva and Viterbo [17] have considered systematically the effects of phase errors of different types on E maps. Both random and systematic errors have been considered and the work shows that small systematic errors can have much greater effects than large random errors. The application of direct methods to structures containing heavy atoms at special or pseudo-special positions has been reported.[18]

Computer Programs.—Programs designed to control a four-circle neutron diffractometer for single-crystal work [19] and for crystal setting and data reduction on a Phillips PW1100 diffractometer [20] have been described.

The design concepts and aims of the XTAL crystallographic computing system have been reported.[21]

Crystal Growth.—Gilmer [22] has described a computer-modelled description of the dynamics of crystal surfaces, which has provided new information on the crystal growth process.

General Protein Crystallography.—James [23] has reviewed the structure determinations of enzymes by *X*-ray crystallography and shown how an understanding of structure can allow full understanding of function.

The irregularities that can occur in biological structures have been classified and analysed for their possible effects on observed *X*-ray, neutron, and electron diffraction patterns.[24]

Protein Crystallography.—The determination of phase angles by anomalous *X*-ray scattering with a four-circle solid-state detector diffractometer has been described [25] and Phillips and Hodgson [26] have presented a methodology for using information of the magnitude of anomalous scattering effects to plan multiple

[10] S. Harkema, J. Dam, G. J. van Hummel, and A. J. Reuvers, *Acta Crystallogr.*, 1980, **A36**, 433.
[11] A. J. C. Wilson, *Acta Crystallogr.*, 1980, **A36**, 929.
[12] B. Busetta, C. Giacovazzo, M. C. Burla, A. Nunzi, G. Polidori, and D. Viterbo, *Acta Crystallogr.*, 1980, **A36**, 68.
[13] C. Giacovazzo, *Acta Crystallogr.*, 1980, **A36**, 74.
[14] A. A. Freer and C. J. Gilmore, *Acta Crystallogr.*, 1980, **A36**, 470.
[15] H. Hauptman, *Acta Crystallogr.*, 1980, **A36**, 624.
[16] J. Karle, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5.
[17] A. M. Silva and D. Viterbo, *Acta Crystallogr.*, 1980, **A36**, 1065.
[18] P. T. Beurskens, P. A. J. Prick, Th. E. M. van den Hark, and R. O. Gould, *Acta Crystallogr.*, 1980, **A36**, 653.
[19] A. Barthélemy and A. Filhol, *J. Appl. Crystallogr.*, 1980, **13**, 101.
[20] M. Biagini-Cingi, G. Bandoli, D. A. Clementi, and A. Tiripicchio, *J. Appl. Crystallogr.*, 1980, **13**, 197.
[21] S. R. Hall, J. M. Stewart, and R. J. Munn, *Acta Crystallogr.*, 1980, **A36**, 979.
[22] G. H. Gilmer, *Science*, 1980, **208**, 355.
[23] M. N. G. James, *Can. J. Biochem.*, 1980, **58**, 251.
[24] J. Woodhead-Galloway, W. H. Young, and D. W. L. Hukins, *Acta Crystallogr.*, 1980, **A36**, 198.
[25] T. Sakamaki, S. Hosoya, and T. Fukamachi, *Acta Crystallogr.*, 1980, **A36**, 183.
[26] J. C. Phillips and K. O. Hodgson, *Acta Crystallogr.*, 1980, **A36**, 856.

wavelength phasing experiments for crystals containing macromolecules. Several different strategies are compared.

Small-angle *X*-ray scattering of ferritin near the iron K absorption edge has been studied using synchrotron radiation.[27]

Fisher and Sweet[28] have described a method of correction for the diffraction data from B-phycoerythrin, which allows the effects of twinning by merohedry to be overcome. They describe methods for determining the volume fractions of the two twins and the effects of the correction on the electron density map are analysed.

Low-angle scattering methods for determining the distance between heavy atoms in protein molecules in solution have been reported.[29]

The effects of low temperatures on the flexibility, temperature factors, mosaic spread, extinction, and diffuse scattering of bovine trypsinogen and Fc fragment have been examined.[30] These parameters are seen to change rapidly in a narrow temperature range indicative of a phase transition, the transition temperature being dependent on the solvent composition.

An automated peak fitting procedure for processing weak neutron diffraction data from protein crystals has been described.[31] The improvements in the data are examined quantitatively.

The results of the International Union of Crystallography microdensitometer project have been published.[32] Fifteen different laboratories processed several precession films and the data have been extensively analysed. The general conclusion is that the average microdensitometer system gives *X*-ray diffraction data at a high level of accuracy.

Structure Refinement.—Vijayan[33] has presented a general theoretical approach to elucidate the effects of errors in atomic positions of known atoms on the positions determined for the unknown atoms. The theory allows an optimal choice of parameters for use in the difference Fourier syntheses.

Konnert and Hendrickson[34] have described a restrained-parameter thermal-factor refinement procedure which allows meaningful anisotropic refinement of macromolecules to be carried out with low-resolution data. The anisotropic thermal parameters obtained using this method should prove of use in the understanding of the dynamics of the functioning of biological macromolecules.

The refinement of sickling deer haemoglobin by restrained least-squares procedures and interactive computer graphics has been reported.[35] The *R*-factor has been reduced from 0.42 to 0.25 and the best strategy to adopt when using these two procedures is discussed.

Crystallographic refinement of actinidin at 0.17 nm by fast Fourier least-squares

[27] H. B. Stuhrmann, *Acta Crystallogr.*, 1980, **A36**, 996.
[28] R. G. Fisher and R. M. Sweet, *Acta Crystallogr.*, 1980, **A36**, 755.
[29] B. K. Vainshtein, L. A. Feigin, Yu. M. Lvov, R. I. Gvozdev, S. A. Marakushev, and G. I. Likhtenshtein, *FEBS Lett.*, 1980, **116**, 107.
[30] T. P. Singh, W. Bode, and R. Huber, *Acta Crystallogr.*, 1980, **B36**, 621.
[31] S. A. Spencer and A. A. Kossiakoff, *J. Appl. Crystallogr.*, 1980, **13**, 563.
[32] S. Abrahamsson, P. Kierkegaard, E. Andersson, O. Lindquist, G. Lundgren, and L. Sjölin, *J. Appl. Crystallogr.*, 1980, **13**, 318.
[33] M. Vijayan, *Acta Crystallogr.*, 1980, **A36**, 295.
[34] J. H. Konnert and W. A. Hendrickson, *Acta Crystallogr.*, 1980, **A36**, 344.
[35] R. L. Girling, T. E. Houston, W. C. Schmidt, jun., and E. L. Amma, *Acta Crystallogr.*, 1980, **A36**, 43.

methods has been described.[36] The *R*-factor has been reduced from 0.429 at 0.2 nm resolution to 0.171 at 0.17 nm resolution with a final estimate of the accuracy of atomic positions as better than 0.01 nm.

A method of detecting and idealizing non-crystallographic symmetry has been reported.[37] The method has been applied to the phase refinement of satellite tobacco necrosis virus and showed both good convergence and the capability of phase extension from 1.0 nm to 0.4 nm resolution.

Graphics Systems and Programs.—A method has been described which allows the derivation of three-dimensional co-ordinates from stereodiagrams of molecular structures.[38] The method has been tested for two proteins, cytochrome b_5 and tomato bushy stunt virus, with r.m.s. deviations of the extracted co-ordinates from the original co-ordinates of 0.19 nm and 0.26 nm respectively. The authors go to some lengths to discuss the ethics of the procedure.

A program system written in Basic and capable of producing high-quality drawings of crystal and molecular structures using only modest hardware has been reported.[39] The operation of the program is fully interactive through user issued console prompts and allows easy preview plotting and correction/editing.

Fitzwater and Scheraga [40] have described a system for fitting a molecular model with fixed bond lengths and angles to a set of Cartesian co-ordinates. The method is particularly applicable to proteins and has been used to fit a model of bovine pancreatic trypsin inhibitor to the co-ordinates derived from the 0.25 nm resolution electron density map. Complete mathematical details of the method are given.

Gund and co-workers [41] have discussed the use of three-dimensional molecular modelling with respect to the design of drugs. The graphics system at Merck Laboratories is described and applications of the system to the study of anti-inflammatory drugs, somatostatin-like compounds, and dihydrofolate reductase inhibitors are summarized.

Electron Microscopy and Diffraction.—Methods for determining the parity in the optical diffraction patterns from structures with helical symmetry have been reported [42] and the fidelity of structure in electron micrographs of negatively stained protein molecules has been examined by comparing micrographs with the known *X*-ray structure of an immunoglobulin.[43]

3 Immunoglobulins and Haptoglobin

Immunoglobulin Kol.—The structures of the intact immunoglobulin molecule Kol and its antigen-binding fragment, Fab, have been refined [44] at 0.3 nm and 0.19 nm resolution respectively to final *R*-factors of 0.24 and 0.26. A range of crystallographic refinement techniques were used and have allowed the structures of the

[36] E. N. Baker and E. J. Dodson, *Acta Crystallogr.*, 1980, **A36**, 559.
[37] C. E. Nordman, *Acta Crystallogr.*, 1980, **A36**, 747.
[38] M. G. Rossmann and P. Argos, *Acta Crystallogr.*, 1980, **B36**, 819.
[39] J. F. de Wet, *J. Appl. Crystallogr.*, 1980, **13**, 625.
[40] S. Fitzwater and M. A. Scheraga, *Acta Crystallogr.*, 1980, **A36**, 211.
[41] P. Gund, J. D. Andose, J. B. Rhodes, and G. M. Smith, *Science*, 1980, **208**, 1425.
[42] S. I. Aizawa and Y. Maeda, *J. Mol. Biol.*, 1980, **137**, 437.
[43] A. C. Steven and M. A. Navia, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4721.
[44] M. Marquart, J. Deisenhofer, R. Huber, and W. Palm, *J. Mol. Biol.*, 1980, **141**, 369.

hinge segment and the hypervariable regions to be clearly visualized. The hinge region is seen to contain a short poly-L-proline double helix and the antigen binding site is compared with the combining site of Fab New. Even after refinement the Fc part of the molecule cannot be located in the electron density map, suggesting a high degree of disorder.

Bence-Jones Protein Mcg.—The crystallization of Bence-Jones protein Mcg in water has been reported [45] and an initial low-resolution, 0.65 nm, electron density map has been calculated. Using a computer graphics system the structures of the protein in the water-grown and ammonium sulphate-grown crystals have been compared. Changes in the spatial relations among the domains are seen including a change in the bend angle between the V and C domains. The light-chain dimer in water-grown crystals adopts a conformation closely related to that of the antigen-binding fragments.

Immunoglobulin G.—Using a combination of accessibility studies, sequence analysis, inhibitor and chemical modification studies a receptor site for complement component Clq on immunologlobulin G has been proposed.[46] The proposed site is two strands of β-sheet which show an area of highly conserved charged amino-acid side chains.

Haptoglobin.—By sequence comparison with the serine proteases a model has been constructed[47] for the structure of the haptoglobin heavy chain, and the implications for haptoglobin–haemoglobin interactions are discussed.

4 Oxygen Carriers and Electron Transfer Proteins

Myoglobin.—The structure of oxymyoglobin has been refined at 0.16 nm resolution using diffractometer data collected at $-12\,^{\circ}C$.[48] The structure has refined to an *R*-factor of 0.159 with internal atom positions being known to an accuracy of 0.01 nm. The haem iron atom lies 0.022 nm out of the plane of the porphyrin, 0.025 nm closer than in the deoxy-form. The F-helix has moved by a similar distance. The oxygen molecule binds to the iron in a bent end-on fashion with an iron—oxygen bond length of 0.183 nm and an iron—oxygen—oxygen angle of 115°. The mean iron to prophyrin nitrogen distance is 0.195 nm, 0.008 nm shorter than in deoxymyoglobin but the difference is not significant compared to the experimental error. The distance from the F-helix histidine imidazole nitrogen, N^{ε}-His 8F, to the iron atom is 0.207 nm, the same distance seen in model compounds. In general, the observed movements of the haem, the iron atom, F-helix, and FG corner upon oxygenation are similar to those found in the T–R state transition in haemoglobin but of a smaller magnitude. Over 300 water molecules and three sulphate ions have been located in the structure.

A model for the kinetics of the refolding of the myoglobin molecule has been presented.[49]

[45] E. E. Abola, K. R. Ely, and A. B. Edmundsen, *Biochemistry*, 1980, **19**, 432.
[46] D. R. Burton, J. Boyd, A. D. Brampton, S. B. Easterbrook-Smith, E. J. Emanuel, J. Novotny, T. W. Radeacher, M. R. van Schravendijk, M. J. E. Sternberg, and R. A. Dwek, *Nature (London)*, 1980, **288**, 338.
[47] J. Greer, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3393.
[48] S. E. V. Phillips, *J. Mol. Biol.*, 1980, **142**, 531.
[49] F. E. Cohen, M. J. E. Sternberg, D. C. Phillips, I. D. Kuntz, and P. A. Kollmann, *Nature (London)*, 1980, **286**, 632.

Human Haemoglobin.—The structure of human carbonmonoxy haemoglobin has been determined at 0.27 nm resolution.[50] The structure was solved by molecular replacement using horse methaemoglobin as the starting structure with an r.m.s. shift for all atoms between the initial model and refined structure of 0.135 nm. The structure shows that the carbon monoxide ligand lies off the normal to the haem in both the α and β subunits, the Fe—C—O group making an angle of 13° to the haem normal and pointing towards the inside of the haem pocket. In the α subunits the iron atoms lie in the haem plane whereas in the β subunits their positions refine to lie 0.022 nm out of the plane. Several specific movements of side chains are described.

Perutz and co-workers [51] have tried to identify the residues responsible for the acid Bohr effect and that part of the alkaline Bohr effect not previously accounted for. The role of the *C*-terminal histidine residue in the alkaline Bohr effect has also been discussed.[52]

Perutz and Imai [53] have considered the regulation of oxygen affinity of human and bovine haemoglobins, chosen as examples of the two classes of mammalian haemoglobins.

The chemical and structural properties of an elongated β chain haemoglobin variant, haemoglobin Cranston, have been reported.[54] The eleven extra amino-acid residues at the *C*-terminus are seen to bind between the two β chains in the central cavity and are relatively protected from the water environment.

Sickling Haemoglobin.—Studies on the identification of the β chain contact sites in haemoglobin S polymers have been described [55] and the phase transformation of deoxygenated haemoglobin S fibres into a new monoclinic crystal form has been reported.[56] Helical crystals of haemoglobin S have been described by Wellems and Josephs.[57]

The production of compounds designed to prevent the sickling of haemoglobin S and their binding to the 2,3-bisphosphoglycerate site have been reported.[58] The bifunctional reagent, bis(3,5-dibromosalicyl)fumarate, crosslinks Lys-82β_1 to Lys-82β_2 and in so doing bridges the 2,3-bisphosphoglycerate binding site. This seems to perturb the Val6β acceptor site sufficiently that sickling is prevented.

Cytochrome b_5.—The orientation of the haem group in cytochrome b_5 has been redetermined by difference Fourier techniques at 0.2 nm resolution.[59] It is now seen that the original less favourable alternative for the orientation was correct and the new orientation is now in agreement with that obtained in a high-resolution n.m.r. study.

[50] J. M. Baldwin, *J. Mol. Biol.*, 1980, **136**, 103.
[51] M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. H. Fogg, P. J. G. Butler, and H. S. Rollema, *J. Mol. Biol.*, 1980, **138**, 649.
[52] J. V. Kilmartin, J. H. Fogg, and M. F. Perutz, *Biochemistry*, 1980, **19**, 3189.
[53] M. F. Perutz and K. Imai, *J. Mol. Biol.*, 1980, **136**, 183.
[54] M. J. McDonald, D. P. Lund, M. Bleichman, H. F. Bunn, A. de Young, R. W. Noble, B. Foster, and A. Arnone, *J. Mol. Biol.*, 1980, **140**, 357.
[55] R. L. Nagel, J. Johnston, R. M. Bookchin, M. C. Garel, J. Rosa, G. Schiliro, H. Wajcman, D. Labie, W. Moo-Penn, and O. Castro, *Nature (London)*, 1980, **283**, 832.
[56] C. C. Chiu and B. Magdoff-Fairchild, *J. Mol. Biol.*, 1980. **136**, 455.
[57] T. E. Wellems and R. Josephs, *J. Mol. Biol.*, 1980, **137**, 443.
[58] J. A. Walder, R. Y. Walder, and A. Arnone, *J. Mol. Biol.*, 1980, **141**, 195.
[59] F. S. Mathews, *Biochim. Biophys. Acta*, 1980, **622**, 375.

Cytochrome *c*.—Tuna ferrocytochrome *c* and ferricytochrome *c* have been refined independently at 0.15 nm and 0.18 nm respectively to *R*-factors of 0.173 and 0.208.[60] Small but significant conformational changes are seen surrounding a buried water molecule. In the oxidized state the water is 0.1 nm closer to the haem and the haem has moved 0.015 nm out of the crevice, both changes leading to a more polar haem environment.

Dickerson [61] has considered the evolution of purple photosynthetic bacteria by examining the cytochrome *c* molecules from the organisms while Osheroff and co-workers [62] have examined the electrostatic interactions in cytochrome *c* that may stabilize the structure of the haem crevice.

A computer simulation of the molecular dynamics of cytochrome *c* and the possible role of these movements in the electron transfer mechanism have been described.[63] This simulation has been compared with information on the internal mobility of cytochrome *c* obtained from thermal factor refinement of the *X*-ray structure.[64] The comparison shows that the two methods give very closely similar results.

Cytochrome c^1.—Cytochrome c^1, a dimeric, high-spin haem protein, from *Rhodospirillum molischianum* has had its structure determined at 0.25 nm resolution.[65] The protein shows little structural resemblance to members of either the cytochrome *c* or the globin structural families, the monomer structure consisting of a left-twisted four α-helix bundle. The subunit interface also consists of a four α-helix bundle and the haem group is covalently attached by two cysteine side-chains to the haem vinyl groups and a histidine to the haem iron atom. The sixth ligand ferric ion co-ordination site is unfilled and this is consistent with the high-spin state.

Ferredoxin.—An *X*-ray study of the structure of a ferredoxin-like protein from *Azotobacter vinelandii* at 0.25 nm resolution has been reported.[66] The molecule contains two Fe—S clusters separated by 1.2 nm and of different sizes. The larger cluster consists of a tetranuclear core, 4Fe—4S, ligated to the protein at each iron atom. The smaller cluster appears almost planar and cannot be modelled by 2Fe—2S or 4Fe—4S centres. The best model for this site is a 3Fe—3S core which forms six contacts with the protein.

The structure of a 2Fe—2S ferredoxin from the blue-green alga *Spirulina platensis* has been described.[67] The 0.25 nm resolution electron density map has allowed the conformation of the molecule and the iron–sulphur cluster to be determined and the authors discuss the structure and compare it with other

[60] T. Takano and R. E. Dickerson, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 6371.
[61] R. E. Dickerson, *Nature (London)*, 1980, **283**, 210.
[62] N. Osheroff, D. Borden, W. H. Koppenol, and E. Margoliash, *J. Biol. Chem.*, 1980, **255**, 1689.
[63] S. H. Northrop, M. R. Pear, J. A. McCammon, and M. Karplus, *Nature (London)*, 1980, **286**, 304.
[64] S. H. Northrop, M. R. Pear, J. A. McCammon, M. Karplus, and T. Takano, *Nature (London)*, 1980, **287**, 659.
[65] P. C. Weber, R. G. Bartsch, M. A. Cusanovich, R. C. Hamlin, A. Howard, S. R. Jordan, M. D. Kamen, T. E. Meyer, D. W. Weatherford, N. H. Xuong, and F. R. Salemme, *Nature (London)*, 1980, **286**, 302.
[66] C. D. Stout, D. Ghosh, V. Pattabhi, and A. H. Robbins, *J. Biol. Chem.*, 1980, **255**, 1797.
[67] K. Fukuyama, T. Hase, S. Matsumoto, T. Tsukihara, Y. Katsube, N. Tanaka, M. Kakudo, K. Wada, and H. Matsubara, *Nature (London)*, 1980, **286**, 522.

ferredoxins. The distribution of variant and invariant amino-acid positions shows the cluster site is quite highly conserved.

Rubredoxin.—An energy minimization study of rubredoxin has been reported.[68] Starting from the refined and unrefined 0.15 nm structures, two similar but not quite identical models of equal energy were obtained. Both these models have a crystallographic *R*-factor midway between the refined and unrefined structures. After alterations to the structure and to the constraints applied, a model with an *R*-factor very close to that of the refined structure was obtained. Details of the active site are well seen in this model except that there is no evidence for one short Fe—S bond.

The crystallographic refinement of rubredoxin at 0.12 nm resolution has also been reported.[69] The final crystallographic *R*-factor is 0.128 and the mean standard deviation in C^{α}—C^{β} bond length is about 0.01 nm. The mean Fe—S bond length in the FeS_4 cluster is 0.228 nm with a range from 0.224 nm to 0.233 nm. Thermal factors for the refined structure are shown to vary over a wide range, the larger values correlating with the structural elements which would be expected to be less rigidly fixed. In the most clearly defined regions of the rubredoxin molecule it is possible to see hydrogen atoms in difference electron density maps.

Cytochrome *c* Peroxidase.—The structure of yeast cytochrome *c* peroxidase has been determined at 0.25 nm resolution [70] using the method of multiple isomorphous replacement. The protein, the first haem-containing enzyme to be studied in detail, consists of a single chain of 293 residues folded into ten α-helical segments and three antiparallel β-pairs. The molecule has two obvious domains with the haem group located in the interdomain crevice. The haem group is sandwiched between two helices with only one edge of the pyrrole ring exposed and the crevice is lined by both aliphatic and aromatic side-chains, several of which make interactions with the haem. There is a proximal histidine haem ligand and a water molecule acts as the sixth iron ligand. From one of the haem crevice helices an arginine, a histidine, and a tryptophan side-chain extend towards the sixth ligand position. The structure of the haem environment has been compared with myoglobin and shows both similarities and differences.

Poulos and Kraut [71] have proposed a model for the cytochrome *c* peroxidase: cytochrome *c* electron transfer complex. Cytochrome *c* peroxidase contains a ring of aspartate side-chains on its surface, which is complementary to the distribution of highly conserved lysines which surround the exposed edge of the cytochrome *c* haem crevice. Model building studies have been used to optimize the interactions and propose a structure in which the haems are parallel with an edge separation of 1.65 nm. The proposed structure is examined in the light of known solution properties and a mechanism of electron transfer is discussed.

This possible mechanism and the stereochemistry of peroxidase catalysis have been described by Poulos and Kraut in a second paper.[72]

[68] D. R. Ferro, J. E. McQueen, jun., J. T. McCown, and J. Hermans, *J. Mol. Biol.*, 1980, **136**, 1.
[69] K. D. Watenpaugh, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, 1980, **138**, 615.
[70] T. L. Poulos, S. T. Freer, R. A. Alden, S. L. Edwards, W. Skogland, K. Takio, B. Eriksson, N. H. Xuong, T. Yonetani, and J. Kraut, *J. Biol. Chem.*, 1980, **255**, 575.
[71] T. L. Poulos and J. Kraut, *J. Biol. Chem.*, 1980, **255**, 10322.
[72] T. L. Poulos and J. Kraut, *J. Biol. Chem.*, 1980, **255**, 8199.

5 Lysozyme and Ribonuclease

Lysozyme.—The crystal structure of tortoise lysozyme, the first reptilian species of the enzyme to be studied, has been reported.[73] The 0.6 nm resolution electron density map shows that the structure is closely homologous to that of the hen egg-white enzyme. The structure also reveals that the crystals of the tortoise enzyme contain a large proportion of liquid, which forms large channels through the crystals. Since the lysozyme active sites face into these channels it is hoped that the tortoise enzyme may be suitable for low-temperature studies of true enzyme–substrate complexes.

The crystal structure of hen egg-white lysozyme has been used to calculate ring-current shifts for the protein, which have been compared with the observed proton nuclear magnetic resonance spectrum.[74]

Ribonuclease.—Crystals of ribonuclease T_1 from *Aspergillus oryzae* have been grown in the presence of 2^1-guanylic acid.[75] Several different crystal forms have been grown and two of these forms show an enzyme : nucleotide ratio of 1 : 1. Isomorphous heavy atom derivatives of each of these forms have been produced and a full structure determination is in progress.

The structure of bovine ribonuclease-A has been studied by a combination of *X*-ray and neutron diffraction techniques and has been refined to an *R*-factor of 0.252 at 0.25 nm resolution.[76] Refinement began based on the partially refined ribonuclease-S co-ordinates and included manual intervention to interpret difference Fourier syntheses using a computer graphics system. Only six side-chains out of 124 are not seen in the electron density map and are most probably disordered. Preliminary information of the neutron study is also discussed.

Creighton[77] has found a three-disulphide intermediate in the refolding of reduced ribonuclease A and has discussed this observation in light of the known structure of the enzyme.

6 Proteolytic Enzymes

***Streptomyces griseus* Proteases.**—The crystal structure at 0.28 nm resolution of the complex between *Streptomyces griseus* protease A (SPGA) and the tetrapeptide inhibitor, chymostatin, has been reported.[78] Reaction of the inhibitor with the enzyme in the crystalline state gives no significant movement of the active-site residues and the difference electron density map confirms the formation of a covalent tetrahedral hemiacetal adduct with the active-site serine residue, Ser-195. The novel structural features of the inhibitor, previously suggested spectroscopically, are confirmed. The authors compare the binding of chymostatin to that seen for another tetrapeptide aldehyde and have shown that the conformation of the His-57 residue is strongly dependent on the nature of the P_2 residue of the bound polypeptide.

[73] R. Aschaffenburg, C. C. F. Blake, H. M. Dickie, S. K. Gayen, R. Keegan, and A. Sen, *Biochim. Biophys. Acta*, 1980, **625**, 64.
[74] S. J. Perkins and R. A. Dwek, *Biochemistry*, 1980, **19**, 245.
[75] U. Heinemann, M. Wernitz, A. Pähler, W. Saenger, G. Menke, and H. Rüterjans, *Eur. J. Biochem.*, 1980, **109**, 109.
[76] A. Wlodawer, *Acta Crystallogr.*, 1980, **B36**, 1826.
[77] T. E. Creighton, *FEBS Lett.*, 1980, **118**, 283.
[78] L. T. J. Delbaere and G. D. Brayer, *J. Mol. Biol.*, 1980, **139**, 45.

The conformation of the native SPGA and its complexes with three synthetic tetrapeptides have been determined and refined at 0.18 nm resolution.[79] *R*-factors for the four structures are in the range 0.122 to 0.142. The results show that the tetrapeptide aldehyde, acetyl-Pro-Ala-Pro-Phe-H, forms a covalent hemicacetal bond with Ser-195, the aldehyde carbonyl carbon–serine O^{δ} distance being 0.173 nm. With the tetrapeptides acetyl-Pro-Ala-Pro-Phe and acetyl-Pro-Ala-Pro-Tyr this distance is 0.258 nm and 0.266 nm respectively and no electron density is seen from the serine O^{δ} to the inhibitor. The three protein regions comprising binding sites S_2, S_3, and S_4 show some conformational changes and the aldehyde inhibitor is seen to move in a concerted manner toward His-57 and Ser-195 as a result of the formation of the hemiacetal bond. With the aldehyde complex large movements of the imidazole ring of His-57 are seen. Two hundred water molecules within the first contact shell of the enzyme have been located, of which only four are internal. Sixteen of these water molecules, which are located in the active site of the native enzyme, are displaced by the tetrapeptides. Possible alterations to the mechanistic pathway based on these results are discussed.

The binding of two tripeptide chloromethylketone inhibitors to *Streptomyces griseus* protease B (SPGB) has been examined.[80] The two inhibitors, *N*-t-butoxycarbonlyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone and *N*-t-butoxycarbonyl-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone, are each bound to the enzyme by three hydrogen bonds from the enzyme main-chain residues 214 to 216 inclusive to the peptide backbones of the inhibitors. Both inhibitors make two covalent bonds with the enzyme, the imidazole ring of His-57 being alkylated and the O^{δ} atom of Ser-195 making a hemiketal bond with the carbonyl carbon atom of the inhibitor. The importance of an electrophilic component in the serine protease mechanism is discussed in the light of the comparison of the binding modes of the two inhibitors.

Actinidin.—The methods used in the refinement of actinidin at 0.17 nm resolution have been described,[36] and the refined structure has been described in detail.[81] The positions of most of the 1666 atoms have been determined with an accuracy better than 0.01 nm, only two residues at the *C*-terminus and one glutamate side-chain not being seen in the structure. A total of 272 solvent molecules have been positioned and the calculated temperature factors have given a good indication of the mobility of various parts of the structure. The geometry of the hydrogen-bonds in the enzyme's secondary structure has been analysed and all are seen to be non-linear. Each of the two domains is built round a hydrophobic core while the interdomain contacts are mostly polar through a network of water molecules. The sulphydryl group seems to be oxidized and the active-site geometry is compatible with the proposed mechanism for papain.

Carboxypeptidase A.—The structure of the complex between carboxypeptidase A and the 39 amino-acid carboxypeptidase A inhibitor from potatoes has been determined at 0.25 nm resolution.[82] The binding of the inhibitor is clearly seen and

[79] M. N. G. James, A. R. Sielecki, G. D. Brayer, L. T. J. Delbaere, and C.-A. Bauer, *J. Mol. Biol.*, 1980, **144**, 43.
[80] M. N. G. James, G. D. Brayer, L. T. J. Delbaere, and A. R. Sielecki, *J. Mol. Biol.*, 1980, **139**, 423.
[81] E. N. Baker, *J. Mol. Biol.*, 1980, **141**, 441.
[82] D. C. Rees and W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4633.

the *C*-terminal peptide bond is, surprisingly, seen to have been hydrolysed with the *C*-terminal glycine residue remaining trapped in the enzyme's binding pocket. The structure of the complex resembles a stage in the catalytic cycle and the side-chain of tyrosine-248 is seen to be oriented in the folded down position and forms interactions with the inhibitor.

Lipscomb [83] has examined possible mechanisms for the functioning of carboxypeptidase in solution and in the solid state.

A 0.28 nm resolution study of the binding of a substrate analogue to carboxypeptidase A has been described.[84] An examination of the structure supports a previously proposed reaction pathway including the role of the enzyme-bound zinc atom.

Pancreatic Trypsin Inhibitor.—A conformational isomer of pancreatic trypsin inhibitor produced by refolding has been described[85] and Creighton[86] has considered the role of the environment on the refolding of this protein.

Trypsin.—A neutron diffraction study which has identified His-57 as the catalytic base in trypsin has been reported,[87] the neutron map at 0.22 nm resolution showing clearly that the mechanistically important proton is co-ordinated to the imidazole side-chain of His-57.

Angiotensin Converting Enzyme Inhibitor.—The crystal structure of 1-(D-3-mercapto-2-methyl propionyl)-L-proline, a specifically designed potent inhibitor of angiotensin converting enzyme, has been determined[88] and compared to the structure of the same compound bound to active-site of penicillopepsin.

DD-Carboxypeptidase.—The structure of the exocellular DD-carboxypeptidase from *Streptomyces albus* G has been determined at 0.45 nm resolution.[89] The molecule is seen to consist of two domains the largest of which seems to bind the zinc ion and also shows a deep cleft near the zinc. Two compounds, a dipeptide inhibitor, and a β-lactam, Δ^3-cephalosporin, have been shown to bind inside the cavity and close to the zinc atom.

Chymosin.—The three-dimensional structure of chymosin at 0.55 nm resolution has been described.[90] The molecule shows a two-domain structure and an obvious depression at the active site, a characteristic of the acid proteases. Studies are continuing to higher resolution.

7 Glycolytic Enzymes

Phosphorylase.—Fletterick and Madsen[91] have reviewed the current knowledge of the structure and function of phosphorylase. Johnson and co-workers[92] have

[83] W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3875.
[84] D. C. Rees, R. B. Honzatko and W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3288.
[85] D. J. States, C. M. Dobson, M. Karplus, and T. E. Creighton, *Nature (London)*, 1980, **286**, 630.
[86] T. E. Creighton, *J. Mol. Biol.*, 1980, **144**, 521.
[87] A. A. Kossiakoff and S. A. Spencer, *Nature (London)*, 1980, **288**, 414.
[88] M. Fujinaga and M. N. G. James, *Acta Crystallogr.*, 1980, **B36**, 3196.
[89] O. Dideberg, P. Charlier, L. Dupont, M. Vermeire, J.-M. Frere, and J.-M. Ghuysen, *FEBS Lett.*, 1980, **117**, 212.
[90] M. G. Safro and N. Andreeva, *Dokl. Akad. Nauk Biochem.*, 1980, **247**, 275.
[91] R. J. Fletterick and N. B. Madsen, *Ann. Rev. Biochem.*, 1980, **49**, 31.
[92] L. N. Johnson, J. A. Jenkins, K. S. Wilson, E. A. Stura, and G. Zanotti, *J. Mol. Biol.*, 1980, **140**, 565.

solved the crystal structure of glycogen phosphorylase *b* in the presence of the weak activator inosine-5^1-phosphate and with bound glucose-l-phosphate at the catalytic site. Using an improved phosphorylase model it has been possible at 0.3 nm resolution to determine the binding interactions of the glucose-1-phosphate. The essential pyridoxal phosphate co-factor lies 0.6 nm away from the substrate site, consistent with previous biochemical data. However, examination of how the pyridoxal phosphate might act in catalysis leads to results that are inconsistent with solution studies. Additionally, it is difficult to accommodate a glycogen substrate with its terminal glucose in the site occupied by glucose-1-phosphate. Model-building has allowed an alternative binding mode for glucose-1-phosphate to be characterized and this alternative easily accommodates glycogen. This binding site has allowed mechanistic proposals to be made, namely that the phosphate group of pyridoxal phosphate acts as a nucleophile while the imidazole side-chain of His-376 functions as a general acid. It is suggested that these are essential features of the mechanism, and the original binding mode of glucose-1-phosphate was in a non-productive manner as a result of the absence of glycogen and AMP. AMP binding is proposed to direct binding to the productive mode.

Hexokinase.—The structure of the complex between hexokinase (form β III) and 8-bromoadenosine monophosphate has been determined at 0.3 nm resolution by difference Fourier methods.[93] The map shows the ribose *anti* to the adenine moiety and the sugar pucker is C-2^1-*endo*. From model building the adenine is seen to bind at a shallow depression on the surface of the large lobe of the enzyme at the entrance to the deep cleft. The adenine N-7 atom and the ribose 2^1 and 3^1 hydroxyls are hydrogen bonded to the enzyme. There is no difference electron density corresponding to the phosphate group. The three phosphates of ATP have been model-built into the active site together with the essential metal ion, and the β- and γ-phosphates are seen to make hydrogen bonds to one serine residue and the backbone nitrogen of another serine. The cobalt ion also forms interactions with this second serine residue. In the closed conformation of the enzyme, which is induced by glucose binding, there would be additional contacts between the small enzyme lobe and the metal ion. This could explain the observation of synergism for ATP and glucose binding to hexokinase in solution. In this model-built structure the γ-phosphate of the ATP is nearly 0.6 nm away from the 6-hydroxyl of glucose and therefore an additional conformational change must occur in the enzyme upon ternary complex formation.

Two papers dealing with the structure of the complex between yeast hexokinase A and glucose have been published.[94, 95] The structure of the hexokinase A–glucose complex has been solved at 0.45 nm resolution by multiple isomorphous replacement and the co-ordinates refined at 0.35 nm resolution.[94] Initially the 0.6 nm resolution electron density map based on one isomorphous derivative was used to orient the native hexokinase B structure in the hexokinase A–glucose unit cell. From this, molecular replacement phases were calculated. The results show a large conformational difference between the complex and the hexokinase B

[93] M. Shoham and T. A. Steitz, *J. Mol. Biol.*, 1980, **140**, 1.
[94] W. S. Bennett, jun. and T. A. Steitz, *J. Mol. Biol.*, 1980, **140**, 183.
[95] W. S. Bennett, jun. and T. A. Steitz, *J. Mol. Biol.*, 1980, **140**, 211.

structure and stopped an attempt to extend the phases from 0.6 nm to 0.35 nm by molecular replacement. The improved 0.45 nm resolution multiple isomorphous replacement map confirmed the bilobal folding of the hexokinase A molecule, the folding within the individual lobes being very similar to that of native hexokinase B. The relative orientations of the two lobes is, however, quite different and a structure refinement to 0.35 nm resolution with an *R*-factor of 0.26 has allowed detailed comparison.

Analysis of the hexokinase A–glucose complex and native hexokinase B structures has revealed several obvious differences.[95] One lobe is rotated 12° with respect to the other and there are several systematic differences in the backbone conformation adjacent to the glucose binding site and the crystal packing contacts. In the glucose-bound complex the active site is narrowed and substantially reduces the accessibility of the active site to solvent. The binary complex structure can be formed by either subunit in the heterologous dimer of hexokinase B. New or altered interactions between subunits, or with ligands bound in the intersubunit ATP site, may be formed when the upper subunit of the dimer is in the closed conformation and may contribute to the co-operative interactions observed in the crystalline dimer and in solution.

D-Glyceraldehyde-3-phosphate Dehydrogenase.—The co-enzyme-free form of lobster D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been prepared and crystallized.[96] The protein crystallizes in a triclinic space-group and some of the zones of the reciprocal lattice show very close similarity to zones observed for human apo- and holo-GAPDH crystals and southern rock lobster holo-GAPDH crystals. The apo-structure was initially solved by comparison with the known lobster holo-enzyme structure. This initial approximate solution was then refined using the 222 molecular symmetry and the molecular replacement technique. Only minor conformational changes are seen after removal of the co-enzyme. The differences that occur are localized in the S-shaped loop and possibly the adenine pocket. Displacements on the S-loop were up to 0.45 nm but only the movement of the Trp-193 residue can be classed as a definite conformational change, other differences being inaccurate fitting of poor side-chain density.

8 Hormones

Hormone Families.—Blundell and Humbel[97] have discussed the existence of hormone families, the members of which show structural and functional relationships. They present four hormone families, which in the pancreas are represented by insulin, glucagon, somatostatin, and pancreatic polypeptide. Relaxin, a polypeptide hormone from the *corpus luteum*, seems to be a member of the insulin family together with the somatomedins or insulin-like growth factors. Sequence and model-building comparisons are presented. Glucagon is a member of a large family of homologous polypeptides including secretin, vasoactive intestinal peptide, and gastric inhibitory peptide, and their relationships are discussed. The possible evolutionary pathways of the pancreatic hormones and their relation to the gastrointestinal and neuroendocrine systems are considered.

[96] M. R. N. Murthy, R. M. Garavito, J. E. Johnson, and M. G. Rossmann, *J. Mol. Biol.*, 1980, **138**, 859.
[97] T. L. Blundell and R. E. Humbel, *Nature (London)*, 1980, **287**, 781.

9 Other Globular Proteins

Amylase.—The crystal structure of amylase-A from *Aspergillus oryzae* has been determined at 0.3 nm resolution.[98] A total of 452 amino-acid residues have been positioned, about 20% of which are present in the eight α-helices. There is a small amount of β-structure and the four disulphide bonds were clearly visible. The molecule is composed of two domains and the *C*-terminal domain containing only β-type secondary structure is much smaller than the main domain. The large domain shows a pronounced cleft in which inhibitors and poor substrates have been demonstrated to bind and at one end of which lie several possible catalytically important residues.

The structure of porcine pancreas α-amylase has been determined at 0.5 nm resolution.[99] The molecule appears as a bilobal structure 7.5 nm × 5.5 nm × 5.0 nm with a 3 nm long cleft on one side. A modified maltotriose molecule has been shown to bind in this cleft and on the surface of the molecule, the cleft being identified as the active site. The site of the loosely bound calcium ion has been identified but as yet the location of the essential calcium ion has not been determined.

Bacteriorhodopsin.—The use of neutron diffraction to locate the positions of valine and phenylalanine residues in the purple membrane structure has allowed the distribution of amino-acids within the bacteriorhodopsin molecule to be determined.[100] The results suggest that the charged and polar groups tend to lie at the molecular interior while the non-polar surfaces are directed outwards. Compared to soluble proteins the structure can be thought of as being 'inside-out'.

The sequence of bacteriorhodopsin has been used to interpret the electron density map of the molecule.[101] Having first selected seven segments of sequence as probable transmembrane helices, all of the 5040 possible ways of fitting the density were considered. Using criteria of connectivity of the non-helical link regions, charge neutralization, and total scattering density per helix, a single most probable model emerged.

A new two-dimensional crystal form of purple membrane has been produced and the projected structure to 0.65 nm resolution determined by electron microscopy and diffraction.[102] It shows an indentical structure to that of the native form of the molecule.

Henderson and Shotton[103] have reported the crystallization of purple membrane in three dimensions. Though not large enough for *X*-ray analysis the crystals appear microscopically crystalline in thee-dimensions with space-group *P*321 or *P*312.

The location of the retinylidene chromophore in bacteriorhodopsin has been

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[100] D. M. Engelman and G. Zaccai, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5894.
[101] D. M. Engelman, R. Henderson, A. D. McLachlan, and B. A. Wallace, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2023.
[102] H. Michel, D. Oesterhelt, and R. Henderson, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 338.
[103] R. Henderson and D. M. Shotton, *J. Mol. Biol.*, 1980, **139**, 99.

determined by neutron diffraction.[104] The retinal is seen to be located between α-helical segments with nearest neighbour separations of 2.6 nm intratrimer and 3.8 nm intertrimer.

Catalase.—The three-dimensional structure of catalase from *Penicillium vitale* has been described at 0.35 nm resolution [105] and at 0.6 nm resolution.[106] At the higher resolution [105] the electron density map for each subunit can be interpreted as a single polypeptide chain of 650 residues with 160 in α-helices and 120 in β-structures. The molecule consists of three domains: a large α/β domain arranged as a β-cylinder, a smaller all-α-domain of about 150 residues, and a *C*-terminal 150-residue domain of α/β structure similar in structure to flavodoxin. Subunit boundaries between the four subunits are not very clear but the two haem groups lie on the non-crystallographic axis of symmetry.

An investigation of the structure of tubular crystals of catalase has been reported.[107]

Aspartate Transaminase.—The structure of chicken heart cytosol aspartate transaminase at 0.35 nm resolution has been described.[108] The subunits of the dimeric enzyme show extensive secondary structure, a total of nine α-helices having been located. One of these helices is 4.8 nm long. The core of the subunits consists of parallel β-structure. The binding site for the pyridoxal phosphate co-enzyme has been identified and seems to involve a helix dipole interaction in the binding. The interactions of substrates with the enzyme have been examined with a view to determining if any conformational changes occur, and these results are discussed.

The 0.28 nm resolution structure of chicken mitochrondrial aspartate aminotransferase has been reported.[109] The subunits are rich in secondary structure with a seven-stranded α/β fold forming the pyridoxal phosphate binding domain. The active sites are located near the deep crevices that form the subunit–subunit interface and it is hoped that examination of the amino-acid side-chains present will lead to an understanding of the mechanism.

Wheat Germ Agglutinin.—The structure of the non-covalent complex between wheat germ agglutinin and *N*-acetyl-D-neuraminic acid has been determined at 0.28 nm resolution.[110] The difference electron density map shows two strong binding sites on the agglutinin dimer located in crevices at the subunit–subunit interface. The saccharide binds with its acetyl group buried whereas the charged carboxylate and the glycerol groups point away from the surface, although they can still interact with surface residues.

[104] G. I. King, P. C. Mowery, W. Stoeckenius, H. L. Crespi, and B. P. Schoenborn, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4726.
[105] B. K. Vainshtein, V. R. Melik-Adamyan, V. V. Barynin, and A. A. Vagin, *Dokl. Akad. Nauk Biochem.*, 1980, **250**, 9.
[106] B. K. Vainshtein, V. R. Melik-Adamyan, V. V. Barynin, A. A. Vagin, Yu. V. Nekrasov, L. V. Malinina, M. F. Gulyi, L. V. Gudkova, and R. G. Degtyar, *Dokl. Akad. Nauk Biochem.*, 1980, **246**, 151.
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[109] G. C. Ford, G. Eichele, and J. N. Jansonius, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2559.
[110] C. S. Wright, *J. Mol. Biol.*, 1980, **139**, 53.

These binding studies have been extended[111] to include *N*-acetyl-D-glucosamine derivatives, 6-iodo-1,4-dimethyl-*N*-acetylglucosamine and *N*-acetylneuraminic acid lactose. The interactions involved in the various binding modes and their implications for the functioning of the agglutinin have been discussed.

The structures of snake venom postsynaptic neurotoxins and the domains of wheat germ agglutinin have been shown to have a remarkably similar folding pattern.[112] This consists of equivalently placed but variably sized loops held together by four similarly positioned disulphide bonds. A similar structure is predicted for two small plant proteins, hevein and ragweed pollen allergen Ra5, on the basis of sequence matching.

Ferritin.—The structure and function of ferritin have been reviewed in detail[113] and an improved interpretation of the 0.28 nm resolution electron density map of horse spleen apoferritin has been reported.[114] The improved map has allowed a more detailed determination of the disposition of secondary structure and a short section of electron density previously unaccounted for has been found. Two alternative helix connectivities are described and compared with the conformations of other known proteins.

Neurotoxins.—A comparison of the neurotoxin and wheat germ agglutinin folds has been published.[112]

The crystal structure of variant-3 toxin from the scorpion has been reported at 0.3 nm resolution.[115] The secondary structure consists of $1\frac{1}{2}$ turns of α-helix and a three-strand stretch of β-sheet, the central sheet strand being connected to the helix by two disulphide bridges. Several loops of chain extend out from this core and there is an identifiable area where several of the conserved residues are clustered.

The 0.28 nm resolution structure of the 'long' neurotoxin from cobra venom has been described.[116] The chain folds into three loops and one tail extending from a globular head. The longer central loop is flanked by two shorter ones and the tail lies behind the central loop. The conformation is determined by four disulphides in the head and one at the tip of the long loop, by a triple-stranded β-sheet involving this loop and by hydrophobic interactions stablizing the other loops. The structure is compared with that of the short erabutoxin b which shows a similar arrangement of structurally and functionally invariant groups.

Uteroglobin.—The crystal structure of oxidized uteroglobin at 0.22 nm resolution has been reported.[117] The molecule, a dimer, is composed of two identical polypeptide chains of 70 residues each. In the crystal form studied these two subunits are related by a two-fold axis and the subunits are held together by two disulphide bridges. The structure consists of a single domain containing about 70% α-helix and no β-sheet. The structure shows a central oblong hydrophobic

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[112] J. Drenth, B. W. Low, J. S. Richardson, and C. S. Wright, *J. Biol. Chem.*, 1980, **255**, 2652.
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[115] J. C. Fontecilla-Camps, R. J. Almassy, F. L. Suddath, D. D. Watt, and C. E. Bugg, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 6496.
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[117] J. P. Mornon, F. Fridlansky, R. Bally, and E. Milgrom, *J. Mol. Biol.*, 1980, **137**, 415.

cavity, which in size is capable of accommodating progesterone. However, in the oxidized protein studied there is no access available for progesterone to enter the cavity and bind.

Ribosomal Proteins L7/L12.—The structure of the *C*-terminal fragment of the protein L7/L12 from *E. coli* has been determined at 0.26 nm resolution.[118] The protein shows a compact plum-shaped structure and consists of 33% of the residues in three α-helices and 14% of the residues in three β-strands. An anion binding site, occupied by a sulphate ion, has been identified and is analogous to that found in a large family of enzymes that bind nucleotides. The site is located between the *C*-terminal side of a β-strand and the *N*-terminus of a helix. This could be the binding site for the phosphates of GTP.

Canavalin.—The three-dimensional structure of canavalin from jack bean has been reported at 0.3 nm resolution.[119] The two major fragments that make up the canavalin monomer are derived from the *N*- and *C*-terminal halves of a precursor protein of molecular weight 42 000. The structures of these two fragments are virtually identical and are related by a near-exact molecular two-fold axis. The two halves of the monomer are themselves composed of two discrete domains, in one case corresponding to the secondary cleavage products of a major fragment. One domain contains the zinc ion binding site and the other domain forms a distinct cleft that may be a substrate-binding region. The molecule is composed almost entirely of β-structure, organized into a series of inter-related sheets, and the positions of the proteolytic cleavage points have been identified.

Levansucrase.—The tertiary structure of levansucrase from *Bacillus subtilis* has been determined at 0.38 nm resolution.[120] The levansucrase molecule is a very elongated ellipsoid with overall dimensions 2.6 nm × 3.2 nm × 11.7 nm and the top of the molecule appears to be formed by four strands of β-sheet surrounded by eight short helices. Below this domain the molecule narrows to a waist, which in solution may be flexible. The bottom of the molecule, which contains the *N*- and *C*-termini, is made up by four strands of β-sheet interconnected by two short helices.

***p*-Hydroxybenzoate Hydroxylase.**—The five peptides produced by cyanogen bromide cleavage of *p*-hydroxybenzoate hydroxylase have been aligned using the 0.25 nm electron density map of the protein.[121] This combination of results has allowed an analysis of the binding of FAD to the enzyme. The AMP moiety is bound to a βαβ unit resembling that found in the dehydrogenases. The ribityl residue and the isoalloxazine ring form several hydrogen bonds to the protein. The structure has been compared with other known flavo-protein structures.

Glutathione Reductase.—The two nucleotide binding domains of glutathione reductase have been examined to see if the similarity of the folds is significant or not.[122] The results indicate that there is an evolutionary relationship as the result of a gene duplication.

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[119] A. McPherson, *J. Biol. Chem.*, 1980, **255**, 10 472.
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[121] J. Hofsteenge, J. M. Vereijken, W. J. Weijer, J. J. Beintema, R. K. Wierenga, and J. Drenth, *Eur. J. Biochem.*, 1980, **113**, 141.
[122] G. E. Schulz, *J. Mol. Biol.*, 1980, **138**, 335.

Elongation Factor Tu.—The structure of the tetragonal crystal form of trypsin-treated elongation factor Tu has been determined at 0.5 nm resolution.[123] The location of the trypsin-removed polypeptide of 14 residues has been located. The protein consists of two domains, the larger one exhibiting considerable α-helical structure but the smaller domain showing no recognizable secondary structure features. The authors present a correlation of the low-resolution structure with the known biochemical properties.

D-Galactose Binding Protein.—The structure of D-galactose binding protein, a receptor for both a high-activity transport system and chemotaxis in *E. coli*, has been solved at 0.41 nm resolution.[124] The molecule is ellipsoidal with dimensions 6.5 nm × 3.5 nm × 3.5 nm and consists of two domains separated by a cleft. Initial chain tracing at this low resolution suggests a structure similar to that of L-arabinose binding protein.

C-Phycocyanin and B-Phycoerythrin.—The structures of C-phycocyanin from *Anabaena variabilis* at 0·5 nm resolution and B-phycoerythrin from *Porphyridium cruentum* at 0.525 nm resolution have been reported.[125] C-Phycocyanin is a light harvesting protein composed of 6α and 6β subunits. The molecule is seen to be 11 nm in diameter, 4 nm thick, and has a 2 nm diameter central channel. B-Phycoerythrin is composed of 6α, 6β, and 1γ subunits and the molecule is found to be 10.7 nm in diameter and 5.5 nm thick. There is a region of low, unstructured density at the centre of the molecule, which appears to be the disordered γ subunit.

Histone Nucleosome Core.—Image reconstruction at 2.2 nm resolution has shown that the histone octamer $(H3)_2$-$(H4)_2$-$(H2A)_2$-$(H2B)_2$ is a left-handed helical spool with a two-fold axis of symmetry.[126] About two turns of a flat superhelix of DNA could be wound onto the spool in the nucleosome. From the observed structure and biochemical studies, the disposition of individual histones has been proposed.

Glyoxalate Oxidase.—The crystal structure of the octameric enzyme glyoxalate oxidase from spinach has been solved at 0.55 nm resolution.[127] The molecules are roughly spherical in shape and are approximately 10 nm in diameter. Large solvent channels run through the crystal. The secondary structure of the subunit appears to consist of an 8-unit β-strand α-helix barrel similar to triose phosphate isomerase. The binding site of a substrate analogue has been located in a deep cleft at one end of the barrel and near its axis.

γ-Crystallin.—The crystal structure of calf lens γ-crystallin III b at 0.5 nm resolution has been described.[128] The molecule is an ellipsoid 5.0 nm × 2.9 nm × 2.5 nm and appears to consist of two domains. Its structure is compared with that of other crystallins.

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[128] Yu. N. Chirgadze, V. D. Oreshin, Yu. V. Sergeev, S. V. Nikonov, and V. Yu. Lunin, *FEBS Lett.*, 1980, **118**, 296.

10 Viruses

Southern Bean Mosaic Virus.—The crystal structure of southern bean mosaic virus (SBMV) has been determined at 0.28 nm resolution[129] using the method of multiple isomorphous replacement. The final electron density was averaged over the ten different icosahedral units within the crystallographic asymmetric unit. The dominant structural feature is a β-barrel structure and five α-helices can be recognized. Great similarity to the tomato bushy stunt virus (TBSV) shell domain is seen but there is little electron density for the position of nucleotides. In addition to the eight-stranded antiparallel β-barrel, 66 residues at the *N*-terminus of the C subunits form a partly ordered arm extending into the centre of the virus. This is also seen in the TBSV structure. The surprising similarity of SBMV and TBSV, despite their differences in physical characteristics, is considered in some detail.

Satellite Tobacco Necrosis Virus.—An electron density map of satellite tobacco necrosis virus (STNV) has been obtained at 0.4 nm resolution using one isomorphous derivative and phase refinement by icosahedral averaging.[130] The particle has 60 protein subunits in the shell and is seen to have a roughly regular icosahedral shape. At the innner boundary of the protein coat there is some evidence of partially ordered RNA structure. The protein subunits consist of one main domain with a three-stranded β-sheet and they have an arm extending towards the centre of the particle. Sequence work suggests that this arm may be involved in nucleotide binding. The phase extension technique has also been reported.[37]

Tobacco Mosaic Virus.—An analysis of the structure of the tobacco mosaic virus (TMV) coat protein has led to a proposal for the possible evolution of the molecule.[131] A primitive dimeric structure for the TMV protein is suggested and a tandem gene duplication is proposed. The involvement of the RNA in the structure is also discussed.

Bacteriophage Pf1.—The structure of phage Pf1 has been determined at 0.7 nm resolution by analysis of fibre diffraction data.[132] The coat protein structure is seen to consist of two α-helical segments, one almost parallel to the particle axis, the other tilted about 25° from the particle axis. The tilted helix lies at higher radius than the parallel helix and between them they form a 2 nm thick double layer of tightly packed and intricately interacting α-helices to protect the viral DNA.

11 Nucleic Acids

tRNA^{Asp}.—The crystal structure of yeast tRNA^{Asp} has been determined at 0.35 nm resolution for two interconvertible crystal forms.[133] The structure is seen

[129] C. Abad-Zapatero, S. S. Abdel-Meguid, J. E. Johnston, A. G. W. Leslie, I. Rayment, M. G. Rossmann, D. Suck, and T. Tsukihara, *Nature (London)*, 1980, **286**, 33.

[130] T. Unge, L. Liljas, B. Strandberg, I. Vaara, K. K. Kannan, K. Fridborg, C. E. Nordman, and P. J. Lentz, jun., *Nature (London)*, 1980, **285**, 373.

[131] A. D. McLachlan, A. C. Bloomer, and P. J. G. Butler, *J. Mol. Biol.*, 1980, **136**, 203.

[132] L. Makowski, D. L. D. Caspar, and D. A. Marvin, *J. Mol. Biol.*, 1980, **140**, 149.

[133] D. Moras, M. D. Comarmond, J. Fischer, R. Weiss, J. C. Thierry, J. P. Ebel, and R. Giegé, *Nature (London)*, 1980, **288**, 669.

to resemble that of $tRNA^{Phe}$ and the complete ribosephosphate chain is clearly visible. Detailed comparison with the $tRNA^{Phe}$ structure and comparisons of the two independently solved $tRNA^{Asp}$ structures are presented.

$tRNA_f^{Met}$.—The crystal structure of an initiator transfer RNA, $tRNA_f^{met}$, from *E. coli* has been reported at 0.35 nm resolution.[134] Comparison with yeast $tRNA^{Phe}$ shows certain noticeable differences, for example the last five nucleotides at the 3^1 terminus curl back towards the acceptor end rather than continuing the helical acceptor stem as is seen in the chain-elongating tRNA. An insertion in the α-region of the D loop and the conformation of the anticodon arm are the only other major differences. The possible functional significance of these differences is discussed.

DNA, Nucleotides, and DNA Complexes.—The polymorphism of DNA double helices has been examined[135] suggesting six additional structures possible for the molecules. Shindo and Zimmerman[136] have described sequence-dependent variations in the backbone geometry of a synthetic DNA fibre and Zimmerman and Pheiffer[137] have examined whether conditions supposed to generate the C conformation of DNA actually do so. The crystal structure of a complete turn of B-DNA has been determined.[138]

Three papers[139–141] have appeared which show DNA polymers adopting a left-handed helical conformation. The binding of proflavine by intercalation between the non-complementary base-pairs of cytidilyl-3^1,5^1-adenosine[142] and the structure of a daunomycin–deoxyhexonucleoside complex[143] have been published.

Chicken Erythrocyte Chromosomes.—Langmore and Schutt[144] have reported a 40 nm periodicity for chicken erythrocytes *in vivo* due to nuclear structure that is directly related to the 30 nm side-by-side packing of chromosome fibres seen by electron microscopy. The periodicity can be preserved for isolated nuclei using the correct techniques.

12 Muscle

A model for the binding of tropomyosin to troponin T has been presented based on structure predictions[145] and Luther and Squire[146] have examined the structure of the myosin filament superlattice in vertebrate muscle A-band.

[134] N. H. Woo, B. A. Roe, and A. Rich, *Nature (London)*, 1980, **286**, 346.
[135] A. G. W. Leslie, S. Arnott, R. Chandrasekaran, and R. L. Ratliff, *J. Mol. Biol.*, 1980, **143**, 49.
[136] H. Shindo and S. B. Zimmerman, *Nature (London)*, 1980, **283**, 690.
[137] S. B. Zimmerman and B. H. Pheiffer, *J. Mol. Biol.*, 1980, **142**, 315.
[138] R. Wing, H. Drew, T. Takano, C. Broka, S. Tanaka, K. Itakura, and R. E. Dickerson, *Nature (London)*, 1980, **287**, 755.
[139] S. Arnott, R. Chandrasekaran, D. L. Birdsall, A. G. W. Leslie, and R. L. Ratliff, *Nature (London)*, 1980, **283**, 743.
[140] J. L. Crawford, F. J. Kolpak, A. H.-J. Wang, G. J. Quigley, J. H. van Boom, G. van der Marel, and A. Rich, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4016.
[141] H. Drew, T. Takano, S. Tanaka, K. Itakura, and R. E. Dickerson, *Nature (London)*, 1980, **286**, 567.
[142] E. Westhof, S. T. Rao, and M. Sundaralingam, *J. Mol. Biol.*, 1980, **142**, 331.
[143] G. J. Quigley, A. H.-J. Wang, G. Ughetto, G. van der Marel, J. H. van Boom, and A. Rich, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 7204.
[144] J. P. Langmore and C. Schutt, *Nature (London)*, 1980, **288**, 620.
[145] K. Nagano, S. Miyamoto, M. Matsumura, and I. Ohtsuki, *J. Mol. Biol.*, 1980, **141**, 217.
[146] P. K. Luther and J. M. Squire, *J. Mol. Biol.*, 1980, **141**, 409.

Structure prediction has also been used to detect a periodicity of α-helix forming potential in the tropomyosin sequence, which correlates with alternating actin binding sites.[147] A study of paracrystals of light meromyosin [148] has allowed the positions of the molecules' ends to be correlated with the observed striation pattern.

X-Ray diffraction studies on leg striated muscles from crab in the relaxed and rigor states have been presented.[149] In the rigor state a basic period of 76.5 nm for the thin filaments gives many distinct layer lines with a few weaker layer lines, with a basic 14.5 nm period being observed from the thick filaments. In the relaxed state most of the thin filaments' layer lines become very weak.

The *X*-ray pattern from frog muscle has been examined using a synchrotron radiation source.[150] As a result of using an *X*-ray image intensifier, TV detector patterns taking 24 h to record with a rotating anode source could be obtained in 1 s. The low-angle reflections could be followed during contraction with a time resolution of 10 ms.

13 Membranes

A method to measure the movement of small molecules across membranes using neutron diffraction has been reported [151] and the conformation of deoxylysophosphatidylcholine monohydrate has been determined by *X*-ray analysis of single crystals.[152] Kataoka and Ueki [153] have shown what information can be deduced about the structure of biomembranes from their diffraction patterns and have derived the necessary equations. The *X*-ray and neutron scattering density profiles of intact erythrocyte ghosts have yielded information on the location and relative distribution of lipids and proteins.[154] Low-angle *X*-ray diffraction patterns have been recorded from frog sciatic nerve both before and after digestion with trypsin and pronase.[155] Enzyme-treated nerve membrane becomes symmetric about its centre as a result of proteolytic cleavage and a subsequent redistribution of protein components. The changes in myelin structure and electrical activity have been examined for frog sciatic nerves treated with n-alkanes.[156] *X*-Ray diffraction has been used to determine the effect of the anaesthetics on the membrane structure during the process.

The direction and angle of molecular tilt in ordered multilayers of dipalmitoyl lecithin have been determined by *X*-ray diffraction.[157] The effect of temperature on the molecular packing has also been examined. The conformation of phosphatidyl ethanolamine in the gel phase has been studied by neutron diffraction.[158]

[147] L. B. Smillie, M. D. Pato, J. R. Pearlstone, and A. S. Mak, *J. Mol. Biol.*, 1980, **136**, 199.
[148] D. Safer and F. A. Pépe, *J. Mol. Biol.*, 1980, **136**, 343.
[149] K. Namba, K. Wakabayashi, and T. Mitsui, *J. Mol. Biol.*, 1980, **138**, 1.
[150] H. E. Huxley, A. R. Faruqui, J. Bordas, M. H. J. Koch, and J. R. Milch, *Nature (London)*, 1980, **284**, 140.
[151] N. P. Franks and W. R. Lieb, *J. Mol. Biol.*, 1980, **141**, 43.
[152] H. Hauser, I. Pascher, and S. Sundell, *J. Mol. Biol.*, 1980, **137**, 249.
[153] M. Kataoka and T. Ueki, *Acta Crystallogr.*, 1980, **A36**, 282.
[154] L. McCaughan and S. Krimm, *Science*, 1980, **207**, 1481.
[155] C. R. Worthington, T. J. McIntosh, and S. Lalitha, *Arch. Biochem. Biophys.*, 1980, **201**, 429.
[156] R. Padron, L. Mateu, and J. Requena, *Biochim. Biophys. Acta*, 1980, **602**, 221.
[157] M. Hentschel, R. Hosemann, and W. Helfrich, *Z. Naturforsch., Teil A*, 1980, **35**, 643.
[158] G. Büldt and J. Seelig, *Biochemistry*, 1980, **19**, 6170.

14 Other Biological Structures

Actin and Microtubules.—The formation of actin tubes into crystalline aggregates has been reported.[159] The use of synchrotron radiation to study the kinetics of microtubule assembly has been described[160] and shows that at 4 °C the *X*-ray pattern arises from a mixture of tubulin rings and dimers. Raising the temperature to 36 °C induces the breakdown of rings followed by the growth of microtubules, which suggests that the microtubules form from oligomers smaller than rings. An investigation of the structure of crystalline actin sheets has also been reported.[161]

Gap Junctions.—A 1.8 nm resolution map of the gap junction has been obtained,[162] which shows that the protein oligomer in the junctional membranes, the connexon, is a cylinder composed of six subunits which are tilted with respect to the cylinder axis. Analysis of the open and closed connexon conformations has allowed the proposal of a model for the transition which suggests how the passage of small molecules betweeen cell interiors may be regulated.

Lipoproteins.—Atkinson and co-workers[163] have presented the results of *X*-ray and neutron scattering studies of the structure of plasma lipoproteins.

Retinas.—A neutron diffraction analysis of the structure of rod photoreceptor membranes in intact frog retinas has been described.[164]

Mollusc Shell.—An *X*-ray diffraction study of the insoluble organic matrix of several mollusc shells has been reported.[165] The observed *X*-ray patterns, though similar, can be grouped into two distinct categories.

Small Molecules of Biochemical Interest.—The crystal structure of a novel potent mutagen isolated from broiled food has been determined,[166] and Hunt and co-workers[167] have described crystallographic and molecular orbital studies on the geometry of antifolate drugs. The *X*-ray structure of the Zn^{II}–ATP–2,2′-bipyridyl complex has been reported[168] and its implications as a possible model for ATP transport have been discussed. The structure of a compound which may act as a model for the interaction between indole and thymine rings has been determined by *X*-ray diffraction.[169]

15 Fibrous Proteins and Synthetic Polypeptides

Collagen.—Eyre[170] has reviewed the current state of knowledge of the structure of collagen.

[159] C. G. Dos Remedios, J. A. Barden, and A. A. Valois, *Biochim. Biophys. Acta*, 1980, **624**, 174.
[160] E.-M. Mandelkow, A. Harmsen, E. Mandelkow, and J. Bordas, *Nature (London)*, 1980, **287**, 595.
[161] U. Aebi, P. R. Smith, G. Isenberg, and T. D. Pollard, *Nature (London)*, 1980, **288**, 296.
[162] P. N. T. Unwin and G. Zampighi, *Nature (London)*, 1980, **283**, 545.
[163] D. Atkinson, D. M. Small, and G. G. Shipley, *Ann. N. Y. Acad. Sci.*, 1980, **348**, 284.
[164] M. Yeager, B. Schoenborn, D. Engelman, P. Moore, and L. Stryer, *J. Mol. Biol.*, 1980, **137**, 315.
[165] S. Weiner and W. Traub, *FEBS Lett.*, 1980, **111**, 311.
[166] S. Yokoyama, T. Miyazawa, H. Kasai, S. Nishimura, T. Sugimura, and Y. Iitaka, *FEBS Lett.*, 1980, **122**, 261.
[167] W. E. Hunt, C. H. Schwalbe, K. Bird, and P. D. Mallinson, *Biochem. J.*, 1980, **187**, 533.
[168] P. Orioli, R. Cini, D. Donati, and S. Mangani, *Nature (London)*, 1980, **283**, 691.
[169] T. Ishida, S. Mitoguchi, Y. Miyamoto, K.-I. Tomita, and M. Inoue, *Biochim. Biophys. Acta*, 1980, **609**, 158.
[170] D. R. Eyre, *Science*, 1980, **207**, 1315.

X-Ray diffraction studies of native and reconstituted rat-tail tendon collagen have shown that the axial structures are very similar.[171] However, the lack of lateral order in the reconstituted fibres suggests that the native lateral packing may depend on other tissue components.

The typical rat-tail tendon diffraction pattern has also been observed for three other types of tissue composed of type I collagen.[172] These non-tail tissues are chicken- and turkey-leg tendon and bovine achilles tendon.

Two groups of workers have reported that skin collagen in the native state shows an unusual periodicity of 65 nm.[173, 174] The spacing for wet collagen is usually 67 nm and this spacing is observed if purified skin collagen is used to form fibrils. Both groups suggest that the levels of proteoglycans or glycosaminoglycans in the native skin may be important for altered periodicity.

Synthetic Polypeptides.—The first observation of cross-β structure for monodisperse linear homo-oligopeptides, in this case hexapeptides, has been presented[175] and fairly complete determinations of the structures have been possible.

16 Protein Conformations – Analysis and Prediction

Conformational Analyses.—Rees[176] has presented an experimental evaluation of the effective dielectric constant of proteins and Srinivasan and Olson[177] have described conformational wheels for four cytochromes and two lysozymes. Dashevskii[178] has presented a lattice model of the three-dimensional structure of globular proteins.

Remington and Matthews[179] have described a systematic method for comparing the backbone conformations of proteins. The method has been tried on dehydrogenases and bacterial and pancreatic proteases in an attempt to determine the effects of insertions and deletions. The influence of the compared probe length has also been tested.

McLachlan[180] has shown that superoxide dismutase contains two paired subdomains and has discussed the significance of the repeated folding pattern. Busetta[181] has performed a conformational analysis of mellitin using the residual representation and Vijayan[182] has described an approach to prebiotic polymerization based on known crystal structures.

Principles of Structure and Prediction.—Takano[183] has presented a novel approach to the prediction of protein tertiary structure. The method is confined to α/β proteins and calculates simplified topological patterns of α/β domains.

[171] E. F. Eikenberry and B. Brodsky, *J. Mol. Biol.*, 1980, **144**, 397.
[172] J.-C. Jésior, A. Miller, and C. Berthet-Colominas, *FEBS Lett.*, 1980, **113**, 238.
[173] R. H. Stinson and P. R. Sweeny, *Biochim. Biophys. Acta*, 1980, **621**, 158.
[174] B. Brodsky, E. F. E. Eikenberry, and K. Cassidy, *Biochim. Biophys. Acta*, 1980, **621**, 162.
[175] P. Spadon and A. Del Pra, *Int. J. Pept. Protein Res.*, 1980, **15**, 54.
[176] D. C. Rees, *J. Mol. Biol.*, 1980, **141**, 323.
[177] A. R. Srinivasan and W. K. Olson, *Int. J. Pept. Protein Res.*, 1980, **16**, 111.
[178] V. G. Dashevskii, *Mol. Biol. (Moscow)*, 1980, **14**, 80.
[179] S. J. Remington and B. W. Matthews, *J. Mol. Biol.*, 1980, **140**, 77.
[180] A. D. McLachlan, *Nature (London)*, 1980, **285**, 267.
[181] B. Busetta, *FEBS Lett.*, 1980, **117**, 277.
[182] M. Vijayan, *FEBS Lett.*, 1980, **112**, 135.
[183] K. Nagano, *J. Mol. Biol.*, 1980, **138**, 797.

Cohen and Sternberg [184] have shown that in structure predictions the r.m.s. deviations of predicted atomic positions from the known positions are proportional to the number of residues. They have presented a mathematical model to explain this observation.

Lesk and Chothia [185] have analysed the structures of nine different globins, the two most distantly related ones showing only 16% homology. The principal determinants of the structure appear to be 59 residues involved in helix–helix or helix–haem interactions. Half of these residues are buried internally and changes in the side-chain volume of buried residues are accompanied by changes in the geometry of helix packing. The implications of these results for protein evolution are discussed.

A method for describing the quaternary structure of tetrameric proteins has been presented [186] and allows the classification of the tetramers as left-handed or right-handed.

Brändén [187] has reviewed the relation between structure and function of α/β proteins. The relationship between α and β secondary structures and pseudosymmetrical amino-acid arrangements has been analysed for 51 polypeptides of known secondary structure and sequence.[188] The data suggest that symmetrical arrangements of amino-acids could result from structural constraints imposed either by the α or β secondary structures.

Janin and Chothia [189] have proposed a model for the packing of α-helices on β-sheets in α/β proteins. The packing involves two smooth surfaces with complementary twists: the surface of a regular β-sheet with a right-handed twist and the helix face formed by residues i, $i + 4$, $i + 8$, *etc.* and $i + 1$, $i + 5$, $i + 9$, *etc.* Helix–sheet and helix–helix contacts in eight proteins of known structure are examined in order to substantiate the model.

Weber and Salemme [190] have described the geometrical properties of the four-α-helical left-twisted bundle structure as seen in haemerythrin, for example. The geometrical properties are used to suggest how they relate the functional and aggregational properties of this class of proteins.

An analysis of β-sheet sandwiches, as seen in the immunoglobulin fold for example, has suggested an algorithm, which successfully predicts the tertiary fold of these proteins from their sequence and secondary structure.[191] The authors propose tertiary structures for β_2-microglobulin and an HLA-B7 antigen fragment.

Lifson and Sander [192] have calculated the frequencies of occurrence of nearest-neighbour residue pairs on adjacent β-strands for 30 known protein structures.[192] Several statistically significant pairings are found and the data may be useful in prediction methods of tertiary structure.

[184] F. E. Cohen and M. J. E. Sternberg, *J. Mol. Biol.*, 1980, **138**, 321.
[185] A. M. Lesk and C. Chothia, *J. Mol. Biol.*, 1980, **136**, 225.
[186] E. J. Milner-White, *Biochem. J.*, 1980, **187**, 297.
[187] C.-I. Brändén, *Q. Rev. Biophys.*, 1980, **13**, 317.
[188] P. Delhaise, C. Wuilmart, and J. Urbain, *Eur. J. Biochem.*, 1980, **105**, 553.
[189] J. Janin and C. Chothia, *J. Mol. Biol.*, 1980, **143**, 95.
[190] P. C. Weber and F. R. Salemme, *Nature (London)*, 1980, **287**, 82.
[191] F. E. Cohen, M. J. E. Sternberg, and W. R. Taylor, *Nature (London)*, 1980, **285**, 378.
[192] S. Lifson and C. Sander, *J. Mol. Biol.*, 1980, **139**, 627.

Némethy and Scheraga[193] have described the eight classes of β-bends and shown how the possibility of forming intrabend hydrogen bonds depends on the type of bend being considered. They show how techniques such as n.m.r. or Raman spectroscopy could be used to distinguish the various types of bend.

The identification of nucleation sites for protein folding has been discussed[194] and a redefinition of the knotting problem of protein folding has been presented[195] and shows that loop penetration is not as rare as currently believed. Rose and Roy[196] have shown that the concept of protein folding starting with secondary structure nucleation is unlikely. They suggest that regions of chain rich in hydrophobic residues serve as small clusters that fold against each other with a concomitant or later fixation of secondary structure. From the crystal structures of 21 proteins Ponnuswamy and co-workers[197] have calculated a new set of hydrophobic indices that provides valuable information with regard to hydrophobic domains, nucleation sites, and loop sites in protein molecules.

Wodak and Janin[198] have presented an analytical method for calculating the accessible surface area of proteins. The method is very fast and produces good estimates of the accessible area.

A Monte Carlo simulation of water structure around a dipeptide has been reported[199] and the structure of ordered water around a deoxynucleoside–drug complex has been described.[200]

Olsen[201] has presented a method for testing the correctness of predicted structures based on internal residue criteria and has used the method to test some predictions. Popov[202] has concluded that theoretical approaches to the determination of protein structures cannot reach the ultimate goal of an *a priori* conformational description of a protein.

Examples of Structure Predictions.—Cohen and Sternberg[203] have shown how chemically derived distance constraints can be applied to structure predictions. Using these constraints the 200 possible structures predicted for myoglobin were reduced to 2 with r.m.s. deviations of 0.448 and 0.453 nm from the crystal structure. The effects of the various constraints which reduced the possible predicted conformations from 10^{32} to 2 are explained.

Argos and Rossmann[204] have predicted secondary structural elements for glycerol-3-phosphate dehydrogenase. The *N*-terminal half of the chain shows a pattern of helices and sheets consistent with an NAD^+ binding domain. The *C*-terminal half of the sequence shows similarities with the catalytic domain of glyceraldehyde-3-phosphate dehydrogenase.

[193] G. Némethy and H. A. Scheraga, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 320.
[194] P. K. Ponnuswamy and M. Prabhakaran, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 1582.
[195] M. H. Klapper and I. Z. Klapper, *Biochim. Biophys. Acta*, 1980, **626**, 97.
[196] G. D. Rose and S. Roy, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4643.
[197] P. K. Ponnuswamy, M. Prabhakaran, and P. Manavalan, *Biochim. Biophys. Acta*, 1980, **623**, 301.
[198] S. J. Wodak and J. Janin, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1736.
[199] A. T. Hagler, D. J. Osgusthorpe, and B. Robson, *Science*, 1980, **208**, 599.
[200] S. Neidle, H. M. Berman, and H. S. Shieh, *Nature (London)*, 1980, **288**, 129.
[201] K. W. Olsen, *Biochim. Biophys. Acta*, 1980, **622**, 259.
[202] E. M. Popov, *Mol. Biol. (Moscow)*, 1980, **14**, 24.
[203] F. E. Cohen and M. J. E. Sternberg, *J. Mol. Biol.*, 1980, **137**, 9.
[204] J. Otto, P. Argos, and M. G. Rossmann, *Eur. J. Biochem.*, 1980, **109**, 325.

A secondary structure prediction of NAD-specific glutamate dehydrogenase from *Neurospora crassa* has been presented[205] and Hayes[206] has reported predictions for the secondary structures of F and Le interferons.

Predictions have also been published for cytoplasmic aspartate aminotransferase[207] and bee venom toxin, apamin.[208, 209] Rackovsky and Scheraga[210] have compared predicted and observed conformations of gramicidin S and suggest that the formation of intermolecular interactions can explain the differences.

PART III: Conformation and Interaction of Peptides and Proteins in Solution *Edited by R. H. Pain, with contributions by B. Adams, A. Benson, T. Brittain, D. P. E. Dickson, P. D. Jeffrey, L. W. Nichol, H. W. E. Rattle, B. Samraoui, R. M. Stephens, M. J. E. Sternberg, and D. J. Winzor*

1 Theoretical Aspects of Protein Conformation

Contributed by B. Samraoui and M. J. E. Sternberg

We report recent publications in the primary literature on theoretical studies on polypeptides and proteins. Our review can be supplemented by the proceedings of the Regensburg[1] meeting on 'Protein Folding', in which both theoretical and experimental studies are reported.

Potential Energy Functions.—The detailed understanding of the structure and function of polypeptides and proteins requires an accurate description of the interatomic interactions. This year the emphasis has been on modelling the hydrophobic effect[2-6] rather than intraprotein interactions.[7, 8]

Protein–water interactions have been investigated by Swaminathan and Beveridge[2] who performed a Monte Carlo simulation of water with two methane molecules separated by various distances. The force vanishes for separations of ~4.2, 5.15, and 7.0 Å. Ninham[3] discusses the relative roles of long-range and short-range interactions of solvent-mediated forces. A different approach to quantify the hydrophobic effect comes from the concept of accessible surface area that was introduced by Lee and Richards.[9] The area is the van der Waals surface of an atom that can be in contact with a hypothetical water probe. Chothia[10] has shown that the accessible area of residue is roughly proportional to the experimentally observed free energy of transfer of that residue from water to a non-polar

[205] B. M. Austen, M. E. Haberland, and E. L. Smith, *J. Biol. Chem.*, 1980, **255**, 8001.
[206] T. G. Hayes, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 872.
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[208] P. N. Mel'nikov and E. M. Popov, *Mol. Biol. (Moscow)*, 1980, **13**, 712.
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[210] S. Rackovsky and H. A. Scheraga, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 6965.
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[2] S. Swaminathan and D. L. Beveridge, *J. Am. Chem. Soc.*, 1979, **101**, 5832.
[3] D. W. Ninham, *J. Phys. Chem.*, 1980, **84**, 1423.
[4] S. J. Wodak and J. Janin, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1736.
[5] M. H. Abraham, *J. Am. Chem. Soc.*, 1980, **102**, 5910.
[6] H. Pfeiffer, G. Zundel, and E. G. Weidemann, *J. Phys. Chem.*, 1979, **83**, 2544.
[7] S. N. Vinogradov, *Int. J. Pept. Protein Res.*, 1979, **14**, 281.
[8] J. E. Douglas and P. A. Kollman, *J. Am. Chem. Soc.*, 1980, **102**, 4295.
[9] B. K. Lee and F. M. Richards, *J. Mol. Biol.* 1971, **55**, 379.
[10] C. Chothia, *Nature (London)*, 1974, **248**, 338.

medium. Previously, accessible area was estimated by numerical integration, but now Wodak and Janin [4] have formulated an analytical approximation to the area. Not only is the approximation computationally fast to evaluate but the function can be differentiated and therefore can be used in energy minimizations.

Energy Calculations on Polypeptides.—A variety of techniques has been used to model polypeptides and solvent structures: *ab initio* calculations,[11-14] Monte Carlo simulations,[15-17] and semi-empirical conformation studies.[18-22]

Several workers using *ab initio* calculations [11-14] investigated non-covalent interactions between peptides. Peters and Peters [11] showed that *ab initio* computations using the Gaussian 70 package are able to classify the C_{10} hydrogen bonds. *Ab initio* quantum chemical analysis by Mehler [13] of non-covalent interactions between peptides as modelled by dimers and a trimer of formamide resulted in an estimation of the interaction energy and dipole moment for hydrogen-bonded complexes of any length.

Several workers [15-17] have studied the effect of solvent on molecular structure. Hagler *et al.*[16] used a Monte Carlo technique to study the solvent structure around the dipeptide *N*-acetylalanyl-*N*-methylamide fixed in the α_R ($\phi = -60°$, $\psi = -50°$) conformation. They found that the effect of the dipeptide on the water structure decreases rapidly with distance.

Genest and Ptak [18] carried out empirical calculations on cyclic dipeptides (L-Asp-L-His) and (L-Glu-L-His). The side-chain conformations were studied for various diketopiperazine (DKP) ring structures and they found that distortions can modify the stability of some conformations by favouring peptide backbone–side-chain or side-chain–side-chain interactions. Scheraga and co-workers [19-22] carried out a study of the conformational properties of collagen-like repeating sequences. The conformational space of regular structures of poly(glycyl-prolyl-prolyl), $(GPP)_n$, poly(glycyl-prolyl-hydroxyprolyl), $(GPH)_n$, poly(glycyl-prolyl-alanyl), $(GPA)_n$, and poly(glycyl-alanyl-prolyl), $(GAP)_n$, were examined for stable triple-stranded complexes. The studies showed that stable coiled-coil triple helices can be formed by poly(tripeptide)$_5$ of the sequence $(GXY)_n$, where G = Gly and X or Y, or both, can be either Pro or Ala (or Y can be Hyp). The interchain energy is essential to the stability of the collagen-like triple helical structure.

Analysis and Prediction of Secondary Structure.—Based on the notion that local sequence determines local structure, present-day algorithms can predict secondary structure from the amino-acid sequence with up to 80% accuracy. This year's work

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[12] D. Peters and J. Peters, *J. Mol. Struct.*, 1979, **53**, 103.
[13] E. L. Mehler, *J. Am. Chem. Soc.*, 1980, **102**, 12.
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[20] M. H. Miller, G. Nemethy, and H. A. Scheraga, *Macromolecules*, 1980, **13**, 470.
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has concentrated on analyses of local structure [23-27] and on the applications of existing prediction schemes,[28-34] rather than on the development of new algorithms.[35]

Three articles [23-25] have emphasized the importance of bends in secondary structure. The first by Chou and Fasman,[23] extending their previous work, predicted a high conservation of chain reversal. The second by Isogai *et al.*[24] defined multiple bends and reported their occurrence and structural characteristics. The third by Nemethy and Scheraga [25] classified the β-bends into eight groups according to the orientation of the three peptide groups comprising the bend. Erham *et al.*[26] studied the amino-acid neighbourhood relationships and developed a method which allows the breakdown of an amino-acid sequence into overlapping doublets, tripeptides, and quadruplets. These peptides may be used to improve the accuracy of the prediction of secondary structure.

Several workers [28-34] carried out secondary structure predictions, most of them using combined predictive methods, but no major improvement has been carried out to increase the accuracy of these methods. The proteins studied include: NAD-specific glutamate dehydrogenase;[28] aspartate amino-transferase;[29,30] the *lac* repressor;[31] the amino-terminal (signal) sequence of 21 secreted proteins;[32] anterior pituitary hormones;[33] and glycerol-3-phosphate dehydrogenase.[34]

Dunfield and Scheraga [35] have used a nearest-neighbour Ising model based on empirical conformational energies for two successive residues to predict the ϕ, ψ conformation of a polypeptide chain. If the residues in α-helical and bend regions are omitted, the backbone dihedral angles of the remaining residues are predicted with 43—60% accuracy. The model can be expanded to include interactions more distant along the chain such as those required to predict α-helical conformations.

Analysis of Tertiary Structure.—This year's studies can conveniently be divided into analyses of the packing [36-41] of α-helices and/or β-strands and investigations of the overall fold [42-50] with emphasis on the location of hydrophobic and polar residues.

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[34] J. Otto, P. Argus, and M. G. Rossmann, *Eur. J. Biochem.*, 1980, **109**, 325.
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[40] J. Janin and C. Chothia, *J. Mol. Biol.*, 1980, **143**, 95.
[41] S. Lifson and C. Sander, *J. Mol. Biol.*, 1980, **139**, 627.
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References continued overleaf

Earlier work by Chothia *et al.*[51] described three dominant packing motifs in globular proteins: the pairing of α-helices (α/α); the stacking of two β-sheets (β/β); and the packing of α-helices on both sides of a predominantly parallel β-sheet (α/β). The recent examinations have considered all of these motifs. Chothia *et al.*[36] considered 50 α/α packings and showed that 38 of them are in good agreement with the model of the intercalation of the ridges and grooves formed by the rows of side-chains on the helix surface. An extension of the model that considers small residues describes the general features of another 10 helix pairings. This work formed the basis for the study by Lesk and Chothia[37] on the variations in helix packing in the globin family of proteins. The common geometric properties of the bundle formed from the antiparallel packing of four α-helices that is found in several globular proteins is described by Weber and Salemme.[38]

The stacking of two primarily antiparallel β-sheets, as is observed in each immunoglobulin domain, was analysed by Cohen *et al.*[39] From the change in surface area that is accessible to solvent (see Energy Functions), it was shown that the non-polar residues that mediate the sheet–sheet interaction trace specific patterns on the sheet surfaces as a result of the twisted nature of the β-sheet. Janin and Chothia[40] have proposed a model for α/β packing based on the association of the twisted β-sheet and two rows of residues on the α-helix. The angular geometry suggested by the model is in agreement with examinations of packing in eight proteins. Lifson and Sander[41] have detailed the frequency of occurrence of nearest-neighbour residue pairs on adjacent antiparallel and parallel β-strands.

Several workers[42–46] have compared the path of the polypeptide chain both between different structures and within parts of the same molecule. Connolly *et al.*[42] have systematically identified topological features of the backbone such as the threading and linking of loops. Remington and Matthews[43] have evaluated the power of their comparison method. Rackovsky and Scheraga[44] have explored the use of differential geometry for structural comparisons. Drenth *et al.*[45] have described a new group of small protein structures organized around a four-disulphide core. McLachlan[46] has located a structural repeat in superoxide dismutase that may have arisen by gene duplication.

The role of hydrophobic residues in the formation of protein structure continues to be emphasized.[47–51] Examinations have considered the location of non-polar residues along the polypeptide chain,[47] in secondary structures,[48] and throughout the globular protein.[49–50]

Prediction of Three-dimensional Structure.—The emphasis of studies[39, 52–61] to predict protein structure has been on combinatorial approaches[39, 52–54] rather

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[52] F. E. Cohen, J. Novotony, M. J. E. Sternberg, D. G. Campbell, and A. F. Williams, *Biochem. J.*, 1981, **195**, 31.

than energy calculations [55] or sequence homology.[56] There has also been interest in characterizing the folding pathway.[59-61]

Several workers [39, 52-54] have predicted structures by the combinatorial approach in which all associations of the actual or postulated α-helices and β-strands are examined and structures eliminated if they violate simple constraints. Using the results of their analysis of the stacking of β-sheets, Cohen *et al.*[39] have developed a combinatorial algorithm to predict the structures of β-sandwiches such as immunoglobin domains. About 10^8 associations of the native (*i.e.* crystallographic) β-strands into the two sheets are generated but many are eliminated by constraints on topology, connectivity, and the burial of non-polar residues. In trials on nine different β-sandwiches, a native-like structure (r.m.s. deviation from the crystal structure of 1.4—5.1 Å) was high (top 6 of 3300) in a list of β-sandwiches rank ordered on hydrogen bonding. In conjunction with the prediction of secondary structure, this approach suggested structures with an immunoglobulin fold for β_2-microglobulin,[39] part of the HLA-B7 histocompatibility antigen,[39] and a cell surface protein thy-1.[52] Engelman *et al.*[53] applied the combinatorial approach to suggest one model for the 5040 possible ways of docking seven α-helices to form the structure of bacteriorhodopsin. Nagano [54] has restricted possible topologies for α/β proteins.

A variety of other approaches have been followed.[55-58] Rashin and Yudman [55] used a simplified model for the protein to evaluate the interactions between monomers and thereby predict the quaternary structure of haemoglogin and α-chymotrypsin. Greer [56] has proposed a model of haptoglobin heavy chain based on structural homology with the serine proteases. Hermans [57] has suggested a model for fibrin.

In contrast to the problem of characterizing the final states, several approaches have considered the kinetic question of the path between the unfolded and native state. Cohen *et al.*[59] have simulated the folding pathway of myoglobin by combining Richmond and Richards'[62] information about helix docking in the native structure with the Karplus and Weaver [63] theory of folding by diffusion–collision–adhesion. Levitt [60] has calculated the difference in energy between each of the prolines in pancreatic trypsin inhibitor adopting a *cis* or *trans* conformation in the native structure. His results are compared with the experimental work of Brandts *et al.*, [64] which reports fast and slow refolding species for this molecule (see p. 179).

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Protein Dynamics.—The recent interest in quantifying the mobility of proteins has continued.[65-69] Karplus[65,66] and his co-workers have performed a 16 ps molecular dynamics calculation of ferrocytochrome *c*. Correlations are observed between the magnitudes of the atomic position fluctuations and the structural features of the protein. The calculated mobility is in broad agreement with the crystallographically determined fluctuations that were obtained from atomic temperature factors derived from refinement of the 1.5 Å structure.

Protein Function.—A variety of techniques have been used to consider the function of proteins[70-78] including quantum mechanical calculations,[70-72] conformational analysis,[73-75] and dynamic simulations.[76]

Warshel and Weiss[70] have developed a simple empirical valence bond approach for comparing potential surfaces of reactions in solution and in enzymes. Conformational energy calculations were performed by Pincus and Scheraga[73] to investigate the most favoured binding modes of oligomers of β-D-*N*-acetylglucosamine to the active site of lysozyme. North and co-workers[74] used conformational energy calculations and an interactive computer display system to model the proportions of the four isomers that are produced when oxygen attacks each of the four methene-bridge carbon atoms in haem proteins.

Conclusion.—We would like to point out two possible areas for further work that arise from this year's publications. First, the analytical approximation to accessible surface area[4] might provide a convenient model for the hydrophobic effect. The advantages gained by the inclusion of this function in conformational energy calculations need to be explored. Second, several workers have used a combinatorial approach[39,52-54] to dock α-helices and β-strands and have thereby predicted rough folds for proteins. This approach requires the locations of the regular secondary structure. It is important, therefore, that the prediction of secondary structure should be improved.

2 Mechanisms of Folding in Globular Proteins

Contributed by B. Adams

There is an excellent book giving an up to date coverage of this field.[79]

Stability.—*Effect of Disulphide Bonding.* Ribonuclease A is capable of forming an active native-like conformation without the completion of all its native disulphide

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bonds.[80-82] This intermediate lacks the disulphide bond 40—95 and is more thermolabile, unfolding at 40 °C rather than 54 °C for the native molecule. Hen egg lysozyme,[83,84] bovine pancreatic trypsin inhibitor,[85] bovine enterokinase,[86] and human antithrombin III[87] also form native-like structures without the presence of all the native disulphide bonds.

In anti-poly(D-alanine) antibodies, cleavage of disulphides has been shown to increase the radius of gyration.[88] Binding to the hapten still occurs, lowering the radius of gyration of the reduced antibody but not of the native antibody.

Seminal ribonuclease has two additional cysteine residues and shows different kinetics of refolding. Modification of these two cysteine residues with either a neutral or a positive alkylating agent causes the refolding kinetics to become more like those of ribonuclease A.[89] The modifications also render the protein more stable to thermal inactivation. The extra cysteine residues may be competing with the formation of the correct disulphide bonds or be forming intermolecular linkages.

In general, it appears that although disulphide bonds do stabilize a protein, the non-covalent interactions are more important in the formation of native conformation.

Effect of Individual Residues. Modification of a histidine residue in glutamate dehydrogenase from *Neurospora crassa* results in loss of activity.[90] High substrate concentrations bring back activity and since the histidine residue is not thought to be near the active site it is suggested to be important in maintaining the conformation of the active site. Fluorescence studies support this hypothesis.

Copo *et al.* suggest that conversion of lysine residues into arginine stabilizes globular proteins.[91] Tritium exchange rates were reduced in all protein studied, except lysozyme, after guanidination. The effect of modification of lysine residues and replacement of tyrosine-74 on the conformation and activity of cytochrome *c* has been studied.[92] All analogues were apparently native, but replacement of the tyrosine by leucine caused a loss of activity. This residue presumably provides a functional rather than a conformational requirement.

Conversion of glycine-211 into arginine or glutamate has little effect on the stability of the α-subunit of tryptophan synthetase as measured by differential scanning calorimetry.[93] However, large changes in the enthalpy of unfolding were

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seen, indicative of an equivalent change in the entropy. The effects of electrostatic forces on the stability of this protein have been studied using two mutant forms.[94]

Effect of Ligands. Substrate has been shown to stabilize lysozyme to thermal and guanidine hydrochloride denaturation[95] in agreement with a prediction by Schellman.[96]

The valency of metal ions binding to myoglobin affects its stability.[97] The effect is thought to be through the imidizole-to-protein association. Binding of terbium to porcine pancreatic elastase enables changes in stability and conformation on binding ligands to be studied.[98]

Sedimentation velocity measurements show large conformational changes in the α-subunit of *E. coli* F_1ATPase on binding ATP,[99] while cross-linking prevents conformational changes induced in penicillinase on binding substrate.[100] Changes in the exposure of aromatic amino-acids of bovine antithrombin occur in the presence of heparin.[101] Metal ions have been shown to induce conformational changes in human prothrombin,[102] demetallized concanavalin A,[103] pyruvate kinase,[104] and G-actin.[105]

The amino-acids L-phenylalanine and L-alanine induce conformational changes in rabbit muscle pyruvate kinase[106] and the change is dependent on the nature of the divalent metal ions present. L-Phenylalanine has also been shown to inhibit stereospecifically the rate of renaturation of pyruvate kinase.[107] The rate of refolding of glyceraldehyde-3-phosphate dehydrogenase has been shown to be enhanced by a covalently bound co-enzyme analogue.[108]

Solvent Effects on the Native State, on Denaturation, and on the Denatured State.—The stabilization of bovine serum albumin by aqueous solvents has been investigated by Damodaran and Kinsella.[109] The effect of pH on the conformation has been investigated using aspartate transcarbamylase,[110] ferricytochrome *c*,[111] and goat α-lactalbumin.[112] The effect of dimethyl sulphoxide and *p*-dioxane on the thermal unfolding of ribonuclease has also been studied.[113]

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Further evidence that a structured core can exist under drastic denaturing conditions comes from two papers. Some structure is apparently present in *Carcinus meanus* haemocyanin in 8 M urea.[114] Further, although most of the structure of thermolysin as seen in fluorescence energy transfer studies is lost at 90 °C in 6 M guanidine, it still binds zinc ions.[115]

Conformational Dynamics.—Temperature-dependent *X*-ray crystallography and a dynamical simulation model were used to examine the distribution of internal mobility of ferricytochrome *c*.[116] Both gave similar pictures.

Wüthrich *et al.*[117] measured the p^2H dependence of the exchange rates of interior amide proteins in native and reduced (at the disulphide 14—28) bovine pancreatic trypsin inhibitor, using proton n.m.r. Rates of exchange were 100 to 1000 times faster in the reduced protein, at all p^2H values, which correlates with the decrease in thermal stability. This is stated to be incompatible with the mechanism of Hilton and Woodward [118] in which the kinetics of proton exchange are described as the sum of two discrete processes, the thermal unfolding and the dynamical process in the folded protein. The latter two-process mechanism is supported by a paper by Knox and Rosenberg.[119]

The dynamics of tyrosine ring rotations in the pancreatic trypsin inhibitor have been investigated by computer simulation.[120] The ring is apparently driven over its rotational potential by nearly impulse collisions with neighbouring atoms. The activation volume of about 50 $Å^3$ for the rotation calculated from high-resolution n.m.r. studies as a function of pressure [121] is consistent with values calculated from theoretical considerations and from *X*-ray data. This indicates that the rotations are the cause of or are caused by 'stirring' motions in the overall protein structure, rather than facilitated by a compressible ring environment.

Two papers have shown, surprisingly, a dependence of protein dynamics on solvent viscosity,[122, 123] suggesting that internal mobility is governed by some diffusion limited process in the solvent.

A study by Lakowicz and Chevek [124] compares the fluorescence lifetime of *N*-acetyl-L-tryptophanamide in solvents of different viscosity and of tryptophan in three proteins. These results show tryptophan to be in an apparently viscous environment, undergoing reorientation on a nanosecond time-scale.

Acrylamide has been used to show a correlation between activity and structural dynamics in phosphorylase *b*.[125] It is suggested that acrylamide fills up cracks in

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[120] J. A. McCammon and M. Karplus, *Biopolymers*, 1980, **19**, 1375.
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[123] D. Beale, L. Eisenstein, H. Frauenfelder, D. Good, M. C. Marden, L. Reinisch, A. H. Reynolds, L. B. Sorenson, and K. T. Yue, *Biochemistry*, 1980, **19**, 5147.
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[125] J. Matko, L. Tron, M. Balazs, J. Hevessy, B. Somogyi, and S. Damjanovich, *Biochemistry*, 1980, **19**, 5782.

the protein matrix and restricts motion, thus inhibiting the catalytic rate of the protein but not substrate binding.

Evidence for large-scale structural changes during catalysis has come from studies on the pyruvate dehydrogenase complex,[126] bacterial thymidylate synthetase,[127] Ile-tRNA synthetase,[128] and actin–myosin binding.[129]

Bovine β-trypsin undergoes a thermal transition at 31 °C[130] as detected by modified binding constants for a synthetic substrate and for an inhibitor. Ribonuclease exhibits similar behaviour at 32 °C.[131] Using temperature jump techniques thermal fluctuations have been seen in methaemoglobin.[132]

Folding Intermediates.—The late intermediate in the folding of ribonuclease has been described above.[80-82] Previous reports show a native conformation in ribonuclease lacking the disulphides 65—72,[133] 65—72, and 58—110.[134] It has been shown, using immunological probes, that there is significant structure in parts of reduced ribonuclease.[135]

Lysozyme is capable of forming an active, native-like structure, with only 2 out of its 4 disulphide bonds formed.[84] The influence of solvent on a 3-disulphide form of hen egg lysozyme produced during reoxidation has been examined.[83] The paper concluded that since this form is absent under one set of conditions it cannot be considered as a manifestation of the obligatory nature of the pathway. Similar studies on pancreatic trypsin inhibitor point to the most highly populated intermediate states being those which have the greatest extent of stabilizing hydrophobic interactions.[85]

The effects of protein-disulphide isomerase on the unfolding of bovine pancreatic trypsin inhibitor and ribonuclease A have been investigated.[136] The nature of trapped intermediates is not altered, but some interactions not normally significant apparently become so in the presence of isomerase. This is supported by a study of the u.v. difference spectra of intermediates trapped in the refolding of pancreatic-trypsin inhibitor.[137]

The refolding of rabbit muscle creatine kinase has been followed by activity and by reactivity of exposed thiol groups and the monomer association by dimethylsuberimidate crosslinking.[138] Folding occurs in three stages: a rapid refolding of the subunit, rapid association to a dimer (yielding 70% activity), and finally slow rearrangement of the dimer taking several hours.

[126] H. J. Grande, A. J. W. G. Visser, and C. Veeger, *Eur. J. Biochem.*, 1980, **106**, 361.
[127] A. Lochshin and P. V. Danemberg, *Biochemistry*, 1980, **19**, 4244.
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Aspartokinase *I*-homoserine [139] and γ-thrombin [140] also refold by similar mechanisms. Porter and Cordenas [107] have shown that L-phenylalanine stereospecifically inhibits the refolding of muscle pyruvate kinase. The amino-acid must be binding to an intermediate and in some way showing a rate limiting step. The L-phenylalanine binding site must be formed early in the folding process.

Theories of Protein Folding.—*Proline Isomerization.* Jullien and Baldwin [141] studied the refolding of the slow refolding species of ribonuclease A. Tyrosine absorbance showed two slow refolding rate constants, both higher than that seen by fluorescence. This supports work using energy calculations on the same protein,[142] in which the proline residues were divided into three classes. One of the proline residues destabilized the native protein by less than one kcal mol^{-1} when in the *cis* conformation, suggesting it can isomerize freely in the native protein. A further two destabilized the native protein by less than 11 kcal mol^{-1}, indicating that initial folding can occur with either isomer, isomerization to the *trans* configuration occurring later. The fourth proline residue is calculated to destabilize the native protein by 33 kcal mol^{-1} when in the *cis* conformation. This would block folding as in the earlier model.[143]

States *et al.* have detected a metastable species with identical bonds to the native trypsin inhibitor but with a different conformation.[144] They suggest this may be formed by a parallel pathway to that leading to the native protein, possibly the result of a different proline isomer.

Garel [145] working on ribonuclease A has reported a slow proline isomerization species. After conversion of the three exposed tyrosine residues into nitrotyrosine, their change in ionization was used to follow refolding kinetics. These also served as reporters for the formation of a slow refolding species, which has similar kinetic properties, guanidine dependence, and activation energy to proline isomerization. A second paper [146] suggests that certain prolines on the polypeptide chain are rate limiting. Kim and Baldwin [147] trapped intermediates at low temperature and looked at these using amide exchange. In conditions strongly favouring the native conformation, intermediates with protected amide protons were seen. In 2—3 M guanidine hydrochloride, pH 7.5 at 10 °C (conditions in which folding goes to completion), no intermediates were trapped. This indicates that the effect of a proline residue on hindering the refolding of the protein depends on the stability of the ordered conformation surrounding the wrong proline isomer. Henkens *et al.* [148] show a temperature dependence of the magnitude of the slow refolding phase. They suggest that this cannot be explained by a fast refolding species having two *cis* proline residues.

[139] J.-R. Garel and A. Dautry-Varsat, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3379.
[140] T. Chang, R. S. Banar, and L. J. Berliner, *J. Biol. Chem.*, 1980, **255**, 3904.
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[143] J. F. Brandts, H. R. Halvorson, and M. Brennan, *Biochemistry*, 1975, **14**, 4953.
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[145] J.-R. Garel, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 795.
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[147] P. S. Kim and R. L. Baldwin, *Biochemistry*, 1980, **19**, 6124.
[148] R. W. Henkens, A. D. Gerber, M. R. Cooper, and W. R. Herzay, *J. Biol. Chem.*, 1980, **255**, 7075.

Trypsinogen folds rapidly to a globular structure that undergoes a slow conformational change.[149] Enthalpies of activation for this slow step are the same as for proline isomerization. It is suggested that proline isomerization may be essential for correct folding, but that it only takes place after extensive folding has already occurred.

There is therefore growing evidence in favour of the scheme for protein folding proposed by Baldwin.[141,150]

Nucleation and the Hydrophobic Cluster Model. Bovine carbonic anhydrase *b*[151,152] has been shown to refold in three steps: a rapid formation of secondary structure, burying of aromatic residues, then a reshuffling to the native enzyme. This is taken to indicate that the nucleation event is probably the formation of an ordered secondary structure.[149]

Evidence for a late ordering event after a rapid condensing step also comes from work on the formation of ordered complexes from cytochrome *c* fragments.[153] Work on ribonuclease, the S-protein, and des(121—124) ribonuclease[154] is taken to indicate an early nucleation event, probably the hydrophobic pocket formed by residues 106—118.

The effect of reduction of the 14—38 disulphide bond on the aromatic ring flipping and the exchange rates for interior amide protons have again been studied[155] and support the concept of hydrophobic clusters.

Fragments of soybean trypsin–chymotrypsin inhibitor have no α or β structure, but a tyrosine residue is apparently in a hydrophobic environment.

There have been many theoretical papers supporting the hydrophobic cluster model. Crystal data have been examined for 21 proteins,[156] from which a new scale of hydrophobic indices has been set up. This gives information on hydrophobic domains, nucleation sites, surface domains, loop sites, and the spatial positions of residues in proteins. The role of hydrophobic regions in protein structures has been studied.[157–160] Rose and Roy[161] show that a linear protein chain fluctuates between minimal and maximal hydrophobicity. The hydrophobic segments tend to be expressed as α-helices or β-strands. This imposes a major geometric constraint upon possible folding events.

Protein Fragments and Domains.—Des(121—124)-ribonuclease is unable to form native structure except in the presence of the *C*-terminal tetrapeptide.[154] These authors have also investigated the stability of the native conformation of various other ribonuclease peptides.[135]

[149] P. McPhie, *J. Biol. Chem.*, 1980, **255**, 4048.
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[151] F. L. McCoy, E. S. Rowe, and K. Wong, *Biochemistry*, 1980, **19**, 1738.
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[159] K. W. O. Isen, *Biochim. Biophys. Acta*, 1980, **622**, 259.
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[161] G. D. Rose and S. Roy, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4643.

Three cytochrome *c* fragments [162] are capable of forming native-like structures and of association to give an active native-like complex.

Isolated domains from the β_2 subunit of *E. coli* tryptophan synthetase stabilize each other.[163,164] A tryptic fragment of cytochrome b_5 folds into a globular conformation.[165]

Structural domains have been shown to exist in anthranilate isomerase,[166] cAMP-dependent protein kinase,[167] human erythrocyte spectrin,[168] *Helix pomatia* βc-haemocyanin,[169] Cu–Zn superoxide dismutase,[170] glutamate dehydrogenase,[171] and kidney lipoate acetyltransferase.[172] Different functions of different domains have been demonstrated in haemoglobin [173] and troponin-C.[174]

It has been suggested that different exons encode functional and structural units (subdomains) in chicken lysozyme.[175]

Synthetic and Semi-synthetic Proteins that Fold.—The principles and rationale behind designing biologically active polypeptides have been reviewed.[176]

Analogues of the 34-residue peptide reported in last year's review have been produced. They are unable to form the disulphide cysteine-10 to cysteine-33.[177] These and a 64-residue dimer were both shown to be capable of binding DNA and cleaving at the 3′ end of cytidylate.

Trypsin has been used to catalyse the production of semi-synthetic active staphylococcal nuclease.[178]

3 Immunological Probes of Protein and Peptide Conformation

Contributed by A. Benson

The highly specific interaction between specific antibody and its antigen partner is a useful tool for the protein chemist interested in the conformation of proteins. This reaction is highly dependent on native arrangement of the amino-acid side-chains and thus on the backbone conformation in the area of interaction.[179] The extent of antigen–antibody binding can be measured, utilizing the formation of a three dimensional lattice between the bivalent antibody and its specific antigen, by

[162] M. Juillerat, G. R. Parr, and H. Taniuchi, *J. Biol. Chem.*, 1980, **255**, 845.
[163] C. R. Zetina and M. E. Goldberg, *J. Biol. Chem.*, 1981, **255**, 4381.
[164] C. R. Zetina and M. E. Goldberg, *J. Mol. Biol.*, 1980, **137**, 401.
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[166] K. Kirschner, H. Szadkowski, A. Henschen, and F. Lottspeich, *J. Mol. Biol.*, 1980, **143**, 395.
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[173] C. S. Craik, S. R. Buchman, and S. Beychok, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1384.
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[175] A. Jung, A. E. Sippel, M. Grez, and G. Schutz, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5759.
[176] B. Robson, *Trends Biochem. Sci.*, 1980, **5**, 240.
[177] R. Jaenicke, B. Gutte, U. Glatter, W. Strassburger, and A. Wollmer, *FEBS Lett.*, 1980, **114**, 161.
[178] M. Z. Atassi and S. Sakata, *Biochim. Biophys. Acta*, 1980, **624**, 575.
[179] J. D. Capra and A. B. Edmundson, *Sci. Am.*, 1977, **236**, 50.

precipitin reactions (see Section 9, p. 232), such as double diffusion in agar gels, radioimmunoassay, and micro-complement fixation.

Changes and Similarities in Protein Conformation.—The ability of subunits or fragments of proteins to retain native conformation can be shown by their ability to cross-react immunologically with the native protein. All antigenic determinants of the native protein may be detectable on the fragments or subunits, as with the β chain of *E. coli* tryptophan synthetase,[180] whose proteolytic fragments F_1 and F_2 cross-react completely with antibody to the native protein, although with greatly reduced affinity, suggesting small conformational differences. Antibodies elicited by the subunits of yeast nuclear RNA polymerases react specifically with these subunits,[181] but inhibition studies indicate that these subunits are not similarly exposed in the native molecule. The antibodies elicited against subunits of human complement components C_3' and C_5' recognize the subunits but fail to recognize the whole enzyme.[182] On denaturation of the native protein full recognition by the respective subunit antibody was attained. Thus, the isolated subunits of these two enzymes were shown to be unstable. Monoclonal antibody to acetylcholine receptor[183] showed cross-reaction between subunits, and that a small region on the α subunit dominated the immunogenicity of the native molecule.

Metal-induced conformational changes can be recognized using conformation specific antibodies. Antibodies specific for the metal-ion stabilized tertiary structure in the bovine prothrombin–calcium complex demonstrated no binding in the absence of Ca^{II},[184] while metal-ion substitution in the prosthetic group of haemoglobin leads to conformational changes detectable by specific antibody.[185]

Function and antigenicity of a protein molecule are not necessarily interdependent. The riboflavin-binding protein from serum egg white and yolk,[186] on sulphonation of four of nine tryptophan residues, remains antigenically analogous to the native protein but loses biological function completely. Chicken liver microsomal terminal desaturase[187] loses antigenicity with function on trypsinization. Antigenicity of a protein may depend on conformational restraints, such as disulphide bridges, the disruption of which causes loss of immunogenicity as in a region of bovine growth hormone.[188] However, the position of such a constraint in relation to the antigenic site is important, and reduction of such a disulphide bond may have no effect on antigenicity,[189] as in proteinase inhibitor I from potato.

It is possible that gross conformational change may occur without a change in immunogenicity being apparent, possibly due to the antigenic determinants retaining their integrity. Human serum lipoprotein CIII[190] shows gross conformational change on chemical treatment, with no loss in immunogenicity.

[180] M. M. Zakin, G. Boulol, and M. E. Goldberg, *Eur. J. Immunol.*, 1980, **19**, 16.
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[183] S. J. Tzartos and J. M. Lindstrom, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 755.
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[187] M. R. Prasad, K. Sreekrishna, and V. C. Joshi, *J. Biol. Chem.*, 1980, **255**, 2583.
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[189] C. Plunkett and C. A. Ryan, *J. Biol. Chem.*, 1980, **255**, 2752.
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Molecular Evolution.—Immunochemical cross-reactions are a function of similarity in sequence and/or conformation of the antigenic sites. This similarity is commonly used to establish relationships between proteins, bearing in mind that the surface features of proteins are subject to much less stringent evolutionary controls than are conformation forming residues. Relationships based on the immune cross-reaction between proteins and enzymes having the same function in different organisms, such as that of carbonic anhydrase III from different mammalian sources,[191] the demonstration of a common antigen on phenylalanine hydroxylase from several mammalian species,[192] glycine decarboxylase from fish, reptile, fowl, and mammals,[193] seed lectins from species of the Solanaceae,[194] and the tetrameric malate dehydrogenase from mesophilic, moderately thermophilic, and extremely thermophilic bacteria,[195] have been shown. These observations may indicate the evolutionary stability of certain structural features, and suggest a common evolutionary precursor.

Family relationships between sea urchins based on the immune cross-reaction of their glucose phosphate dehydrogenases have been studied,[196] relationships between Echinoderms established on immune cross-reactions between hexose 6-phosphate dehydrogenase and glucose 6-phosphate dehydrogenase,[197] suggesting a common ancestral molecule for both enzymes, and the evolutionary relationship of turtles has been suggested by the cross-reactions of their plasma albumins.[198]

Studies on myoglobins have shown the ability of antibodies elicited against sperm whale myoglobin to recognize the structure of myoglobins from many sources,[199] while an anti-beef myoglobin antibody fraction contains some antibodies which bind equally well to sheep or beef myoglobin.[200] These results suggest that the immune response to myoglobin is not species specific. In a bovine serum albumin study, mouse antibodies were found to recognize the same antigenic sites as rabbit antibody.[201]

Antibody Binding Effects.—Two mechanisms have been proposed to interpret the effects induced by antibody binding to enzyme,[202] firstly a direct mechanism involving steric hindrance and/or electrostatic effects between antibody and the substrate, and secondly an allosteric mechanism involving destabilization of some conformation of the enzyme. The oxidation of azurin by cytochrome oxidase from *Ps. aeruginosa* is inhibited by specific antibody[203] and specific antibody inhibits the terminal desaturase activity in chicken microsomes,[187] while monoclonal antibody to microsomal cytochrome P-450 LM2 inhibited the benzo[*a*]pyrene hydroxylation of the enzyme.[204]

[191] S. Jeffery and N. Carter, *Comp. Biochem. Physiol.*, 1980, **66B**, 439.
[192] R. G. H. Cotton, I. C. Jennings, K. H. Choo, and K. Fowler, *Biochem. J.*, 1980, **191**, 777.
[193] K. Hayasaka, H. Kochi, K. Hiraga, and G. Kikuchi, *J. Biol. Chem.*, 1980, **88**, 1193.
[194] D. C. Kilpatrick, C. E. Jeffree, C. M. Lockhart, and M. M. Yeaman, *FEBS Lett.*, 1980, **113**, 129.
[195] T. K. Sudaram, I. P. Wright, and A. E. Wilkinson, *Biochemistry*, 1980, **19**, 2017.
[196] N. Matsuoka, *Comp. Biochem. Physiol.*, 1980, **66B**, 605.
[197] N. Matsuoka and S. H. Hori, *Comp. Biochem. Physiol.*, 1980, **66B**, 191.
[198] B.-Y. Chen, S.-H. Mao, and Y.-H. Ling, *Comp. Biochem. Physiol.*, 1980, **66B**, 421.
[199] S. S. Twining, H. Lehman, and M. Z. Atassi, *Biochem. J.*, 1980, **191**, 681.
[200] I. J. East, P. E. Todd, and S. J. Leach, *Mol. Immunol.*, 1980, **17**, 519.
[201] S. Sakata and M. Z. Atassi, *Biochim. Biophys. Acta*, 1980, **625**, 159.
[202] F. Celada and R. Strom, *Quart. Rev. Biophys.*, 1972, **5**, 395.
[203] M. C. Silvestrini, A. Colosimo, M. Brunori, C. Citro, and R. Zito, *FEBS Lett.*, 1980, **113**, 85.
[204] S. S. Park, A. V. Perasen, M. J. Coon, and H. V. Gelboin, *FEBS Lett.*, 1980, **116**, 231.

Differences in immuno-inhibition can demonstrate differences in enzymes with the same function. Antibody against soluble guanylate cyclase from bovine brain completely inhibited its activity[205] whereas no inhibition was seen with Triton dispersed particulate guanylate cyclase from the same tissues. Antibody raised against Wistar rat liver UDP-glucuronyltransferase slightly inhibited its activity towards l-naphthal, whereas the same antiserum greatly inhibited Gunn-rat liver UDP-glucuronyltransferase activity towards the same substrate,[206] suggesting conformational differences reflected by lower Gunn-rat enzyme specific activity. The effect of multiple antibody binding to antigens on radioimmunoassay binding curves has been investigated,[207] and shown that multivalent antigens do not produce steeper binding curves, but actually tend to produce slightly less steep curves.

Conformational Equilibria.—Isolated synthetic peptide fragments attaining native-like three dimensional structure in isolation have been used to confirm the predicted structure of antigenic sites on human serum albumin[208] as well as on hen egg white lysozyme.[209] A series of synthetic β-endorphin analogues were used to suggest that the immunologically active conformation of β-endorphin has common features with that adopted in secondary structure promoting environment.[210] Antibodies specific for α_2 antiplasmin have been used to show changes in antigenic specificity during complex formation with plasmin.[211] Four antigenic subsets, set I not modulated by complex formation, sets III and IV seen on antiplasmin but not on the complex, and set II sensitive to plasmin proteolysis, are indicated by immunological experiments.

It is suggested that the immunological detection of sequence homologies is more discriminating when unfolded proteins are used. Antibodies against denatured glyceraldehyde 3-phosphate dehydrogenase[212] from *E. coli* K12 cross-react with denatured enzyme from mammalian, avian, fish, and yeast sources whereas antibody against native enzyme recognizes only homologous enzyme from *B. stearothermophilus*. Antibodies raised against denatured actin[213] recognized only antigenic sites on polymerized or partly denatured actin, but not on G-actin, showing that antibodies detect differences in conformation of actin in these different states.

4 Nuclear Magnetic Resonance

Contributed by H. W. E. Rattle

One of the chief characteristics of the n.m.r. method is the very large amount of information that is theoretically available but in practice inaccessible; each n.m.r.

[205] M. Nakane and T. Deguchi, *Biochim. Biophys. Acta*, 1980, **631**, 20.
[206] P. J. Weatherall, S. M. E. Kennedy, and B. Burchell, *Biochem. J.*, 1980, **191**, 155.
[207] T. E. Creighton, *Biochemistry*, 1980, **19**, 4308.
[208] S. Sakata and M. Z. Atassi, *Mol. Immunol.*, 1980, **17**, 139.
[209] Y. Tagaki, A. Hirayama, H. Fujio, and T. Amano, *Biochemistry*, 1980, **19**, 2498.
[210] L. Graff, M. Hollosi, I. Barna, I. Hermann, J. Borvendeg, and N. Ling, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1623.
[211] E. F. Plow, B. Weman, and D. Collen, *J. Biol. Chem.*, 1980, **255**, 2902.
[212] M. M. Zakin, C. Hurth, J.-R. Garel, and C. N. Cohen, *Mol. Immunol.*, 1980, **17**, 1373.
[213] M. Dosseto and C. Goridis, *Mol. Immunol.*, 1980, **17**, 1219.

experiment has to be carefully designed to isolate, and hence extract, one specific piece of data and the skill of the experimenter is revealed both in the genuine usefulness of the information obtained and in the elegance of the method used to get it. In the following panorama of a year's output on the application of n.m.r. to amino-acids, peptides, and proteins, the author has attempted some value judgements in the selection of references for description; for the sake of completeness, however, references which are relevant to a given paragraph or section, but which were not specifically described in the text, are given in parentheses at the end of the section. In such cases the titles of the papers are included with their references.

Techniques.—The assignment of n.m.r. signals in an 18-residue neurotoxin according to the position of the amino-acids in the sequences has been described.[214] Heteronuclear decoupling of the natural-abundance carbonyl ^{13}C and α-carbon proton of adjacent residues was employed, with additional irradiation to suppress interactions of the carbonyl ^{13}C with protons of the same residue. The difficult task of assigning backbone amide proton resonances of small proteins was approached[215] by decoupling them from α-CH resonances while exchange for deuterons was taking place; the authors term this 'on-the-fly' decoupling. The well known reluctance of hydrogen-bonded peptide NH hydrogen to exchange for deuterium in D_2O solution may be of additional use here, but makes it all the more surprising that[216] hydrogen-bonded NH is found to exchange much more readily with chlorine than solvent-exposed NH groups. (See also ref. 217.)

The 'two-dimensional' n.m.r. experiment has been further developed by its chief exponents in the biochemical field. With a single instrument setting, it is capable of yielding a complete network of nuclear Overhauser enhancements[218] or of *J*-connectivities and cross-relaxation pathways involving labile protons[219] in a macromolecule, thus opening another way to the investigation of three-dimensional structure. From the same laboratory comes a discussion of the correlation between the stability and internal mobility of a protein, viewed as being (in solution) a dynamic ensemble of rapidly interconverting structures,[220] backed by a study of the rotational motion of buried ring structures in proteins measured as a function of applied hydrostatic pressure. Large activation volumes were observed, implying that ring flipping occurs in unoccupied volume provided by fluctuations of the overall protein conformation.[221] Further information on internal motion in proteins may be obtained using the fact that peak intensities are affected by the application of off-resonance r.f. fields, and that the effect is related to an induced relaxation rate which complements the usual $1/T_1$, line width, and

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nuclear Overhauser enhancement (NOE) data in internal motion determination.[222] If the system under investigation is an enzyme activated by both monovalent and divalent cations, a new method for inter-ion distance determination using relaxation effects is possible;[223] the divalent cation is replaced by a paramagnetic ion, and the resultant paramagnetic effect on the longitudinal relaxation of the monovalent ion measured separately for two isotopes of the monovalent ion. Suitable monovalent ion pairs are $^{6}Li^{+}$ and $^{7}Li^{+}$, $^{15}NH_4^{+}$ and $^{14}NH_4^{+}$, and $^{85}Rb^{+}$ and $^{87}Rb^{+}$. Application of the Solomon–Bloembergen equation leads to unambiguous distance data. (See also refs. 224—227.)

Among other new techniques reported in 1980 are a method for the quantitative determination of the total protein content of natural products using a copper relaxation reagent [228] and, rather the opposite, the suppression of the total haemoglobin spectrum in proton spectroscopy of intact erythrocytes by using selective transfer of saturation by spin diffusion, in order to reveal the spectra of other components of the system.[229] A new possibility for the study of enzyme mechanisms involving phosphorus is opened by confirmation that, for most phosphate derivatives of biochemical interest, a broadening effect due to the presence of a neighbouring ^{17}O nucleus is detectable; this effect can be combined with direct ^{17}O resonance measurements to study the interaction of diamagnetic enzyme-bound metal ions with nucleotides.[230]

Amino-acids and Synthetic Peptides.—N.m.r. studies of amino-acids are now restricted, mostly aimed at providing model data for ultimate application to proteins.[231–239] The same may be said of synthetic polypeptides; poly-L-lysine [240] is a useful model polyelectrolyte for investigating the ability of protein to modulate

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bilayer/non-bilayer transitions in phospholipids, and in mixed methanol–water solution,[241] for investigation of variations in rotational diffusion through the α-helix–random coil transition. (See also refs. 242—246.)

A review of structural studies of peptides, including many using n.m.r., may be found in the Proceedings of the 6th American Peptide Symposium.[247] The development of ^{15}N spectroscopy for peptide and protein studies continues, and some of the advantages of this relatively new probe into the peptide backbone are now becoming apparent: in proline-containing peptides, the ^{15}N nucleus is very sensitive to conformational changes induced by *cis–trans* isomerism of the proline, and these effects are long-range and depend on both the amino-acid side-chains and the solvent.[248] Strong neighbouring residue effects have been seen in random co-polymers of Gly, Leu, and Val; the spectra resemble a superposition of the corresponding binary co-polymers.[249] In a series of tripeptides of the form Gly-Gly-L-X a combination of double-resonance and difference spectroscopy gave values for $J^{15}N'H$ and ^{15}N chemical shifts, though not yet sufficient for a systematic analysis of their behaviour.[250] Attempts to improve structure analysis using shift reagents on ^{15}N samples were not entirely successful;[251] solvent effects proved more useful, while an attack on the sensitivity problem for ^{15}N by Overhauser enhancement[252] using the INEPT pulse sequence to transfer spin polarization from amide protons to ^{15}N produced improvement over unenhanced spectra of factors of 8 for proton decoupled and 15 for proton coupled spectra, a very worthwhile improvement. (See also refs. 253—255.)

A number of proline-containing peptides have been investigated by n.m.r. methods: the model peptide pivaloyl-Pro-Pro-Ala-NHMe exhibited a *trans–trans* isomeric structure in solutions, with successive 4 → 1 intramolecular hydrogen bonds (β-turns) leading to an incipient 3_{10} helix.[256] β-Turns were also found in a series of tetrapeptides with proline as residue 2,[257] while 3 → 1 intramolecular

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hydrogen bonds (γ turns) were a characteristic feature of the structure of both the cyclic tetrapeptides [Ala^4]-desdimethylchlamydocin and *cyclo*(-D-Phe-Pro-D-Phe-Pro-) in deuteriated chloroform–dimethyl sulphoxide solvent mixtures.[258] The rapid conformational flexibility of Cγ of proline residues is largely inhibited in hydroxyproline, leading to a much more rigid structure with much more puckered rings; hydroxyproline residues thus play a key role in the stability of the triple-helical peptides of collagen[259] (but see refs. 482—484). In experiments on two cyclic (Tyr-Ile-Pro-Leu) diastereoisomers, which are simplified analogues of a phytotoxic peptide produced by *Cylindrocladium*, a unique *trans–trans–cis–trans* conformation was deduced, the Ile-Pro bond being *cis*.[260]

Other studies involving small synthetic peptides include a series[261–263] on the binding of various divalent cations to the tripeptide Asp-Ala-His-*N*-methylamide, which is the *N*-terminus of the human serum albumin molecule, with clear evidence for metal co-ordination in each case. A combination of transfer of saturation and selective saturation recovery methods has been used to estimate amide H exchange rates, and hence to some extent conformational mobility, in a pentapeptide that represents the active fragment of thymopoietin and that was found to be in a mobile conformational equilibrium between several conformations.[264, 265] (See also refs. 266—277.)

Small Natural Peptides.—*Peptide Hormones.* The pentapeptide neurotransmitters methionine- and leucine-enkephalin have aroused a great deal of interest of late.

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[271] Temperature and pH dependence of proton n.m.r. of glutamine in peptides. R. M. Zanacchi and W. J. Moore, *Aust. J. Chem.*, 1980, **33**, 1505.
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Selective deuteriation of the *N*-terminal tyrosine residue [278] permitted analysis of rotamer populations of its side-chain. Other studies also concentrated on modifications of the molecule: ^{15}N enrichment of the *N*-terminal tetrapeptide,[279] formation of an acetaldehyde adduct,[280] and substitution of D-alanine for glycine-2, which produces a relatively rigid backbone.[281] The native molecule exists in its dipolar form in water near neutral pH.[282]

The hormone somatostatin is a fourteen-residue peptide with a single intramolecular disulphide bridge. Complete assignments for its proton [283] and proton and ^{13}C spectra [284] are presented, both papers concluding that the molecule has a number of preferred conformations and exchanges rapidly between them, with possibly a region of higher stability in the part of the molecule furthest from the disulphide bridge. The smaller pituitary hormone oxytocin also has a disulphide bridge, and a series of specifically designed and synthesized isotopic isomers containing ^{13}C and ^{15}N nuclei at selected sites in the two half-cystyl residues was used to show that the torsion angle χ^1 has the eclipsed value of $-120°$ for half-cystyl 1 and approximately $+120°$ for half-cystyl 6.[285] ^{13}C Labelling at the *meta* positions of tyrosine-2 of oxytocin revealed that the tyrosine undergoes hindered rotation when oxytocin is bound to neurophysin.[286] Further light on the binding of peptides to neurophysin will be cast by the application of newly described spin-labels capable of binding to its hormone-binding sites;[287] first results suggest that residue 3 of the hormone is > 14 Å from tyrosine-49 in the neurophysin. The linear octapeptide hormone angiotensin is a potent hypertensive agent which stimulates the smooth muscles of blood vessels. It also mediates the transport of manganese ions across phosphatidylcholine bilayers, which, studied by n.m.r., may lead to a clearer understanding of the role of metal ions in its physiological activity.[288] (See also refs. 289—292.)

Peptide Antibiotics, Toxins, and Inhibitors. Nitrogen-15 spectroscopy has been applied to solutions of gramicidin-S in organic solvents in order to distinguish

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between solvent-exposed and solvent-shielded peptide groups. Three methods are described:[293] solvent dependence and temperature dependence of ^{15}N chemical shifts, and lability of the N–H proton in the presence of added base. Another relatively little-used resonant nucleus, ^{23}Na, has been used to study the dynamics of the transport of sodium ions through membranes *via* the malonyl gramicidin channel.[294] The cyclic depsipeptide valinomycin, incorporated into small phospholipid vesicle bilayers, has a similar conformation to that in non-polar organic solvents, suggesting a location in the interior of the bilayer,[295] while several analogous molecules have been studied in terms of their ability to transport alkali-metal ions into the organic phase of a two-phase system.[296] Reports will also be found of n.m.r. studies of siomycins,[297] tuftsin,[298] and bacitracin A in its complex with a zinc ion.[299]

The structures of the crystalline form of the erabutoxins a, b, and c from the sea-snake *Laticauda semifasciata* have been determined by *X*-ray methods and used [300] to assign a large number of resonances in the 270 MHz proton resonance spectrum, including the lysine ε-NH_2 resonances and all of the valine, leucine, and isoleucine methyls. These assignments will undoubtedly be of value in n.m.r. studies of other snake venom toxins, of which there are many variants with closely related structures. Hydrogen–deuterium exchange rates indicate [301] that some 17 backbone and 9 side-chain NH atoms exchange slowly, indicating that the erabutoxin b molecule in solution does in fact have the number of H-bonds indicated by the crystal data. Apamin, a toxin from bee venom, would seem to present even greater difficulties, but it has nevertheless been subjected to extensive n.m.r. and model-building studies that suggest an α-helix from residues 6—13 coupled with three β-turns, giving a very plausible tertiary structure for this 18-residue peptide.[302, 303]

The basic pancreatic trypsin inhibitor (BPTI, mol. wt. 6500) continues to provide a useful model system for proteins of intermediate size, as well as having its own intrinsic interest. Among its internal motions, there is a small contribution to relaxation by low-frequency distortional motion of the protein backbone [304, 305] and the following figures are given: for overall rotational motions, $T_R = 4 \times 10^{-9}$ s; for librational wobbling of backbone α atoms, $T_W = 1 \times 10^{-9}$ s; for librational motions of sidechains, $T_W = 4 \times 10^{-10}$ to

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[305] R. Richarz, K. Nagayama, and K. Wüthrich, *Biochemistry*, 1980, **19**, 5189.

3×10^{-9} s; for methyl rotation, $T_F < 1 \times 10^{-11}$ s.[305] Specific labelling of the carbonyl carbon of lysine-15 of the inhibitor with ^{13}C permitted studies of the reactive-site peptide bond Lys 15—Ala 16 in complexes with trypsin; the results show that no formation of a covalent bond to this carbonyl carbon takes place during formation of the complex.[306] A similar conclusion is drawn from another careful study, this time involving ^{13}C labelling near the Arg 63-Ile 64 reactive-site peptide bond of soybean trypsin inhibitor.[307] Formation of a non-native, but stable, conformer of BPTI on refolding the protein with its normal disulphide bridges[308] may cast some light on the sources of the conformational stability of the protein. (See also refs. 309—311.)

Enzymes.—*Class 1: Oxidoreductases.* Two models for the active site of liver alcohol dehydrogenase have been proposed; *X*-ray data imply a direct co-ordination between substrate and active-site zincs, whereas n.m.r. studies on the Co^{2+} derivative deny such direct binding. New n.m.r. work[312] supports the latter conclusion, being consistent with a model in which a metal–water ligand forms a bridge between substrate and metal. Various attempts to explain the discrepancy between n.m.r. and *X*-ray results are presented[313, 314] (*cf.* ref. 315). The ability of a number of dehydrogenase and other enzymes to bind modified NADH as co-enzyme, where the modification caused the nucleotide to be in the *syn*, rather than the *anti*, conformation, is discussed.[316] Binding of co-enzymes is the subject of a number of interesting papers on dihydrofolate reductase from *Lactobacillus casei*[317–319] and *Escherichia coli*,[320] showing differences between the two enzymes; rates of interaction and some steric details are presented, along with the effects of binding trimethoprim and methotrexate. Various aspects of dihydrofolate reductase ligand binding are reported by the same investigators: photo-CIDNP measurements revealed ligand-induced conformational changes in the enzyme[321] and other techniques applied include selective deuteriation,[322] satu-

[306] R. Richarz, H. Tschesche, and K. Wüthrich, *Biochemistry*, 1980, **19**, 5711.
[307] M. W. Baillargeon, M. Laskowski, D. E. Neves, M. A. Porubcan, R. E. Santini, and J. L. Markley, *Biochemistry*, 1980, **19**, 5703.
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[310] Solvent accessibility and microenvironment in *Streptomyces subtilisin* inhibitor. Y. Satow, Y. Watanabe, and Y. Mitsui, *J. Biochem. (Tokyo)*, 1980, **88**, 1739.
[311] Acid denaturation steps of *Streptomyces subtilisin* inhibitor. S. Fujii, K. Akasaka, and H. Hatano, *J. Biochem. (Tokyo)*, 1980, **88**, 789.
[312] B. E. Drysdale and D. P. Hollis, *Arch. Biochem. Biophys.*, 1980, **205**, 267.
[313] I. Andersson, D. R. Burton, H. Dietrich, W. Maret, and M. Zeppezauer, *Metalloproteins, Autumn Meet. Ger. Biochem. Soc.*, 1979, p. 246.
[314] D. T. Jones and R. G. Khalifah, *Adv. Exp. Med. Biol.*, 1980, **132**, 77.
[315] D. C. Anderson and F. W. Dahlquist, *Biochemistry*, 1980, **19**, 5486.
[316] D. A. Lappi, F. E. Evans, and N. O. Kaplan, *Biochemistry*, 1980, **19**, 3841.
[317] E. I. Hyde, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, *Biochemistry*, 1980, **19**, 3738.
[318] E. I. Hyde, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, *Biochemistry*, 1980, **19**, 3746.
[319] B. Birdsall, A. S. V. Burgen, and G. C. K. Roberts, *Biochemistry*, 1980, **19**, 3723.
[320] P. J. Cayley, J. Feeney, and B. J. Kimber, *Int. J. Biol. Macromol.*, 1980, **2**, 251.
[321] J. Feeney, G. C. K. Roberts, R. Kaptein, B. Birdsall, A. Gronenborn, and A. S. V. Burgen, *Biochemistry*, 1980, **19**, 2466.
[322] J. Feeney, G. C. K. Roberts, J. W. Thomson, R. W. King, D. V. Griffiths, and A. S. V. Burgen, *Biochemistry*, 1980, **19**, 2316.

ration transfer,[323] modification with *N*-bromosuccinimide,[324] and histidine titration[325] of a histidine resonance required by the sequence of the protein but not previously observed.

An interesting structural inference may be drawn from the relaxation rates of protons and ^{17}O nuclei of water in the presence of the copper protein laccase from *Rhus vernicifera*;[326] the relative inertness of the water oxygen atom to paramagnetic relaxation enhancement, contrasted with the much stronger effect on ^{1}H nuclei, implies that the type 2 and 3 copper sites are buried in such a way as to be accessible only to protons.[327] The copper–zinc enzyme superoxide dismutase, which is known specifically to inhibit adrenaline autoxidation, has been studied by several groups; results include the assignment of a number of histidine resonances,[328] exchange studies on histidine NH protons, which show that only one of the four histidines is not ligated to the Zn atom,[329] the binding of adrenaline,[330, 331] and the binding of anions to the copper atom.[332] Other enzymes of class 1 that have been studied include the cuproprotein diamine oxidase; the proton magnetic relaxation dispersion shows two values for $1/T_1$, at 16 and 75 MHz, whereas $1/T_2$ shows a minimum at 20 MHz. The implication is that the two Cu^{2+} ions of the protein are in quite different chemical environments.[333]

Class 2: Transferases. A ^{19}F probe, 5-fluoro-2^{1}-deoxyuridylate, has been used[334] to study binding to thymidylate synthase. ^{19}F Chemical shift changes on binding vary with protein preparation methods, and are greatly enhanced by the formation of a ternary complex with methylenetetrahydrofolate. In a selective ^{13}C labelling experiment on the binding of ATP (effector) and CTP (inhibitor) to aspartate transcarbamylase[335] it was shown that, while three histidine residues reacted identically to ATP and to CTP binding, two phenylalanines were affected only by CTP. The bovine galactosyltransferase–manganese–UDP–galactose ternary complex apparently exists in two forms, an initially formed, rapidly exchanging conformer, effective in enhancing the relaxation of solvent water protons, which slowly converts to a second form in which the metal centre is much less accessible to solvent.[336] The effect of a Mn^{2+} centre on the T_1 values of a large number of monovalent cations[337] in pyruvate kinase permitted accurate distance measure-

[323] B. Birdsall, J. Feeney, G. C. K. Roberts, and A. S. V. Burgen, *FEBS Lett.*, 1980, **120**, 107.
[324] J. W. Thomson, G. C. K. Roberts, and A. S. V. Burgen, *Biochem. J.*, 1980, **187**, 501.
[325] P. Wyeth, A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, *Biochemistry*, 1980, **19**, 2608.
[326] M. Goldberg, S. Vuk-Pavlovic, and I. Pecht, *Biochemistry*, 1980, **19**, 5181.
[327] A proton nuclear magnetic relaxation study of tree laccase and related copper proteins. A. Rigo, E. F. Orsega, P. Viglino, L. Morpurgo, M. T. Graziani, and G. Rotilio, *Metalloproteins, Autumn Meet. Ger. Biochem. Soc.*, 1979, p. 29.
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[329] A. R. Burger, S. J. Lippard, M. W. Pantoliano, and J. S. Valentine, *Biochemistry*, 1980, **19**, 4139.
[330] L. M. Schubotz and U. Weser, *Inorg. Chim. Acta*, 1980, **46**, 113.
[331] L. M. Schubotz and U. Weser, *Metalloproteins, Autumn Meet. Ger. Biochem. Soc.*, 1979, p. 127.
[332] I. Bertini, C. Luchinat, and A. Scozzafava, *J. Am. Chem. Soc.*, 1980, **102**, 7349.
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[334] M. J. Beckage, M. Blumenstein, and R. L. Kisliuk, *Mol. Cell. Biochem.*, 1980, **32**, 45.
[335] A. C. Moore and D. T. Browne, *Biochemistry*, 1980, **19**, 5768.
[336] P. J. Andree and L. J. Berliner, *Biochemistry*, 1980, **19**, 929.
[337] F. M. Raushel and J. J. Villafranca, *Biochemistry*, 1980, **19**, 5481.

ment from less than 4 Å to 20 Å between the paramagnetic ion and the bound cation; the method may find application in many other proteins. In bovine heart protein kinase, the mechanism of regulation has been investigated by n.m.r.; it appears that the regulatory subunit acts by physically blocking the substrate binding site.[338] (See also refs. 339 and 340.)

Class 3: Hydrolases. When the ten histidine residues of alkaline phosphatase are labelled at the γ-carbon with ^{13}C, the resultant ^{13}C spectrum has nine resonances spread over 14 p.p.m.[341] Only four of the histidines titrate with pH; the other six appear to be deeply buried, three ligated to active-site metal ion(s) and two at or near the active site. Unambiguous identification of the three ligated histidines was possible using ^{113}Cd—^{13}C spin–spin coupling.[342, 343]

Investigation of the ribonuclease S-protein–S-peptide complex has become more sophisticated; the enthalpy of binding of residues 1—15 to the S-protein is 1.7 kcal mol^{-1} less than that of the full 1—20 S-peptide[344] with a 5 × greater dissociation constant, while a hybrid between rat S-peptide and bovine S-protein confirmed earlier findings that the catalytic properties of the native enzyme are modulated by the S-protein region of the molecule.[345] The tautomeric states of the histidines of RNAase are reported[346] along with a probable hydrogen-binding scheme, and it is shown[347] that the intermediate states of regeneration of RNAase A from its reduced state are more disordered than the reduced form itself. (See also refs. 348—353.) Assignment of the proton n.m.r. spectrum of lysozyme now extends to some 70 resonances from 25 residues.[354]

AMP nucleosidase catalyses the hydrolysis of the *N*-glycosidic bond of AMP, and requires a metal–ATP^{2-} complex as an allosteric activator. A combination of

[338] J. Granot, A. S. Mildvan, K. Hiyama, H. Kondo, and E. T. Kaiser, *J. Biol. Chem.*, 1980, **255**, 4569.
[339] The role of folding units in the kinetic folding of globular proteins. B. Adams, R. J. Burgess, E. A. Carrey, I. R. Mackintosh, C. Mitchinson, R. M. Thomas, and R. H. Pain, *Protein Folding, Proc. Conf. Ger. Biochem. Soc. 28*, 1979, p. 447.
[340] Phosphorus-31 n.m.r. quantitation of the displacement of equilibriums of arginine, creatine, pyruvate, and 3-phosphoglycerate kinase reactions by substitution of sulphur for oxygen in the β phosphate of ATP. C. L. Lerman and M. Cohn, *J. Biol. Chem.*, 1980, **255**, 8756.
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[347] Y. Konishi and H. A. Scheraga, *Biochemistry*, 1980, **19**, 1316.
[348] Guanyl-specific ribonuclease from the fungus *Penicillium chrysogenum* studied by n.m.r. G. I. Yakovlev, M. Ya. Karpeiskii, S. I. Bezborodova, O. P. Beletskaya, and V. G. Sakharovskii, *Eur. J. Biochem.*, 1980, **109**, 75.
[349] Ribonuclease hydration and its thermal stability in water and deuterium oxide solutions. G. M. Mrevlishvili, V. I. Lobyshev, G. Sh. Dzhaparidze, V. M. Sokhadze, D. A. Tatishvili, L. V. Orvelashvili, and Yu. G. Sharimanov, *Biofizika*, 1980, **25**, 44.
[350] Proton nuclear magnetic relaxation and thermodynamic parameters of ribonuclease solutions under thermal denaturation. G. M. Mrevlishvili and Yu. G. Sharimanov, *Biofizika*, 1980, **25**, 338.
[351] Proton magnetic relaxation of proteins in the solid state: molecular dynamics of ribonuclease. E. R. Andrew, D. J. Bryant, and E. M. Cashell, *Chem. Phys. Lett.*, 1980, **69**, 551.
[352] Solvation of lysozyme in water–dioxane mixtures studied in the frozen state by n.m.r. spectroscopy. T. Izumi, Y. Yoshimura, and H. Inoue, *Arch. Biochem. Biophys.*, 1980, **200**, 444.
[353] Water–protein interactions. N.m.r. results on hydrated lysozyme. R. G. Bryant and W. M. Shirley, *Am. Chem. Soc., Symp. Ser.*, 1980, **127**, 147.
[354] F. M. Poulsen, J. C. Hoch, and C. M. Dobson, *Biochemistry*, 1980, **19**, 2597.

n.m.r. and e.p.r. methods reveals that the catalytic and allosteric sites are at least 25 Å apart.[355]

The active-site methionine-192 of chymotrypsin is an open invitation to labelling experiments; it has been *S*-[^{13}C]methylated,[356] giving two resonances, one of which did not appear in the phenylmethylsulphonyl derivative of the enzyme, and also ^{19}F labelled[357] (*cf.* refs. 358—360). The conformational transition from trypsinogen to trypsin has been carefully investigated using a series of ring-current shifted high field resonances and calibrated Johnson–Bovey calculations; it seems clear that activation involves subtle changes of conformation and flexibility in certain regions of the molecule not detectable within the precision of *X*-ray crystallographic studies.[361] Fragments 1 and 2 of bovine prothrombin appear, from their 270 MHz ^{1}H n.m.r. spectra, to be random coils containing a small amount of tertiary structure, probably in the structurally homologous Kringle regions;[362] a number of binding sites for Eu^{3+}, falling into at least two types, exist on prothrombin fragment 1.[363] Strong evidence for the existence of an ion-pair interaction between the active site Cys-25 and His-159 in papain is discussed in two papers; the p*K* of the histidine is ~8.6 in the active enzyme (succinylated to improve solubility at high pH).[364, 365] The inactivation of β-lactamase I (penicillinase) by 6β-bromopenicillanic acid is associated with acylation of serine-70 and with rearrangement and cyclization of the inhibitor;[366] the binding of Co^{2+} to the enzyme is discussed in ref. 367. Reference to other n.m.r. studies of Class 3 enzymes may be found in refs. 368—372.

Class 4: Lyases. A number of earlier studies of carbonic anhydrase have used metal substitutions. It is pointed out[373] that co-ordination of carboxymethylated

[355] W. E. DeWolf, G. D. Markham, and V. L. Schramm, *J. Biol. Chem.*, 1980, **255**, 8210.
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[357] B. H. Landis and L. J. Berliner, *J. Am. Chem. Soc.*, 1980, **102**, 5350.
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[359] Fluorine n.m.r. studies of poly(*N*-acryloyl-β-alanine)–α-chymotrypsin conjugates. J. T. Gerig and D. T. Loehr, *Biopolymers*, 1980, **19**, 1827.
[360] A proton n.m.r. study of *N*-trifluoroacetyl-L-alanyl-L-phenylalaninal binding to α-chymotrypsin. P. Wyeth, R. P. Sharma, and M. Akhtar, *Eur. J. Biochem.*, 1980, **105**, 581.
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[365] S. D. Lewis, F. A. Johnson, and J. A. Shafer, *Biochemistry*, 1981, **20**, 48.
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[367] A. Galdes, H. A. O. Hill, G. S. Baldwin, S. G. Waley, and E. P. Abraham, *Biochem. J.*, 1980, **187**, 789.
[368] Proton n.m.r. and CIDNP studies on bovine pancreatic phospholipase A_2. M. R. Egmond, A. J. Slotboom, G. H. De Haas, K. Dijkstra, and R. Kaptein, *Biochim. Biophys. Acta*, 1980, **623**, 461.
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histidine-200 takes place only to the native active site Zn^{2+} ion and possibly to Co^{2+}, but not to Cd^{2+} or Hg^{2+}; caution is advised in using metal replacement techniques for this protein. The effects of pH and bicarbonate on ^{113}Cd-carbonic anhydrase cadmium resonance spectra show that there is a rapid equilibrium between hydroxide, water, and bicarbonate occupancy of the open co-ordination site of the metal ion.[374] According to other workers,[375] the Cd^{2+} enzyme is inactive in the reversible hydration of acetaldehyde. Photo-CIDNP studies of the binding of sulphanilimide inhibitor to carbonic anhydrase [376, 377] enable the direct observation of bound and free ligand (*cf.* refs. 378 and 379).

Phosphorus-31 resonance following the binding of an analogue of the co-factor pyridoxal phosphate to D-serine dehydratase showed shifts of p*K* value which are useful in studying the binding of co-factors.[380] The mechanism of action of 5-aminolaevulinic acid dehydratase has been elucidated by ^{13}C n.m.r.[381]

Class 6: Synthetases. ^{31}P Resonance was used [382] to investigate the catalysis of the enzyme-bound methionine–MgATP $\rightleftharpoons$ methionine–AMP–Mg–pyrophosphate reaction by methionyl-tRNA synthetase. An upper rate of 360 s^{-1} was found for the left-to-right reaction. Similar techniques were used [383] on the action of carbamoyl phosphate synthetase, supporting the formation of two intermediates, carboxyphosphate and carbamate, in the overall reaction catalysed. The role of enzyme-bound Mn^{2+}, an essential activator bound at the active site of phosphoribosyl pyrophosphate synthetase, was also probed by ^{31}P and proton relaxation; the conformation of the bound nucleotide had a torsional angle at the glycosidic bond which differed by at least 20° from that found in solution. The arrangement of the substrates at the active site was determined.[384]

Haem Proteins.—A major discrepancy between the results of an n.m.r. study [385] of the haem crevice in cytochrome b_5, a cytochrome present mainly in animal microsomes, and *X*-ray studies has now been resolved. The n.m.r. results revealed that the orientation of the haem group as previously reported was 180° out, and subsequent *X*-ray re-analysis at 2 Å resolution confirmed this.[386] There was no evidence of any difference in peptide conformation near the haem between crystal and solution structures. Another cytochrome belonging to the *b* group in which the haem is not covalently bound to protein is P-450. Proton resonance

[374] N. B. H. Jonsson, L. A. E. Tibell, J. L. Evelhoch, S. J. Bell, and J. L. Sudmeier, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3269.
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[378] N.m.r. studies of carbonic anhydrase. J. M. Pesando and R. K. Gupta, *Biophys. Physiol. Carbon Dioxide, Symp.*, 1979, p. 273.
[379] Interaction of bovine carbonic anhydrase with (neutral) aniline, phenol and methanol. G. S. Jacob, R. D. Brown, and S. H. Koenig, *Biochemistry*, 1980, **19**, 3754.
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[382] G. Fayat, S. Blanquet, B. D. N. Rao, and M. Cohn, *J. Biol. Chem.*, 1980, **255**, 8164.
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[385] R. M. Keller and K. Wüthrich, *Biochim. Biophys. Acta*, 1980, **621**, 204.
[386] F. S. Mathews, *Biochim. Biophys. Acta*, 1980, **622**, 375.

spectroscopy of high-spin ferrous P-450 models is reported,[387] and water relaxation time measurements were used to compare the solvation spheres of cytochromes P-450 and b_5 in the presence of acetanilide and imidazole;[388] water proton relaxation enhancement found in the presence of P-450 but not b_5 was removed by imidazole. Doubt has been cast on the efficacy of water relaxation measurements to determine whether water is a sixth ligand of the iron atom.[389] While acetanilide had no effect on water T_1, its own phenyl or methyl protons experienced a selective relaxation enhancement in the presence of various cytochrome P-450's, indicating at least a close approach of these groups to the metal centre;[390] a close approach to the metal of P-450 was also observed by ^{3}H n.m.r. for the labelled region of [6-^{3}H]benzo[*a*]pyrene.[391]

Cytochromes of the *c* class, in which haem side-chains are covalently linked to the protein, are reported to induce non-bilayer structures in cardiolipin-containing model membranes.[392] Substitution of various diamagnetic and paramagnetic metal ions for the Fe atom in horse cytochrome *c* revealed a small conformational change on oxidation;[393] from the same laboratory comes a lengthy sequence of other papers on the n.m.r. of cytochrome *c*, including the assignment of aromatic[394] and aliphatic[395] residues, pH and temperature dependence of ferro-[396] and ferri-[397] cytochrome *c*, and comparison between horse, tuna, and various eukaryotic cytochromes.[398, 399] Given suitable *X*-ray data, it will be interesting to compare these spectra with those predicted, using various ring-current models for the haem ring.[400] The chirality of the axial methionine co-ordinated to the iron atom has been shown to differ[401] between cytochrome *c* and cytochrome *c*-551, apparently explaining previous reports of different electronic haem structures between the two proteins. Anion binding to cytochrome *c*[402, 403] and electron spin relaxation[404] have been discussed.

The low-potential, low-spin cytochrome c_3 from *Desulphovibrio gigas* has been studied through reoxidation[405] and in its interaction with rubredoxin and flavodoxin;[406] some doubt has been cast, however, on the state of n.m.r. studies of this protein, since an earlier outline structure based on sequence and n.m.r. data of

[387] R. C. Parmely and H. M. Goff, *J. Inorg. Biochem.*, 1980, **12**, 269.
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[395] G. R. Moore and R. J. P. Williams, *Eur. J. Biochem.*, 1980, **103**, 503.
[396] G. R. Moore and R. J. P. Williams, *Eur. J. Biochem.*, 1980, **103**, 513.
[397] G. R. Moore and R. J. P. Williams, *Eur. J. Biochem.*, 1980, **103**, 523.
[398] G. R. Moore and R. J. P. Williams, *Eur. J. Biochem.*, 1980, **103**, 533.
[399] G. R. Moore and R. J. P. Williams, *Eur. J. Biochem.*, 1980, **103**, 543.
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[405] I. Moura, J. J. G. Moura, M. H. Santos, and A. V. Xavier, *Cienc. Biol. (Coimbra)*, 1980, **5**, 189.
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cytochrome c_3 from *Desulphovibrio desulphuricans* has been shown not to fit the recently acquired *X*-ray data.[407] The pH and temperature dependence of chemical shifts in the 270 MHz proton resonance spectrum of cytochrome c_1 from *Rhodospirillium rubrum* show haem methyl resonances with p*K* values of 5.8 and 8.7, and spectral changes that correlate with visible spectral changes.[408, 409] (See also refs. 410—413.)

Selective deuteriation of protohaemins and deuterohaemins has enabled workers to resolve 17, and unambiguously assign 12, of the 22 possible haem resonances in native sperm whale myoglobin.[414, 415] Different hyperfine shift patterns for the low-spin and high-spin states were concluded to arise from differential sensitivities of the dominant spin transfer mechanisms to the same rhombic perturbation.[416] For haemoglobin, the possibility of looking at valency hybrids has been exploited to assign two proximal histidyl imidazole exchangeable proton signals at 64 and 76 p.p.m. to individual α and β subunits.[417] (Complexes between imidazole derivatives and methaemoglobin and metmyoglobin have been studied.[418]) In another study of far-downfield resonances from valency hybrids, resonances at 58.5 and 71.0 p.p.m. downfield of the water signal were assigned to proximal histidine exchangeable NH resonances from α and β chains respectively.[419] Histidine-β146 of human adult haemoglobin has been the subject of an investigation of the alkaline Bohr effect[420] with the following conclusions: in 0.2 M phosphate, 0.2 M NaCl, a salt bridge between His-β146 and Asp-β94 is broken during the quaternary structural transition, and the β146 is partly responsible for the Bohr effect, whereas in 0.1 M bis-tris neither of these statements is true. Thus the alkaline Bohr effect varies in its detailed mechanism according to the experimental conditions (*cf.* refs. 421—423). Interest continues in the mechan-

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ism of aggregation of the haemoglobin S responsible for sickle-cell anaemia; evidence is presented[424] from relaxation measurements for the formation of small molecular aggregates as precursors to the fully gelated form, thus providing the possibility of investigating the intermolecular contacts responsible for the aggregation. An n.m.r. method for measuring the amount of sickle Hb polymer within sickled erythrocytes as a function of oxygen saturation is presented in ref. 425. (See also ref. 426.)

Water proton relaxation studies of the haem environment of horseradish peroxidase are reported;[427] deuterium labelling[428] of selected positions of haemin and deuterohaemin yielded hyperfine shift patterns consistent with a Fe^{3+} porphyrin exhibiting appreciable $S = 3/2$ character. Reconstituted horseradish peroxidase with deuterohaemin revealed a 180° rotation of the porphyrin relative to the native protein.[429]

Cytochrome *c* oxidase is difficult to place in this review, being classified as both an enzyme and a cytochrome, and also containing copper. Specific trifluoroacetylation at single lysine side-chains of cytochrome *c* showed that only those lysines near the haem crevice affected reaction rates on modification, but that their ^{19}F relaxation was unaffected on binding of the oxidase, indicating that no detectable conformational changes occurred.[430] Proton resonance studies at 360 MHz of cytochrome *c* oxidase revealed resonances spread over a range of 96 p.p.m., with dramatically pH-dependent behaviour.[431] (See also refs. 432—438.)

Other Proteins.—*Membrane Proteins.* Turning to proteins associated with membranes, it is shown[439] that monomeric melittin is predominantly in an extended flexible form, with fragments 5—9 and 14—20 more highly structured. Formation

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of a tetramer of melittin [440] causes it to take up a conformation similar to that found when the protein is associated with detergent micelles. Truncated-driven nuclear Overhauser enhancement difference spectroscopy is suggested as a powerful method for investigating lipid-bound proteins.[441] The ^{13}C relaxation times of phosphatidylcholine vesicles were unaffected by cytochrome *c* but reduced, for ^{13}C nuclei near the bilayer centre, by myelin basic protein, indicating a penetration of the bilayer by this protein.[442] Membrane-bound ATPase has been investigated by ^{2}H n.m.r.[443] and also by e.p.r. and n.m.r. using paramagnetic probes;[444] in the latter case the Mn^{2+}-to-Cr^{3+} distance in the ATPase–Mn–Cr–ATP complex was 8.1 Å. Proton resonance of the 93-residue porcine pancreatic colipase A [445] showed that tyrosines-56 and -57 and histidine-86 were close to each other; these residues are at the ends of two hydrophobic β-sheet regions (residues 49—57 and 77—85) which may play a role in the association of colipase with the lipid–water interface.

Lipoproteins. Lipids are transported in blood by lipoproteins. Comparative studies of human high-density lipoprotein fractions HDL_2 and HDL_3 suggest that the motions of phospholipids with correlation times in excess of 10^{-6} s were more restricted in the latter, [446] while lipoprotein-X, one of the low-density lipoproteins, has been studied by ^{1}H, ^{31}P,[447] and ^{13}C [448] spectroscopy to show spectra quite different from other low-density lipoproteins. Relaxation measurements suggest that the motions of cholesterol rings and fatty acid side-chains are more restricted in LP-X than either HDL_3 or LDL. A strong temperature dependence of the mobility of carbon nuclei within the steroid ring of cholesteryl esters is reported.[449] The involvement of the sequence around methionine-38 in phospholipid binding by apolipoprotein C-1 has been probed by both nitroxide labelling for e.s.r. and ^{13}C labelling for n.m.r.; significant structural change in the region of this residue was observed both on the binding of phospholipid and on denaturing the protein.[450] Two different types of complex were found in the binding of apolipoprotein A-1 with sonicated vesicles of dimyristoylphosphatidylcholine.[451] Glycophorin is one of the intrinsic proteins of erythrocyte membranes, a glycoprotein whose structure falls into three domains. Proton resonance revealed very different mobilities in the three regions, particularly in the central intramembranous hydrophobic region, which was extremely resistant to normal

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denaturing conditions, eventually submitting to the gentle ministrations of trifluoroacetic acid.[452] Other glycoproteins studied by n.m.r. include fibrinogen,[453] human plasma α_1-acid glycoprotein,[454] and antifreeze glycoproteins from the Antarctic cod.[455]

Ligand Binding Proteins. Moving on to proteins that specifically bind and/or transport other ions or molecules, an interesting stoicheiometry of two molecules of uteroglobin, a progesterone-binding protein, to one molecule of progesterone, has been confirmed;[456] the mechanism of interaction critically involved histidine-8 of the protein, which is not at the active site but influences the protein conformation through the charge carried on its imidazole ring. The iron-transferring proteins ovotransferrin and serum transferrin lose their Fe-binding activity on periodate treatment, and oxidation of ~4 tyrosine side-chains is apparently responsible;[457] it appears that histidyl residues are also involved in metal-ion binding in ovotransferrin.[458] Metallothionein, which binds up to six Zn ions per molecule, has now been shown to exist in a well defined folded form with metal ions bound, but as a random-coil structure in its apoprotein form.[459] Much less specific binding of other molecules is exhibited by the albumins; among the small molecules bound to albumins reported in 1980 are hexacyanocobaltate,[460] 5-fluoro-L-tryptophan,[461] nickel ions,[462] and cobalamin.[463] The regulatory protein α-lactalbumin has become the first for which laser-CIDNP techniques have detected all three polarizable aromatic residues (tyrosine, tryptophan, and histidine) on the surface of the molecule.[464] Of course, the ultimate in recognition proteins may be considered to be the immunoglobulins. In a detailed study, the binding of tetra-L-alanine haptens, each enriched with ^{13}C in a single methyl group, to Fab' fragments of purified sheep anti-(poly-L-alanine) was studied. Downfield shifts of 2.8 p.p.m. were observed on antibody–hapten binding, presumably due to van der Waals interactions and, while the methyl groups were rotating freely, the backbone of the peptide appeared to be firmly bound.[465] Data from n.m.r. work on a number of λ-type Bence-Jones proteins were compared with the *X*-ray structure of the Fab fragment of human immunoglobulin, and showed the probability of close similarities between solution and crystal structures of the constant domain of the λ-chain.[466]

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Nucleic Acid Binding Proteins. Moving to proteins which recognize and bind to nucleic acids, the interaction of the *N*-terminal DNA-binding domain of the *lac* repressor with oligo-d(AT), investigated by photo-CIDNP, showed that two of four tyrosines, and the only histidine residue in the region, are involved in the binding.[467] Deuteriation work on gene-5 protein from phage M13 shows it[468] to interact with oligo-d(CG) by a mechanism involving a tyrosine and >1 phenylalanine residues *via* stacking with base pairs. This type of interaction, however, is not thought to occur when histones bind to DNA in the eukaryotic chromosome. Interest in the core histones has largely given way to work on the three-domain histone H1, which has a trypsin-resistant folded region, now shown to exist also in the sea urchin sperm histone ϕ 1.[469] N.m.r. data showing the single tyrosine in histone H1 to be buried has been challenged following the attachment of a spin label to it for e.p.r.;[470] however, even small substituents at this residue disrupt the folded structure, so this conclusion is unlikely to be correct. Unlike histones, the basic clupeine proteins of salmon sperm do not appear to fold in solution.[471] Some of the so-called 'high mobility group' proteins which have been implicated in the structure of active chromatin have been subjected to preliminary n.m.r. analysis.[472,473] Ribosomal protein data are reported for proteins S4,[474] S16,[475] and L11,[476] and for whole ribosomes.[477] The L11 study confirms earlier reports that the conformation of isolated ribosomal proteins depends critically on their previous treatment.

Structural Proteins. Under the broad heading of structural proteins we may include viral coat proteins. The aggregation of tobacco mosaic virus coat protein has been compared with that of mutant versions[478] and the major coat protein of the filamentous bacteriophage fd characterized by ^{1}H and ^{13}C resonance.[479,480] A method of improving selection of non-protonated carbon resonances in such spectra has been described.[481] Collagen fibrils that have been specifically biosynthetically labelled with deuterium and ^{13}C have been studied;[482-484] among the main conclusions is that the contact regions between the helices in collagen fibrils

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are fluid and that there is no fixed unique set of interactions between side-chains. Cross relaxation between hydrated collagen and water molecules is discussed.[485] N.m.r. studies on elastin[486] show that the protein is a network of mobile chains whose motions are strongly influenced by protein–solvent interactions.

A ^{31}P n.m.r. study of rabbit skeletal muscle myosin showed that the phosphoserine 14 or 15 probably acts, analogously to the phosphoserine in troponin-T, to prevent interactions with other parts of the molecule.[487] G-Actin binding has been shown to quench internal motions in myosin subfragment 1,[488] and the high-affinity sites on actin for a divalent metal and a nucleotide were shown to be separated by $\geqslant 16$ Å.[489] Anomalous splitting in histidine resonances of tropomyosin were used to show the co-existence of several conformational states of the molecule.[490] Troponin C is a calcium-binding protein; binding of calcium was shown[491] to result in a subtle alteration of the tertiary fold of its *N*-terminal half, and proposals were made as to the regions engaged in its interactions with the other proteins of the troponin complex.[492] It is believed that troponin-C has homologous calcium-binding regions with other calcium-binding proteins such as calmodulin and the parvalbumins; paramagnetic probe methods which will enable the testing of this idea are presented[493,494] for carp parvalbumin, and work on calmodulin is described.[495–497]

Miscellaneous. A variety of proteins have been investigated by n.m.r., which do not fall easily into any of the sections outlined above; these are listed here for completeness: protein elongation factor Tu from *Thermus thermophilus*;[498,499] haemerythrin;[500] ferredoxin;[501] crambin, a hyperstable hydrophobic protein;[502] phytochrome chromopeptides;[503] concanavalin A;[504] peptidolipin NA;[505] dental phosphoprotein;[506,507] the copper protein plastocyanin,[508–510] and the behaviour of water in protein powder systems.[511]

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5 Infrared and Raman Spectroscopy

Contributed by R. M. Stephens

Model Compounds.—Far i.r. spectra of poly(*p*-phenylene terephthalamide), polyamide fibres and fabrics have been shown to be similar to an undrawn nylon 6 fibre. The important characteristics were that the amide VII band of the pseudo-hexagonal structure was weak and centred at $\sim 205\,cm^{-1}$ compared with $220\,cm^{-1}$ for the α-crystal structure and $274\,cm^{-1}$ for the γ-crystal structure and that the strong absorption at $200\,cm^{-1}$ lies between the corresponding absorptions of the α- and γ-crystals at $294\,cm^{-1}$ and $317\,cm^{-1}$ respectively.[512] Differential scanning calorimetry and i.r. spectroscopy have been used to investigate the melting behaviour of monoamides having the general formula RCONHR′ (R and R′ = normal alkyl). The lower values of melting entropies, compared to those for linear hydrocarbons with the same number of conformationally flexible chain bonds, were attributed to a reduction in the number of conformations available to the hydrocarbon portion of the molecule. The continued presence of a network of H bonds in the melt of the diamides reduced the conformational freedom of the chain segments more than for the monoamides.[513] Raman spectra of the cyclic hexapeptide *cyclo*(-L-Pro-Gly-)$_3$ and its Na^+, K^+, and Ca^{2+} complexes have been analysed in the solid state and for samples in solution. Spectra of uncomplexed ionophore in solution were consistent with previously proposed solution conformations and permitted the identification of spectral lines characteristic of proline-containing peptide bonds in the *trans* and *cis* conformations. Upon cation complexation the prolyl carbonyl stretch bands sharpened and moved to much higher frequencies. The glycyl carbonyl stretching band was unaffected by Na^+ complexation, shifted by $+15\,cm^{-1}$ (to a higher frequency) by K^+ complexation and by $-20\,cm^{-1}$ for Ca^{2+} complexation. Arguments supporting the involvement of prolyl carbonyl groups in cation complexation are discussd.[514] A detailed examination of the N—H stretching frequency in the i.r. spectra of *N*-acetyl-N^1-methylamides of glycine, L-alanine, and L-leucine in $CHCl_3$ showed no evidence of strongly bonded NH groups in the C_{7eq} conformation and it was attributed to the conformational flexibility of these molecules. Absorption bands due to the extended C_5 conformation were observed in $CHCl_3$. The blocked single residues adopted several conformations in $CHCl_3$.[515] The i.r. and Raman spectra of solid L-cysteine, DL-cysteine, and their *N*- and *S*-deuteriated derivatives were measured in the 4000 and $200\,cm^{-1}$ region, and the influence of the structure on the vibrational spectra was studied. An assignment of most of the fundamental vibrations of the monoclinic and orthorhombic L-cysteines and of the two phases of DL-cysteine was proposed.

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The presence of only one rotational isomer was indicated in the Raman spectra of aqueous solutions.[516] The conformations of Me_3CO_2C-(Met)$_n$-OMe (n = 2—7) in $CHCl_3$ have been invesigated by i.r. and n.m.r. spectroscopy. Extensive n.m.r. concentration-dependent chemical shift studies combined with i.r. results were used to delineate the involvement of the various methionine NH protons in intra- and/or inter-molecular hydrogen bonding. At low concentrations where peptide aggregation was absent, the dipeptide was disordered, and the tetra- to hepta-peptides possessed intramolecular hydrogen-bonded seven-membered rings. The number of internal rings and oligopeptide self association increased with increasing peptide chain length. At intermediate concentrations, associations of peptide molecules with folded structures occur in the *C*-terminal region. At high concentrations associated extended β structures were formed.[517] Studies of synthetic alamethicin fragments and model peptides containing α-aminoisobutyric acid (Aib) have revealed that tripeptides and larger fragments exhibited a strong tendency to form β turns, stabilized by hydrogen bonds. Dipeptides showed less well defined structures, though C_5 and C_7 conformations were detectable. Conformational restrictions imposed by Aib residues resulted in those peptides populating a limited range of states. Integrated intensities of the H-bonded N—H stretching band were used to quantitate the number of intramolecular hydrogen bonds. Predictions made from i.r. data were in excellent agreement with n.m.r. and *X*-ray diffraction studies. Assignments of the methane and tertiary amide carbonyl groups in the free state were made in model peptides. The 1—6 segment of alamethicin adopted a 3_{10} helical structure stabilized by four intramolecular hydrogen bonds. However, the fragments Boc-Leu-Aib-Pro-Val-Aib-OMe (12—16) and Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (11—16) had structures involving 4 → 1 and 5 → 1 hydrogen bonds.[518]

Model Calculations.—Polarized Raman spectra of DL-serine, DL-[*N*,*O*-$^2H_4$] serine single crystals, and i.r. spectra of polycrystalline samples along with a normal coordinate analysis have been evaluated for the optically active intramolecular and intermolecular vibrations. Large factor-group splittings observed for the CO_2^- antisymmetrical stretching mode were explained using model potential functions that include coupling terms due to the interaction between the transition dipoles induced by the vibrations. This type of interaction was also used to explain the variations in sign and magnitude of the antisymmetric and symmetric CO_2^- stretching modes of crystalline α-glycine, L-alanine, and DL-alanine.[519] Band intensities in the polarized Raman spectra were measured for the CO_2^- stretching, CH_2 stretching and bending modes of α-glycine crystals, as well as for the CO_2^- stretching and the CH_3 symmetric deformation modes of L- and DL-alanine crystals. Variations in the relative band intensities on the change of the scattering geometry were elucidated by applying an oriented gas model to the local CH_2, CH_3, and CO_2^- groups of these amino-acid crystals. The calculated values of the components of the derived polarizability tensors were consistent with the geometry

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of the group. The depolarization ratios established from the calculated anisotropy of the polarizability tensors were compared with those observed for aqueous solutions.[520] The far i.r. and Raman spectra of the crystalline α and β modifications of glycine were recorded in the region from 500 to 50 cm^{-1}. A normal co-ordinate analysis of the lattice vibrations was performed using Wilson's G. F. matrix method and the rigid-molecule approximation. Intermolecular force constants associated with the hydrogen bonds were obtained. The translational and rotational lattice vibrations were assigned to the observed bands.[521] Transition moments and absolute intensities of the vibrational spectra of L-alanine as single crystals and in solution have been evaluated from the polarized i.r. spectra. The intensities obtained in solution were interpreted by means of a polar model describing the dipole moment in terms of two types of parameters: electron density around each atom and the intensity variation of this density with bond length, in singly bonded atoms.[522,523] Assignment of the Raman bands from L-alanine and deuteriated anaolgues has been based on a normal co-ordinate analysis and a 34 parameter valence-type force-field defined in terms of local symmetry co-ordinates. For non-deuteriated L-alanine, the vibrations above 1420 cm^{-1} and below 950 cm^{-1} may be described as localized group vibrations. By contrast, the eight modes in the middle frequency range, *viz* the three skeletal stretching, the COO^- symmetrical stretching, one NH_3^+ rocking, the symmetrical CH_3 deformation, and the two methyne CH deformation vibrations, were very strongly coupled to one another. Some decoupling appears to take place in the perdeuteriated molecule and all but five modes can be described as localized group vibrations.[524] Experimental i.r. and Raman spectra of carbobenzoxyl-Gly-Pro-Leu-Gly, a tetrapeptide known to form a type $I^4\beta$ turn, were compared with the normal vibrational calculations on the tetrapeptide modelled by MeO-CO-Gly-Ala-Ala-Gly-OMe. The β-turn conformation appeared to be identifiable by high amide II mode frequencies (1568 cm^{-1}), an amide III mode at 1291 cm^{-1} (observed at 1294 cm^{-1}), and amide V modes calculated as 583 and 609 cm^{-1} (observed at 599 cm^{-1}). Close agreement between calculated and observed frequencies confirms the validity of the force field and mechanism of transition dipole coupling in predicting the characteristic amide modes of β turns.[525]

Proteins.—*Muscle Proteins.* Pre-resonance Raman spectra of actinomycin D have been measured using the exciting lines from an Ar^+ laser. The analysis of the excitation profiles provided information on the origin of the electronic states; in particular absorption between 400 and 500 nm was interpreted as due to a vibrational structure of a single electronic state. In addition, on the basis of the excitation profiles, the number of observed Raman bands, and their frequencies, it was possible to propose a vibrational assignment to the chromophoric framework

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of the drug.[526] The 500—1800 cm^{-1} of the Raman spectra of intact single muscle fibres from the giant barnacle indicated that the contractile proteins adopt a predominantly α helical structure and were not affected when the contractile state of the fibres was changed from relaxed to contracted state by addition of ATP and Ca^{2+}. The contraction decreased the scattering intensity of some of the Raman bands caused by acidic and tryptophan side-chains, showing that these amino-acids are involved during the generation of tension.[527] Structural changes accompanying the polymerization of monomeric G-actin from rabbit muscle to the fibrous form have been recorded. The i.r. spectra indicate a small but positive increase in the β structure content of F-actin relative to G-actin and both c.d. and i.r. spectra supported the assumption that there was no fundamental change occurring in the secondary structure of actin on polymerization.[528]

Proteins in Visual Pigments. Resonance Raman multi-component spectra of bovine rhodopsin, isorhodopsin, and bathorhodopsin have been obtained at low temperatures. Application of the double-beam pump-probe technique gave the complete bathorhodopsin spectrum from the mixture in both protonated and deuteriated media. The spectrum of bathorhodopsin was significantly different to that from either parent pigment, supporting the notion that a geometric change in the chromophore is an important component of the primary photochemical event in vision. A normal mode analysis was evaluated to determine the frequency of the C—N stretching vibration. The increased frequency of this mode in protonated relative to unprotonated Schiff basis was due to coupling between C—N stretching and C—N—H bending modes.[529] Fourier-transform i.r. spectroscopy has been used to study the structure of bovine photoreceptor membrane. The rhodopsin component appeared to contain an extensive helical structure arranged predominantly perpendicular to the membrane plane. Spectra of delipidated rhodopsin and rhodopsin membranes reconstituted from dioleylphosphatidylcholine were compared with native photoreceptor membranes from rod outer segments to facilitate peak assignments. Spectroscopic peaks characteristic of several protein and lipid groups were assigned. Dehydration did not appear to alter grossly the rhodopsin structure although it may have been affected by delipidation.[530] Experiments on iodopsin and bacteriorhodopsin have suggested that light energy is stored as a result of the interaction of a photon with rhodopsin. Resonance Raman spectra of the batho intermediate in several rhodopsins indicated that the $—CNH^+$ stretching vibration did not vary in going from rhodopsin to bathorhodopsin, suggesting that there was still a double bond between the C and the N and a positive charge on the N after the photonic event. Furthermore, the $C—CH_3$ vibration indicated that in bathorhodopsin there are changes in the vicinity of the Me groups. These C atoms were tertiary centres which could stabilize charge. Even though the $—C—NH^+$ vibration did not appear to be affected by the photon, the C—C stretching vibration was significantly altered. Thus, light energy appears to

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be stored in a charged polarized state, and at least in bathorhodopsin light emission is quenched in this state.[531] Kinetic studies using resonance Raman techniques of native and isotopically labelled bacteriorhodopsin has provided evidence that there is a significant alteration in the rate constants for the evolution of various intermediates, when non-exchangeable protons on the membrane are replaced by deuterons.[532]

Haemoglobin. The influence of quaternary structure on the low-frequency molecular vibrations of the haem group within deoxy-Hb and oxy-Hb using resonance Raman scattering methods has shown that the Fe—O_2 stretching frequency was essentially identical between the high-affinity (*R*) state and the low-affinity (*T*) state. However, in deoxy-Hb only one of the polarized lines showed an appreciable frequency shift upon a change of quaternary structure. The 216 cm^{-1} line from deoxy-Hb was proposed to be associated, primarily with the Fe—N_ε (His F8) stretching mode. Accordingly this Fe—N_ε bond is stretched in the *T* state owing to strain exerted by globin.[533] Detailed measurements of the SH vibrational group from α-104 cysteine in human Hb and α chain dimer using Fourier transform i.r. spectroscopy have shown the effects of haem ligation and protein quaternary structure on α chain tertiary structure and its role in the biological control of oxygen transport. The frequency of the SH absorption band increased in the order $HbCO < HbNO < HbN_3 < HbO_2 < Hb^+$. The $\alpha_2\beta_2$ tetramer showed a much greater range of SH shift in centre frequency than isolated α chains. Hb had a higher SH absorption frequency than did the α chain. The SH absorption frequency in Hb was further increased by inositol hexaphosphate, and its α chain tertiary structure may be described as a super *t*-state.[534] The transient picosecond resonance Raman spectrum of HbO_2 had been attributed to a reorganization of the porphyrin ring core through a change in the electron distribution resulting from the departure of the oxygen ligand. The time required for structural reorganization of the porphyrin core was < 30 ps. The ps time-resolved resonance Raman spectra of Hb were related to the haem structural state with the Fe atom pentaco-ordinated in an *R* protein conformation before its return to the *T* stable position. The haem structure was not very dependent on the protein conformation and the porphyrin backbone structure was not very flexible. Thus the protein may influence the oxygen affinity of Hb by acting directly on the charge of the Fe atom but not on the geometric state of the haem.[535] The configuration of the haem-carbonyl group upon binding of CO to crystals of sperm whale myoglobin has been evaluated on the basis of i.r. spectroscopy. Multiplets of the totally symmetric C—O stretching mode were observed for the haem-bound ligand near 1933, 1944, and 1967 cm^{-1} corresponding to three different haem-carbonyl conformers. Variations in the relative proportions of these conformers were induced by incorporation of small fractions of met-Mb or deoxy-Mb into MbCO crystals. The configurations of the FeCO with respect to the immediate co-ordination environment of the haem Fe was assigned for each N(CO) stretching

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frequency on the basis of detailed comparison of the 3-dimensional structures of the haem environments of MbCO, met-Mb, and deoxy-Mb defined by crystallographic methods. The structures of the 3 haem-carbonyl conformers account for the ν(CO) i.r. absorption bands that can be observed in MbCO in solution.[536] Surface-enhanced resonance Raman (SErR) spectra are reported for myoglobin and cytochrome *c* spontaneously adsorbed from micromolar solutions on to a Ag electrode. The enhancement factor was $\simeq 10^5$ above that produced by the conventional rR effect. The signal intensities in both proteins are anodization and potential dependent. All resonance Raman bands are depolarized ($\rho > 0.5$) on the electrode. These results indicate the great potential of combined SErR and rR spectroscopy for bioanalytical applications such as the study of highly dilute preparations.[537]

Gramicidin. Spectroscopic investigations of gramicidin A revealed the existence of at least three distinct conformations. In the solid state, CH_3OH and C^2H_3OH solutions, the amide I frequencies suggested a β-parallel H-bonded π_{LD} helical conformation. In films cast from $CHCl_3$ the conformation is consistent with an antiparallel double-stranded π_{LD} helix. The conformation in Me_2SO and $(C^2H_3)_2SO$ solutions was probably π-helical. The data on head- and tail-modified gramicidin A molecules indicated that their conformations were only slightly different from that seen in the solid state.[538,539] Absorption data between 300 and 4000 cm^{-1} from some chemically modified analogues of gramicidin A have indicated that the most likely channel conformation consisted of a helical dimer with an extended chain parallel hydrogen bonding network between the helical turns. The localized C=O stretching mode was discussed in terms of a perturbation model.[540]

Riboflavin. The vibrational spectra of flavin and semiquinone forms of flavin bound to riboflavin binding protein (RBP) have shown that the natural semiquinone of riboflavin–RBP complex in H_2O solution has an intense line at 1617 cm^{-1} which was not observed for oxidized riboflavin bound to RBP. The line did not shift upon deuteriation. The absorption spectra of semiquinone bound to RBP had maxima at 586, 396, and 344 nm and rR spectrum doublets lines at 1623 and 1615 cm^{-1}. In ^2H_2O the 1623 cm^{-1} line did not shift, but the 1615 cm^{-1} line shifted to 1604 cm^{-1}. Consequently, the line around 1620 cm^{-1} from the flavin semiquinone is useful in the determination of the redox state of flavin.[541] Other rR studies of lumiflavin and 8-substituted riboflavins[542] as well as protein–ligand interactions in lumazine protein and indesulphovibrio flavodoxins[543] have also been reported.

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Carboxypeptidase A. Resonance Raman spectroscopy was used to investigate the environment of the azotyrosine-248 residue (I) of arsanilazotyrosine-248 carboxypeptidase A in solution and in crystal phases. In solution at pH 6.0 (I) is protonated and studies of model compounds indicate that (I) was in an aqueous environment. At pH 8.5, the spectrum of the arsanilazoenzyme correlates well with those of model azophenols forming complexes with Zn. The Zn is bound to the phenolic O and an azo N-atom of (I), with the azo-group in the planar *trans* conformation. At pH 11 this intramolecular complex is dissociated and (I) then exists as the ionized azophenolate species. Resonance Raman studies of *o*-hydroxyazobenzene models for (I) show that in solution the azophenol forms of these molecules co-exist in two different conformations, which differ with respect to the presence or absence of an intramolecular H bond between the phenolic proton and an N-atom of the azo-group. Each of these conformations exhibits characteristic ν^{NN} and $\nu^{\phi N}$ bands. The relative intensities of both the pair of ν^{NN} and $\nu^{\phi N}$ bands in the rR spectrum of azocarboxypeptidase provide specific information about the state of H bonding of the phenolic proton of (I), which had been thought to be transferred to the substrate during catalysis. In solution (I) was predominantly H-bonded intramolecularly and exists in an aqueous-like environment. Crystallization apparently induced a conformational change that enabled the phenolic proton of tyrosine-248 to form an intermolecular H bond to a group in the protein. This interaction may be related to the marked reduction of the enzyme activity brought about by crystallization.[544]

Chymotrypsin and Trypsin. The spectra of both enzymes from 7% aqueous solutions and lyophilized solids containing residual SO_4^{2-} showed that a large amount of β-pleated sheet structures was present in both molecules but that trypsin contained substantially more α-helical conformation. The tyrosines were all or nearly all weakly H-bonded in both, as was the binding of SO_4^{2-} in the lyophilized solids.[545]

Ribosomes. The Raman spectra obtained from aqueous solutions of ribosomes, ribosomal subunits, ribosomal proteins, and rRNA extracted from both rat liver and *E. coli* cells did not contain sufficient detail to reach firm conclusions about the conformations of the ribosomal proteins or their mutual interactions. All rRNA molecules were shown to have highly ordered secondary structures, in which the backbone conformations were predominantly of the α-helix type. RNA molecules within the ribosomal particles remained highly ordered during various stages of ribosome disassembly, and their conformations were generally invariant on perturbation of ribosome structure, including dissociation into subunits, EDTA treatment, and partial deproteinization in a CsCl density gradient. When total protein extension occurred on the ribosomes and subunits, small but significant changes in rRNA secondary structures were detected. The type and magnitude of secondary structure change were different for different ribosomal particles.[546]

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Tobacco Mosaic Virus, TMV. A viral protein (A-protein) and an infectious viral RNA have been isolated from TMV and their structural properties investigated by laser Raman spectroscopy. The amide I and III bands in the spectra of solid and aqueous samples of the viral protein indicated that the peptide backbone of the isolated protein had an α-helical conformation similar to that of the protein within the intact virus particle. The —SH group in TMV is masked to chemical probes but is easily detected from the Raman spectrum by the characteristic 2567 cm^{-1} —SH vibration. Structural prediction calculations from the sequence of the A-protein gave the percentage of α-helix, β-sheet, β-turn, and random coil as 32, 31, 22, and 12% respectively. The infectious viral RNA component had 51.2% ordered structured as determined from the ratio of the intensities at 815 and 1080 cm^{-1}. The spectrum of the intact virus particle appears to be dominated by contributions from the protein component rather than the RNA component.[547]

Histones. The Raman spectra of aqueous solutions of histones H3 and H4 from calf thymus and from rye reflect the high degree of conservation from species to species of the primary and secondary structures of these proteins. The amount of β-sheet structure in H4 was estimated to be 40% and in H3 to be 33%. These results were based on amide I and III frequencies obtained from solutions with high protein concentrations, which could result in aggregation of the proteins. The amount of β confirmation present was also independent of salt concentration. The intensity ratio of the tyrosine doublet at 852 and 826 cm^{-1} indicated that the four tyrosine residues in H4 were exposed to the solvent or weakly bound to positively charged groups of basic amino-acids, whereas in H3 $\geqslant$ 1 tyrosine was buried inside the protein and tightly bound to a carboxylate group. The results also show that the secondary structure of H3 was slightly influenced by the state of oxidation of the two cysteine residues it contains.[548]

Proteins in Milk and Grain. Spectroscopic data have been recorded from a variety of milks to estimate and compare the fat, protein, and lactose concentrations present with the object of determining performance specifications for milk analysis using i.r. spectroscopy.[549, 550]

The feasibility of measuring lysine and protein N in barley using i.r. reflectance techniques has been investigated. A very rapid technique requiring no manipulative skills and measuring several components simultaneously using milled grains was used. The instrument was sensitive to changes in lysine and protein N and was calibrated to measure these components to a high degree of accuracy. The gross morphological and biochemical abnormalities of high-lysine mutants did not effect these measurements, indicating that the method was suitable for rapid screening of material from breeding programmes designed to improve the lysine content of barley proteins.[551]

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Other Proteins. The amide III′ region can be used effectively to estimate the conformation of ^2H_2O-exchanged proteins if difference spectrum analysis is made. For lysozyme RNase A, concanavalin A, and phage protein the computed spectral bands for helical and β-sheet conformations were better resolved in the amide III′ than in the amide I region. Estimates of secondary structure derived from the amide III′ region did not agree as well with *X*-ray estimates as did the estimates from the amide I region; however, they compared favourably with other Raman and with c.d. results.[552]

A procedure utilizing a variable path length absorption cell made possible the recording of what appears to be the first well resolved, compensated near i.r. spectra of intact proteins in aqueous solution. Individual spectra, corresponding to (a) absorbance by the protein plus bound water and (b) the solvent volume excluded by the hydrated protein, were obtained using the same experimental sample. Calculations of bound water and excluded volume from these spectra were compared to other results in the literature. The validity of the spectral method was supported by comparisons with the spectra of proteins in films, where there is no excluded volume effect and where the amount of water present was determined independently by gravimetry. Evidently, the bound water detected by the near i.r. spectra has an absence or deficiency of molecules with quasi-free OH groups (relative to bulk water) and in conjuction with results of other methods these water molecules may represent those that are most firmly or more completely bonded to the protein surface.[553]

6 Circular Dichroism

Contributed by T. Brittain

General.—*Reviews.* General reviews have appeared,[554-557] plus those concerned with newer developments in induced and infrared circular dichroism,[558, 559] and its use to follow fast reactions.[560, 561] More specialized works have been written on fluorescence detected c.d.,[562] the effect of solvent,[563] and the modification of chiral properties due to interaction with small molecules and ions.[564]

Theory and Analysis. Equations have been derived for a free electron on a large helix, which are in good agreement with experimental measurements, and allow estimation of the limits of application of the usual Rosenfeld approximation.[565]

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One approach to computer assisted estimation of protein secondary structure from c.d. spectra has been the reduction of data to the sum of the various Gaussian components.[566] Another has been a statistically valid analysis of mean residue ellipticity between 210 and 240 mm to obtain a measure of α-structure. Application to data from lactate dehydrogenases gives good agreement with the values obtained from *X*-ray diffraction studies.[567] In contrast a manual method for the rapid determination of protein secondary structure has appeared, equivalent to the least squares method,[568] which seems generally applicable and surpasses an earlier method, which was in error.[569] The use of chromophoric systems attached to polypeptides allows secondary structure determination, by the 'chiral conformational probe' method,[570] as do more conventional methods.[571]

A full polarizability treatment of the π–π^* absorption and c.d. spectra of α-helical polypeptides near 190 nm for the right-handed helical forms of $(X)_n$ (X = Gly, Ala, D-Ala; $n \leqslant 12$) using NCO polarizability and transition parameters found for simple amides showed good agreement with experimental data employing NCO parameters centred on the NC bond with a π–π^* oscillator strength twice that of amides. The calculation for $(Gly)_{12}$ showed negative (204 nm) and positive (191 nm) maxima, but the normally predicted negative peak at 180 nm was absent. Calculated hypochromicity (0.26—0.40) and π–π^* band ($4080\,cm^{-1}$) splitting resembled experimentally determined values of 0.3—0.36 and $4370\,cm^{-1}$.[572]

The application of qualitative MO perturbation theory to inherently achiral chromophores, chirally perturbed by their surroundings, has identified generalized sector, chirality, and helicity rules.[573] The dynamic perturbation effects of polarizable chromophoric perturbers of the c.d. intensity arising from absorption transitions of an arbitrary chromophore aggregate in the Frenkel exciton model have been formulated on the basis of an external linear response model.[574] The moments of the c.d. of an isotropic non-conducting medium and their possible application as a necessary constraint on experimental data were discussed.[575]

Instrumental. A number of advances in instrumentation have appeared. The report of photoacoustic detection holds significant prospects for the determination of signals from opaque samples.[576] Its extension to the use of polarization-modulated photoacoustic spectroscopy (PAcd) has appeared, and expressions and calculations appropriate to the analysis of data in terms of c.d. developed within the framework of Rosenewaig and Gersho.[577] The first report of synchroton radiation as a source of vacuum u.v. c.d. measurements appeared. This source, due

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to its very high intensity, improves signal to noise ratios and gave the best ever measurements in the 1325—2050 Å region.[578]

Advances in the use of rapid c.d. measurements include a new rapid scanning (up to 5000 nm s^{-1}) c.d. and m.c.d. spectropolarimeter, which uses a non-collinear acoustic optical filter of TeO_2 as a monochromator. It has the advantage that the light beam is linearly polarized by the optical activity and birefringence inherent in the TeO_2 itself. The new spectropolarimeter is stopped-flow compatible.[579, 580] Stopped-flow c.d. to detect rapid protein conformational changes has been described[581] and conversion of a Cary 60 spectropolarimeter, using a 18 kHz piezo-optical birefringence modulator, allowed investigation of conformational changes in proteins in the ms time range.[582]

A significantly improved, microprocessor controlled near infrared spectrometer was described, using a commercial photoelastic modulator with infrasil quartz optical elements, allowing operation over the range 0.5—2.1 μm. Phase and frequency sensitive detection are employed and control and data handling are by minicomputer.[583] Another computerized system capable of simultaneously obtaining absorbance, c.d., and fluorescence data from a single sample, and of controlling wavelength, temperature, pH, and addition of reagents to the sample, has been developed for higher resolution in conformational studies.[584] The reported determination of c.d. in an oriented system accounts for the stray birefringence of the modulator.[585] The reproducibility and features of solid state spectra measured by a Nujol null method have been discussed.[586]

Small Molecules, Model Compounds, and Synthetic Polymers.—*Amino-acids and Derivatives.* The absolute configuration of amino-acids may be determined *in situ*; the method relies on formation of derivatives with *o*-phthalaldehyde in the presence of 2-mercaptoethanol. Derivatives of L-amino-acids give an intense positive maximum (340 nm); the D-amino-acids show the opposite effect. Exceptions are L-Ala, L-Trp, and L-Asp.[587] Conformational uniformity, local ordering, and crystal lattice interactions mean the solid state vibrational c.d. spectra of Ala and Ser show much larger signals than in solution.[588] The β-nitrostyrene adducts of Cys exhibit characteristic Cotton effects at 285 nm, dependent entirely on the configuration of the new asymmetric centre produced. *X*-Ray analysis shows that the β-nitrostyrene adduct of Ac-L-Cys-OH has the (*R*,*R*) configuration.[589] Spectra of *N*-5- and *N*-6-benzofuroxanoyl-L-α-amino-acids (1) and (2) (X = Gly, Ala, Val, Leu, Phe, or DL-Phe; R = H or Me) exhibit Cotton effects around 365, 320, 265, and 233 nm, with positive maxima at the extreme wavelengths in the L-aliphatic series

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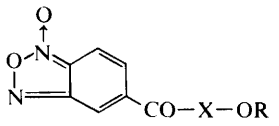
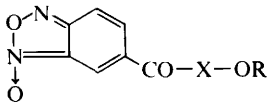
and negative maxima in the L-aromatic series.[590] A method reported for the determination of absolute configuration of α-amino-acids without product isolation utilizes their reaction with fluorescamine to form pyrrolinone type chromophores. The characteristic Cotton effects (324—300 and 290—263 nm) relate to the absolute configuration of the parent amino-acid.[591]



(1)



(2)

The presence and optical purity of an α-amino-acid and its methyl ester in their mixtures can be determined by c.d. measurements on the products of reaction with the cobalt complex of *NN'*-ethylenebis(acetylacetonimine), without prior separation. This reagent enhances the rotatory powers of the amino-acids and esters to different extents, and finds application to mixtures derived from Leu, Val, Phe, Pro, and Trp.[592] C.d. permitted structure assignment to isomers of the mixed ligand Co^{III} complexes of the form [Co(L-Pyala)A] where H_2A = L-His, L,D-Asp, or iminodiacetic acid and L-PyalaH = L-pyridylmethylglycine. The distribution of isomers suggests that pyridyl groups avoid being *trans* to each other but prefer to be *trans* to an amino-group.[593]

Correlation of spectra of reference compounds with those of Co(acac)(A) where A = D-Phe, D-Trp, or D-Tyr led to assignments of δ and λ conformations of the acac ligand in $Co(acac)_2A$ and Co(acac)A respectively (Hacac = acetylacetone).[594] C.d. measurements on single crystals of all-*cis* and all-*trans* amine bis(histidinato)Co^{III} and independently verified spectral assignments support earlier evaluations of cubic ligand field parameters for the co-ordinating histidinate, amine, imidazole, and carboxylate groups.[595]

In the region 620—630 nm complexes of the form Cu^{II}-L-His-L-AA, where His is L- or D-His and AA is L-Thr, L-, D-Asn, L-Glu, L-Ser, L-HomoSer, or L-Cit(L-citullinate, show positive extrema at pH 7—8, suggesting that similar co-ordinating groups occupy the Cu^{II} co-ordination plane.[596] Investigations show $Ni(acac)_2$ reacts with NiL_2 (HL = D-Phe, D-Tyr, or D-Trp) to give Ni(acac)L, the acetylacetone ligand having a folded δ-conformation.[597] Experiments on *trans*-$[Pt\{(R_N)ProH\}(NH_3)Cl_2]$ have shown the absolute configurations of the asymmetric N and C atoms to be *R* and *S* respectively.[598]

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Dipeptides and Oligopeptides. Spectra of the angiotensin fragments H-Val-Tyr-Val-R (R = OH or NHMe) and related compounds have been studied to elucidate the chiroptical properties of the parent molecule. Spectra in various solvents show that the NHMe and OH derivatives have substantially different conformations. However, although increasing the chain length (R = Gly-OMe) did not change the conformational equilibrium, the spectra of R = Ala-OH or Ile-OH were intermediate between those of the forms in which R = OH or NHMe.[599] The cyclic tetrapeptide [Ala^4]-desdimethylchlamydocin, in $CHCl_3$ solution, exists in an all-*transoid* ring conformation containing γ-turns.[600] Reports show that $Cu^{II}[HISH_2]_2$ [where HISH = *cyclo*(-L-His-L-His-)] exists as two thirteen-membered chelate rings co-ordinated to the copper atom *via* two N atoms of the imidazole moieties.[601]

$Na[Co(sar)_2(NO_2)_2]$ (where Hsar = sarcosine) has been resolved into two isomers (Δ and Λ), which were identified by c.d.measurements[602] In PtL(HL)Br (HL = Pro) HL is monodentate and L bidentate. The diastereomers are distinguished by the absolute configuration of the asymmetric N atom of the co-ordinated HL. Comparison with the c.d. spectra of *trans*-PtL(HL)X (X = Cl or Br), *trans*-PtL(Gly)X, and *trans*-PtL(NH_3)Cl gave the contribution, sign and intensity, of monodentately co-ordinated HL to the individual bands.[603] C.d. data allowed quantitative estimates of dihedral and bond angles for the disulphide bond in oxidized glutathione.[604] The binuclear oxidized glutathione–Cu^{II} complex $Na_4[Cu_2L].6H_2O$ (H_8L = glutathione) has also been studied.[605]

Polypeptides. Spectra of L-Glu oligomers as a function of d.p. and degree of neutralization show β-structure to exist only at d.p. 8—10 and the α-helical structure to be stable for d.p. > 30; a small proportion, however, exists at d.p. > 10.[606] An extended principal component analysis has been reported for the helix–coil transition of poly(α-L-Glu); u.v. c.d. intensity as a function of pH was fitted to a Henderson–Hasselbach equation by the introduction of a further parameter α.[607] The ordered form of poly(γ-ethyl-*N*-methyglutamate) was found to be more stable than poly(*N*-methylalanine).[608] The conformations of terminal peptide units of α-helical (γ-benzyl-L-Glu), examined by induced c.d. of covalently attached chromophores, show that in $CHCl_3$ the helix exists as a head-to-tail dimer. The chromophores exhibit strong c.d. induced by asymmetric perturbation, which almost disappears on addition of 3% dichloroacetic acid. The helix–helix junction structure, which accounts for the incorporation of terminal peptides into the helical conformation, is discussed.[609] The c.d. of azobenzene-amidated

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poly(Glu) in $(MeO)_3PO$ and H_2O has been reported.[610] Poly[γ-(*p*-methylbenzyl)-L-Glu] in CH_2Cl_2 and CF_3CO_2H undergoes a temporary change from α-helix to random coil with increasing acid concentration.[611] At neutral pH co-polymers of Tyr and Glu form an α-helix on addition of Zn^{2+} and Cu^{2+}.[612]

A conformation phase diagram, constructed for poly(L-Lys) in solutions of sodium 1-octanesulphonate, shows that at temperatures >10 °C an increase in surfactant concentration induces a stepwise change from random coil to β-form to helical conformation. At lower temperatures the range of concentration at which the β-form exists decreases.[613] Conformational changes in poly(L-Lys) by complex formation with sulphonated polystyrenes (PSS) for a range of conditions have been compared with those induced by sulphonated poly(vinylalcohols) (PVS). The coil to helix transition observed with PVS at neutral or acid pH was absent from studies with PSS. Complex formation with PSS thermally stabilized the α-helical form of poly(L-Lys), although at pH > 11 PSS caused some breakdown in the helical structure.[614] The induced c.d. of poly(L-Lys)–poly(vinyl alcohol)sulphate–Acridine Orange complex in acid aqueous solution indicated a right handed superhelical arrangement of the Acridine Orange molecules about the right handed α-helix of the peptide complex.[615] The biologically important process of glycosylation of proteins has been modelled using poly(L-Lys)–saccharide complexes. Measurements in aqueous MeOH showed the α-helix of poly(L-Lys)–sacc (sacc = D-glucose or D-galactose) to be more stable than in poly(L-Lys)HBr, but the aldose condensed polymer was inactive in blood platelet aggregation or inhibition of collagen-induced platelet aggregation.[616] Poly(-L-Val-L-Val-L-Lys-) exists in β, α, and random forms in solution, the β-forms being favoured by ionization of the peptide and lower polarity solvents.[617] However, in poly(-L-Lys-L-Val-L-Lys-) the introduction of Val residues into the chain containing α-helix-forming residues favours β-formation,[618] as is the case for poly(ε-*N*-benzyloxycarbonyl-L-Lys-L-Val-ε-*N*-benzyloxycarbonyl-L-Lys).[619]

In $NaClO_4$ solution, high concentrations of EtOH or MeOH, but not SO_4^{2-}, HPO_4^{2-}, or $P_2O_7^{4-}$, induce a random coil to α-helix transition in poly(-L-homoArg-). The common monovalent anions do not induce α-formation, whilst complex formation with poly(-Glu-) or polyacrylate does to a lesser extent. In contrast to poly(-Arg-), poly(-homoArg-) does not form an α-helix by interaction with SDS in solution.[620]

Poly(-D-Phe-) in 96% H_2SO_4 has a random coil structure.[621] Cu^{II}–(L-Asp)$_n$ forms two complexes in a two-step process. The first at pH 4.5 contains two carboxyl metal ligands and the bound metal inhibits α-helix destruction. The

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second complex contains a further two N ligands and exhibits patterns opposite to those where a five-membered chelate ring is formed.[622]

Co-polymers of β-benzyl-L-Asp and β[*p*-(phenylazo)phenethyl]-L-Asp with <40% azo amino-acids are left handed helices whereas those with >40% are right handed in 1,2-dichloroethane. The former polymers undergo left hand helix to random coil transitions on addition of hexafluoropropan-2-ol but the latter undergo the reverse. All the polymers show negative c.d. in the n–π^* region, whilst the π–π^* c.d. band was observed only in polymers with 67% or higher azo amino-acids. These results were interpreted in terms of solvent induced changes in orientation or mobility of the side-chain phenylazo moieties.[623]

Proteins.—*'Non-chromophoric' Proteins.* A series of publications are concerned with assignment of parameters to pure α, β, and random structures to allow accurate estimation of these elements in an unknown protein. Within the criteria proposed by Finkelstein *et al.* and Levitt and Greer the α- and β-regions have been determined for five reference proteins, namely myoglobin, lysozyme, RNase A, papain, and lactate dehydrogenase. The experimental data with the *f* factors for these proteins have allowed the calculation of the spectra of the pure conformational forms. The calculated spectra agree well with poly(Lys) standards and *X*-ray data for the proteins studied.[624] Extension to estimate the contribution of the β-bend to the c.d. spectra is possible by comparison of the α and β c.d., with the *X*-ray data for the reference proteins.[625] In one further step, by a similar process, it was possible to evaluate the c.d. spectra of parallel and antiparallel β-structures and β-bends. The total of these data has been used for the analysis of the secondary structure of ten globular proteins.[626] Another improvement in secondary structure estimation has been claimed for c.d. measurements in the vacuum ultraviolet down to 165 nm. Estimations are in close accord with the *X*-ray data for a number of proteins, but with rubredoxin it has been possible to identify a conformational change in the protein on going from the crystal to solution.[627] Further vacuum u.v. c.d. studies have allowed the characterization of the β-turn in this region using standard sequences; $(Ala_2\text{-}Gly_2)_n$, L-Pro-D-Ala, *N*-acetyl-L-Pro-Gly-L-LeuOH. It is necessary to make a correction for aromatic residue contributions for proteins with a high aromatic content. Again, good agreement with published *X*-ray data is found, except in the case of rubredoxin.[628] Linear programming techniques yield the best values of conformational parameters for c.d. spectra.[629]

In air oxidation of disulphide reduced curare-like peptides monitored by far u.v. c.d. techniques, a significant difference in the kinetics of the process in nine such toxins has been proposed as arising from a single amino-acid insertion. Support for this proposal is derived from temperature studies of the renaturation

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kinetics.[630] The u.v. c.d. spectral properties of pyruvate kinase from rabbit muscle, sturgeon muscle, and yeast correlate well with kinetic observations on the three enzymes, both in the presence and absence of allosteric effectors. The results suggest that there are two or more conformational states of the enzyme induced by the binding of substrate and/or effectors.[631] C.d. studies on a major protein from human liver, metallothionein-2, show the presence of $<5\%$ α-helix and β-sheet.[632] pH difference c.d. spectra indicate the presence of a zinc mercaptide chromophore with high ellipticity, ascribed to an exciton interaction between pairs of asymmetrically positioned S ligands within the co-ordination sphere of each metal complexing site.[633] Copper thioneins from rat, bovine foetus, and chicken apparently have a similar copper chemical environment.[634] A stopped-flow c.d. study of the acid unfolding of apomyoglobin indicates a single relaxation at <2 ms.[635] C.d. temperature variation studies of four neurotoxins from snakes suggest a common, delicately balanced conformation independent of their amino-acid sequence. The possible relevance to toxicity of the conformational freedom, defined by the secondary structuring and characteristic disulphide bond formation is discussed.[636] Cleavage fragments of the biologically active Bowman–Birk soybean inhibitor show no secondary structure. Negative c.d. intensity above 260 nm has been assigned mainly to disulphide bonds, and the total spectrum between 230 and 340 nm has been compared with the intact inhibitor and interactions giving rise to c.d. bands have been discussed.[637] Denaturation of Kunitz soybean trypsin inhibitor by MeOH, EtOH, Pr^nOH, trifluoroethanol, ethylene glycomonobutyl ether, and 1,4-dioxane causes disappearance of the 226 nm positive c.d. band and the formation of a negative c.d. band at 239 nm, assigned to the n–π^* transition of the amide bonds. The process of denaturation can be envisaged as a three-step process, leading to the broad flat negative c.d. band at 275 nm, due chiefly to disulphides. The unique folding of the polypeptide main chain appears to be important to the proteins' ability to inhibit trypsin.[638]

The c.d. spectra of the major storage protein of sesame seeds, α-globulin, show under normal conditions $\sim 25\%$ β-structure and 5% α-helix. Under denaturing conditions the percentage of α-helix rises initially to 20% in 1×10^{-2} M SDS, but all periodic structure disappears at high pH and becomes finally 100% aperiodic in 6M urea.[639] Collagenase from *Achromobacter iophagus, Clostridium histolyticum,* and thermolysin have high fractions of α-helix and the effects of pH and organic solvents on the protein structures have been investigated.[640] In contrast to rabbit liver sulphatase A the enzyme from ox liver does not alter its secondary structure significantly when inactivated during the course of its enzymic role.[641] Only poor

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correlations have been obtained between c.d. data and pH and metal-ion induced kinetic changes for rabbit muscle pyruvate kinase. However, good correlations are found between L-Phe and L-Ala induced u.v. c.d. changes and kinetic observations.[642]

C.d. spectra of tryptophan synthase show that following limited tryptic proteolysis the α-subunits of the enzyme are cleaved into two fragments, α-1 and α-2, but retain an ordered structure. Further evidence for a folded structure of the α-1 fragment has been obtained from c.d. observations on solvent perturbation of tyrosine residues. The two fragments, produced by cleavage at Arg 188, show full activity when reconstituted with holo-β-subunits.[643] A comparison has been made between the c.d. parameters of tryptophan synthase α-subunits from wild type *Escherichia coli* and from four mutant proteins, trpA88(Glu-49 → Tyr), trpA3(Glu-49 → Val), trpA33(Glu-49 → Met), and trp11(Glu-49 → Gln). The c.d. spectra of the trpA88 protein show more intense positive bands at 270 nm than the other proteins, reflecting the contribution of the additional Tyr residue. The other three mutants were considerably different from the wild type. Difference spectra of trpA88 protein *vs.* wild type showed well resolved bands, corresponding to Tyr in a non-aqueous environment, at 286, 280, 270, and 263 nm. Difference spectra from trpA3 and A33 (substituted by more hydrophobic residues at position 49) were similar to those of trpA88. One or more of the seven tyrosines in each protein are near position 49 and interact strongly with this residue.[644]

C.d. revealed that the conformational change associated with activation of properdin is relatively small, as compared to the conformational variations produced by conditions not altering its biological activity.[645]

Calculations of the secondary structure of porcine pancreatic colipase A, using the method of Chou and Fasman, give 5% α-helix (39—44), 25% β-sheet (7—11, 49—57, and 77—85), and 8 β-turns and are in good accord with experimental c.d. measurements except with regard to β-turns. The unusual positive band at 225 nm has been assigned to the close proximity of Tyr-56 and Tyr-57 to His-86.[646]

The structural changes accompanying the polymerization of rabbit muscle monomeric G-actin to the helical fibrous form F-actin have been followed by c.d. spectroscopy. The c.d. spectra of chemically modified, non-polymerizing F-actin and actin at subcritical concentrations were identical with those of G-actin. However, on polymerization a small, but significant, increase in β-structure was observed, although the α-helical content was essentially constant. Previously observed conformational alterations, obtained by different methods, were ascribed to local structural changes occurring during polymerization.[647]

Near u.v. c.d. analysis has suggested that albumin interacts with hyaluronic acid and chondroitin sulphate, and in so doing alters its secondary structure, but whether or not the glycosaminoglycans alter their conformation on interaction

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was not detected.[648] The interaction of bovine plasma albumin with SDS has been investigated using c.d. over a range of SDS : protein ratios of $\leqslant 31$ to $\geqslant 206$. At ratios as high as 150 the protein retains a helicity of 50%, indicating little alteration in structure. The c.d. shoulder at 290 nm, due to two solvent exposed Trp residues, disappears at a ratio of 31, whilst negative bands at 260—270 nm, due to Phe residues, remain visible up to a ratio of 206. Changes in the aromatic region with the retention of helical structure have been explained on the basis that the basic residues have an enhanced ability to propagate ordered structure in the presence of SDS, whereas the non polar region changes in conformation by hydrophobic interaction with the alkyl chain of the surfactant.[649]

A correlation has been found to exist between the c.d. ellipticity of the aromatic bands and the physiological activity of partially denatured and abnormal prothrombins. Complexation of the proteins with Ca causes little change in the aromatic c.d., which is in total contrast to the effect of Ca on prothrombin fragment 1. A model for the activity of fragment 1 has been proposed, in which the binding of Ca to the dicarboxyglutamate residue causes an ionization of one or more Tyr residues and a consequent activation of the protein.[650]

The c.d. spectrum of cytoplasmic pyruvate decarboxylase from *Saccharomyces carlsbergensis* in the 250—320 nm range shows a multiple two-signal band. A study of this region, as a function of pH and temperature, has allowed the calculation of a range of thermodynamic and ionization constants to be made, which have been of use in understanding the dimerization and activity of the enzyme.[651]

Dolichas biflorus, Helix pomatia, Lotus tetragonolobus, Phaseolus vulgaris, Picum sativum, Sophora japonica, and *Ulex europaeus* lectins show mainly pleated sheet conformations. On perturbation with SDS the bands arising from Tyr and Trp residues diminish and reorganization of the main chains into new forms containing 15—40% α-helix occurs. In all cases SDS was most effective at inducing reorganization in acid solution at a concentration greater than the critical micelle concentration.[652]

The c.d. spectra of M and N blood group glycoproteins indicate the presence of a low content of α-helix. Tryptic digests, both sialylated and desialylated, are similar with respect to positive ellipticities in the range 210—240 nm. The presence of β-turns is suggested.[653] Support for the proposed β-turn conformation of glycoproteins has been obtained from c.d. studies on aspartate linked glycoproteins in the vacuum u.v.[654]

'Chromophoric' Proteins. Imidazolylmet haemoglobin in the presence of inositol hexaphosphate does not undergo an $R \rightarrow T$ transition and the observed c.d. spectral changes can be accounted for by the replacement of the low-spin ligand by H_2O.[655] Titration of both adult aquomet haemoglobin A and foetal haemoglobin

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with inositol hexaphosphate shows identical total ellipticity changes, although the foetal protein exhibits an inherently negative u.v. c.d. spectrum. A dissociation constant of 16 μM has been obtained for the foetal protein and its negative c.d. assigned to an amino-acid substitution in its γ chains.[656] C.d. spectra have been presented for the haemoglobin, and several of its derivatives, obtained from the marine annelid *Glycera dibranchiata.* The differences observed between the monomer and polymer components in the 195—650 nm region result from changes in the haem environment and geometry, together with the co-ordination states of the central haem Fe atom. Liganded monomer derivatives have a negative Soret band c.d. and the haem band at 260 nm is absent. Oxidation state does not affect the secondary structure, but polymerization decreases the α-helical content from 70 to 50%. Removal of haem leads to a drastic reduction in helical content.[657] The haem environment of leghaemoglobins has been studied by c.d. measurements on artificial leghaemoglobins derived from apo-leghaemoglobin and meso-, deuterio-, and diacetyldeuteriohaems. Substitution of the 2,4 side-chains of haem induced changes in optical activity, reflecting alterations in haem environment, and the effect on the conformation of aromatic amino-acids around the haem correlated with the sixth axial ligand and spin state of Fe.[658] Soret rotational strengths have been calculated and dissymmetry ratios used to reveal hidden transitions for cobaltomyoglobin, oxycobaltomyoglobin, nitrosyl-cobaltomyoglobin, cobaltometmyoglobin, and the corresponding haemoglobin derivatives. It was found that the Soret c.d. is sensitive to the metal oxidation state, ligation, and local environment, but not to quaternary structural changes or magnetic moment.[659] It has been shown that the signal strength in the vibrational c.d. of azidomethaemoglobin is extraordinarily high owing to the presence of chirally arranged lone pairs.[660] In erythrocruorins of *Nereis diversicolor* and *Arenicola marina* the c.d. spectra of the deoxygenated derivatives have shown two large Cotton effects of opposite sign. On addition of O_2 or CO the bands reverse in sign and shift to lower wavelength. In spite of the differences in the detailed appearance of the c.d. spectra of chlorocruorin of *Spirographis spallanzanii,* their common features suggest a similar conformation around the haem as well as in the nature of the amino-acids in its vicinity.[661] The fact that milk lactoperoxidase, its fluoride and cyanide derivatives, and Fe^{II}-lactoperoxidase and its carbonyl and cyanide compounds all exhibit split ellipticity bands has been suggested as evidence for a narrow haem pocket, which prevents ligands forming linear iron–ligand bonds. The secondary structure of lactoperoxidase, as derived from far u.v. c.d., contains 65% β-structure, 23% α-helix, and 12% unordered structure.[662]

Low energy bands, attributable to *d–d* transitions in a flattened (D_{2d}) Cu^{II} centre, have been observed in the near i.r. c.d. at 5000, 950, and 11 200 cm^{-1} in plastocyanin from bean, 5250, 8100, and 10 500 cm^{-1} in stellacyanin from *Rhus*

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vernicifera, and 5800 and 10 200 cm^{-1} in azurin from *Pseudomonas aeruginosa*. Based on a comparison of absorption and c.d. intensities the bands at 13 000, 16 000, and 22 000 cm^{-1}, in these blue copper proteins, were assigned to metal charge transfer transitions $\pi S(Cys) \rightarrow d_{x2-y2}$, $\sigma S(Cys) \rightarrow d_{x2-y2}$, and $\pi N(His) \rightarrow d_{x2-y2}$ respectively.[663] The molar c.d. coefficients have been derived for each of the three laser radiation induced paramagnetic species of human ceruloplasmin (I_a, I_b, II).[664] α-Crustacyanin in phosphate buffer exhibits two c.d. extrema with negative and positive bands at 690 nm and 583 nm respectively, which have been interpreted in terms of dipole–dipole coupling between transition moments of the two astaxanthin molecules per subunit. Further calculations suggest the existence of a dimeric chromophore unit with a mutual orientation angle of 90° and with each chromophore 13 Å apart. C.d. intensity is destroyed in 2M NaCl, and SDS denaturation completely abolishes the long wavelength splitting.[665]

The purple haemocyanin formed on addition of a large excess of ethylene glycol to *Sepioteuthis lessoniana* oxyhaemocyanin is considered to be an equilibrium state of ~60% mildly denatured and ~40% extensively denatured protein.[666] The c.d. spectrum of bovine serum amine oxidase displayed a broad band at 350 nm, a positive extremum at 660 nm, and negative extrema at 450, 540, and 810 nm.[667] On the basis of visible region c.d., inherently chiral chromophores were proposed for the covalently linked chromophores in C-phycoerythrin and its α- and β-subunits. The α-helical contents of the α- and β-apoproteins have been estimated at 60% and 40% respectively. The native C-phycoerythrin c.d. spectrum is a linear superposition of the α- and β-subspectra, indicating that no substantial conformational change occurs on subunit binding. Unfolding of the protein occurs at >60° and slowly leads to irreversible denaturation. Urea denaturation is complete at 8M and is associated with a reduction of c.d. intensity by a factor of ten.[668] The interaction of *Rhus* laccase with O_2, and its reduction intermediates, leads to characteristic alterations in its c.d. spectrum. Activity at 300—400 nm is associated with systems assumed to involve enzyme bound O_2 reduction intermediates and is most intense for the peroxy-derivative, which has the same anisotropy factor as oxy-tyrosinase and oxy-haemocyanins. Aerobic reduction of laccase by ascorbate yields a transient O-bound spectrum, which decays with first order kinetics to a metastable oxidized form of the enzyme. A catalytic scheme has been proposed that allows for the reactions of O_2 with molecules of laccase reduced to different extents.[669]

Complex formation between ferredoxin and ferredoxin–nitrite reductase, or between ferredoxin and ferredoxin–NADP reductase, is accompanied by alterations in the c.d. spectra of the proteins. These alterations indicate changes in both prosthetic group environment and secondary structure. Addition of nitrite to

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nitrite reductase results in visible c.d. changes, but does not affect secondary structure.[670] The molar ellipticities of human NADH–cytochrome b_5 reductase correlate well with enzyme activity and flavin content. Flavin analogues were found to lead to loss of activity and a decrease in the ellipticity of the c.d. bands at 280 nm and 460 nm.[671] C.d. spectra of succinate dehydrogenase indicate a major modification in the immediate vicinity of the flavin and Fe–S centres of the enzyme, when transformed between its active form and oxaloacetate complex. A model was suggested in which control of catalytic activity is exercised *via* redox potential modification by protein–isoalloxazine interactions.[672] Cholesterol oxidase from *Schizophyllum commune* and *Alcaligenes* show similar intensity visible c.d. spectra but of opposite sign, in both the oxidized and reduced forms. These findings are suggested to reflect similar flavin binding environments in both enzymes, but different mutual orientation between the transition moment of flavin and that of its environment.[673] The primary photoevent in vision, namely the conversion of rhodopsin to bathorhodopsin caused by photoisomerization of retinal, has been confirmed by c.d. measurements. The c.d. spectrum of rhodopsin shows two positive peaks at 335 and 500 nm, whereas bathorhodopsin shows one positive peak at 334 nm and one negative peak at 540 nm. The change of sign of the α band c.d. is taken as support for the hypothesis that the conversion of rhodopsin is due to rotation of retinal about the C-11–C-12 double bond.[674] The c.d. signals obtained from the light harvesting chlorophyll *a*/*b* complex are of equal magnitude, but of different sign to those from intact chloroplasts. The aggregate form is viewed as an artifact and is considered to be an example of when a large c.d. signal is generated by macromolecular association. The asymmetric organization in the chloroplast has an opposite sense to that of the aggregate, but affects only chlorophyll *a*.[675]

Extrinsic Chromophores. When less than 0.75 moles of bromophenol blue (BPB) was bound per mole of lactic dehydrogenase (LDH) the induced c.d. showed a positive band at 600 nm and three bands of alternating sign in the 300—400 nm region. Higher levels of BPB split the 600 nm band into positive and negative lobes of increased amplitude. Two interpretations have been suggested for these changes in c.d. The couplet could arise from BPB binding to the co-enzyme and substrate, while conformational changes increase the c.d. amplitude. Alternatively exciton coupling could be responsible.[676] Cibacron Blue and Congo Red bind to several dehydrogenases, not specifically but with similar conformation, making them valuable conformational probes of nucleotide binding enzymes.[677]

Hormones.—The conformations of four analogues of Met-enkephalin and 4,5-pro-enkephalinamide, as indicated by c.d. in the 210—270 nm region, are dependent on the protonation state of the terminal tyrosyl amino-group. The effect on the analgaesic potency of the analogues, arising from the tyrosyl

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alkylammonium–peptide carbonyl interaction, of K, Na, Ca, and Mn was investigated by c.d.[678] Phosphatidylserine, sodium phosphatidate, ganglioside G_{M1}, and cerebroside sulphate, when solubilized in cetyl(oxyethylene) ether, induce a helical conformation in β-endorphin due to interactions with the protein lysine groups. This has significance for the possible brain opiate receptor structure.[679] Molar ellipticity data for the rigid 2,5-substituted Met-enkephalins have been related to opiate agonist activity in various tissues.[680] The circular dichroism of pituitary hormones has been surveyed.[681] Somatostatin and a number of substituted analogues show the presence of a type II β turn, which includes residues 7—11.[682] Thyrotropin releasing factor produces a 1 : 1 complex with Cu *via* coordination to the nitrogen atoms of imidazole, pyroGlu, and a peptide bond.[683] Luteinizing hormone releasing factor appears to exist as an ensemble of conformers with different temperature and solvent sensitivities.[684] Although substance P has no ordered structure in solution, its u.v. c.d. spectrum is sensitive to the state of ionization of the protein.[685] Bradykin appears to spend a maximum of 20% of its time in a partially ordered state containing a γ-turn.[686] Human gastrin and its shorter analogues, down to a tetragastrin, can show a helical or random structure depending on the solvent used.[687] The biological activity of angiotensin, its agonists and antagonists, appears to relate to the controlling influence of Ile^5 in aligning the central Tyr-Ile-His segment.[688]

Membrane Proteins.—When oriented, the photoreceptor membrane c.d. increases four-fold in the 500 nm region, but is absent in the 340 nm region, whilst a large negative peak at 275 nm has been assigned to Tyr.[689] Induced c.d. of β-carotene, present in reconstituted cores of low density lipoproteins, has been used to characterize the properties of the natural cores.[690]

Nuclear Proteins.—The secondary structure of the histone 2A binding protein ubiquitin shows only a low percentage of α-helix or β-sheet. Its interactions with chromatin components are discussed.[691] U.v. irradiation of chromatin leads to an increase in protein c.d. at 225 nm.[692] The finding that the high salt form of the

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H3–H4 histone complex has a higher α-helix content than the low salt form has been related to dynamic equilibria of nucleosome structure.[693] Mononucleosome structure has been discussed on the basis of c.d. measurements of unperturbed and H1 and H5 depleted species.[694]

7 Magnetic Circular Dichroism

Contributed by T. Brittain

Reviews.—Reviews have appeared concerned with m.c.d. in general terms,[695, 696] as well as its application to haemproteins,[697, 698] and the vacuum u.v.[699]

Theory and Analysis.—The application of advanced group theoretical techniques to m.c.d. has been surveyed.[700] Possible manifestations of electric quadrupole contributions in m.c.d. have been studied theoretically for the $A_{2g} \rightarrow T_{1g}$ transitions of octahedral systems, and for the $\Sigma_g^+ \rightarrow \Pi_u$, Π_g, and Δ_g transitions of homonuclear molecules under a variety of conditions, and the identification and characterization of such transitions discussed.[701]

Model Compounds.—The effects of pH, temperature, and solvent on phenylalanine, *N*-acetylphenylalanine, and ethyl-*N*-acetylphenylalanine m.c.d have been reported.[702] Spectra of L-, D-, and DL-Phe have been measured at room temperature and 77 K.[703]

Proteins.—In oxidized lipoamide dehydrogenase and glutathione reductase, the flavin associated B term at 27 000 cm^{-1} has the opposite sign to that of the free flavin as a result of interaction of the disulphide with the short axis dipole of FAD. The half reduced enzymes display A terms at 18 180 cm^{-1}, attributable to a charge transfer complex with a donor thiolate anion stabilized by a protein residue.[704] Native catalase at pH 7 is characterized by high spin Fe^{III} and is similar to Fe^{III}myoglobin and Fe^{III} horseradish peroxidase. Catalase compound I has the same ground state haem π system as horseradish peroxidase compound I, but the corresponding compounds II possess very different m.c.d., even though both show spectra resembling metalloporphyrins, which lack charge transfer transitions and *d*-orbitals decoupled from the porphyrin π system.[705] The results of variable temperature m.c.d. studies on nitrosylferro-Mb, Hb, peroxidase, and protoporphyrin IX have been discussed in terms of the theoretical interpretations of Mineev and Sharanov and the Jahn–Teller effect.[706] The effects of addition of

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inositol hexaphosphate on the spin equilibria in human ferric haemoglobins have been reported.[707]

Magnetic field saturation studies on cytochrome *c* oxidase indicate that any coupling between the low spin ligand bound haem a_3 and the e.s.r. undetectable Cu^{2+} must be very small.[708] However, i.r. m.c.d. data indicate that low spin ligand bound haem a_3 does interact with other metal centres within the protein.[709]

The near i.r. m.c.d. bands at $10\,600\,cm^{-1}$, $8800\,cm^{-1}$, and $10\,500\,cm^{-1}$ in plastocyanin, stellacyanin, and azurin, respectively, suggest the presence of copper centres, which are tetrahedral with square planar distortion.[663]

8 Mössbauer Spectroscopy

Contributed by D.P.E. Dickson

The primary sources of literature information for this review are 'The Mössbauer Effect Reference and Data Journal' published by the Mössbauer Effect Data Center, University of North Carolina, Asheville, NC 28804, U.S.A., and 'The Index of Publications in Mössbauer Spectroscopy of Biological Materials' by L. May, Catholic University of America, Washington, DC 20017, U.S.A.

During 1980 work has been reported on haem proteins, iron–sulphur proteins and enzymes, and iron-storage proteins. Much of this work has been on more complex systems than isolated molecules and has frequently used previous Mössbauer data on particular biomolecules and centres to identify their presence within a more complex system.

Almost all of the studies have been of iron-containing systems and have used the ^{57}Fe Mössbauer nuclide. One investigation, however, has used ^{119}Sn Mössbauer spectroscopy to investigate inorganic tin derivatives of amino-acids.[710] It was found that the nature of the products formed from aqueous $SnCl_2$ and sulphur-containing amino-acids depends on the conditions of preparation. The complexes investigated were with cysteine, penicillamine, and phenylalanine amino-acids.

Shaitan and Rubin[711] have investigated the abnormally low Mössbauer recoil-free fraction observed for ^{57}Fe in proteins between 200 and 250 K. This is accounted for by a model for gamma quantum emission and absorption by an oscillator in a highly viscous medium, which considers the coefficient of friction for the movement of different regions of the molecule with respect to each other. This coefficient is directly proportional to microviscosity and the movement appears to be like a limited diffusion over about 0.07 nm rather than to be truly oscillatory.

Haem Proteins.—The measurement of Mössbauer parameters of haem proteins is of interest in that by comparison with values calculated from theoretical models it is possible to derive information on the conformation of the iron atom, which may be related to the biological function of these proteins. Willems *et al.*[712] have made

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[710] P. A. Cusack, P. J. Smith, and J. D. Donaldson, *Inorg. Chim. Acta*, 1980, **46**, L73.

[711] K. V. Shaitan and A. B. Rubin, *Mol. Biol. (Moscow)*, 1980, **14**, 1323.

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a Mössbauer study of single crystals of deoxymyoglobin using polarized gamma rays. These measurements produce constraints in the computer analysis of the spectra, which are valuable in obtaining precise values of the hyperfine parameters and their orientations relative to each other and to the haem group.

Interactions between protein molecules may be an important factor in the way in which they operate. Mössbauer measurements of partially reduced cytochrome c_3 at 4.2 K show that there is a strong haem–haem magnetic interaction between the cytochrome c_3 molecules.[713]

The haem group, with suitable ligands and in different molecules, can stabilize the iron atom in many of the possible valence and spin states. Mössbauer measurements of the ferricytochrome *c'* from *Chromatium* indicate that, depending on the value of the pH, it can either be in a normal high-spin state or in a mixed-spin state with an unusually low value for the magnetic hyperfine field.[714] Mössbauer spectra of samples of the haem undecapeptide of cytochrome *c*, which were prepared and purified in a new way, show the presence of iron mainly in a low-spin ferric form, which is in contrast to earlier reports.[715] Although some of the haem iron appears to be in a different site, there is no evidence of a temperature-dependent spin–spin equilibrium. Gunter *et al.*[716] have investigated the mixed-metal complex *meso*-α,α,α,α-tetra-*o*-nicotinamidophenyl as a possible model for cytochrome oxidase. Mössbauer data show the existence of two electric states of the ferric iron atom which are probably $S = \frac{3}{2}$ and $S = \frac{5}{2}$.

Keller and Debrunner[717] have obtained information on the dynamics of the iron atom in frozen aqueous oxymyoglobin by analysis of the temperature dependence of the Mössbauer spectrum. Their data are interpreted in terms of the conformational and diffusional mean square displacement of the iron atom. Krupyanski *et al.*[718] have investigated the dynamics of metmyoglobin by the Rayleigh scattering of Mössbauer radiation. This provides a means of obtaining information on the dynamics of the whole molecule and the data obtained show that the dynamics are strongly related to the water content of the protein, with marked differences between the protein in crystalline and frozen solution forms.

Mössbauer spectroscopy was used to monitor the rate of reduction of cytochrome *c* in the presence of hydrogenase.[719] The rate constant for the reduction was derived from the time dependence of the area ratio of the two components of Mössbauer spectra corresponding to the ferri- and ferro-forms of the protein.

Iron–Sulphur Proteins and Enzymes.—This section covers Mössbauer spectroscopic studies on iron–sulphur proteins, enzymes containing iron–sulphur centres, and synthetic analogues, which may provide information that will help in understanding the more complex proteins and enzymes.

[713] M. Utono, K. Ono, K. Kimura, H. Inokuchi, and T. Yagi, *J. Phys. Colloq.*, 1980, **41**, C1—957.
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Iron–Sulphur Proteins. The simplest type of iron–sulphur protein is rubredoxin, which contains a single iron atom tetrahedrally co-ordinated to four sulphur atoms of cysteine groups in the amino-acid chain of the protein. Desulphuredoxin from *Desulphovibrio gigas* is an iron–sulphur protein containing two iron atoms in a molecule of relative molecular mass 7900, which is a dimer with two identical subunits. Previous measurements indicate that there are certain differences between the iron sites in desulphuredoxin and in rubredoxin. Mössbauer studies by Moura *et al.*[720, 721] have shown that the two iron atoms are indistinguishable by both Mössbauer spectroscopy and electron paramagnetic resonance. The Mössbauer parameters indicate that there are geometrical differences between the iron sites in the two proteins, as indicated by the differences in the quadrupole splitting and asymmetry parameter, and that there is a larger degree of covalency of the iron atom in desulphuredoxin, as indicated by the lower value of the saturated Mössbauer hyperfine field.

A large class of iron–sulphur proteins contain centres with four iron atoms and four labile sulphur atoms arranged in a cubane structure. Within this class there are two types of protein, as far as their redox properties are concerned. The larger group, the bacterial ferredoxins, are in the oxidized state in their native form and undergo a one-electron reduction to the reduced state, and the other group, the high-potential iron proteins (HiPIPs), are in the reduced state in their native form and undergo a one-electron oxidation to the oxidized state. In the oxidized ferredoxins and reduced HiPIPs the centre iron atoms are in an equivalent valence state, intermediate between ferric and ferrous. The differences between the centres in the two types of protein are of considerable interest in that it may elucidate the way in which the centres are affected in such a way as to change drastically the redox properties. The Mössbauer spectra of both reduced and oxidized *Chromatium* HiPIP have been analysed using computer fits to theoretical spectra derived from a spin-Hamiltonian.[722] A consistent set of hyperfine parameters was obtained from fits to spectra taken over a range of temperatures between 4.2 and 195 K and in applied magnetic fields up to 10.0 T. The results for the reduced protein confirm that its four iron atoms are essentially equivalent with a valence state intermediate between ferrous and ferric and with the spins coupled anti-ferromagnetically to give the centre zero net spin. The oxidized centre has one less electron, which at low temperatures appears to have come from one pair of iron atoms, which thus become ferric, with the other pair remaining substantially unchanged. It is clear from the magnetic hyperfine parameters obtained from the fits to the low-temperature applied field spectra that a larger magnetic moment is associated with the ferric–ferrous pair of iron atoms than with the ferric pair. This apparently anomalous result (ferric atoms have a larger spin than ferrous atoms and therefore normally have a larger magnetic moment) also explains the g-values with an average value of greater than 2 which are observed in e.p.r. measurements. At higher temperatures the differences in the electronic charge densities at the different iron atoms of the oxidized centre become smeared out.

[720] I. Moura, B. H. Huynh, R. P. Hausinger, J. Le Gall, A. V. Xavier, and E. Münck, *J. Biol. Chem.*, 1980, **255**, 2493.
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[722] P. Middleton, D. P. E. Dickson, C. E. Johnson, and J. D. Rush, *Eur. J. Biochem.*, 1980, **104**, 289.

The eight-iron bacterial ferredoxin from *Clostridium pasteurianum* contains two four-iron centres. Complementary Mössbauer, e.p.r., and magnetic susceptibility studies by Bogner *et al.*[723] show that it is necessary to take into account the spin–spin interaction between the two $S = \frac{1}{2}$ centres in each molecule in order to build up a suitable level scheme to explain the magnetic properties of the protein.

For many years it was accepted that the main types of centre in the iron–sulphur proteins contain one, two, or four iron atoms. However, recently, there has been considerable evidence for a new group of iron–sulphur proteins containing centres with three iron atoms. Mössbauer spectroscopic data have provided an important part of this evidence. The tetrameric form of a *Desulphovibrio gigas* ferredoxin, named FdII, mediates electron transfer between cytochrome c_3 and sulphide reductase. Mössbauer spectroscopy and electron paramagnetic resonance data have been presented by Huynh *et al.*[724] to demonstrate the presence of a spin-coupled structure containing three iron atoms as well as labile sulphur in this protein. The spectrum of the oxidized ferredoxin at 77 K shows essentially a single quadrupole-split doublet indicating that at this temperature all of the iron atoms are equivalent. At 4.2 K the reduced protein shows a spectrum consisting of two quadrupole-split doublet components with an intensity ratio of 2:1. In the presence of applied magnetic fields the oxidized ferredoxin gives a spectrum which requires three equal intensity components to fit it while the spectrum of the reduced ferredoxin requires two components with an intensity ratio of 2:1. The authors conclude from this data that the centre contains three iron atoms. The ferredoxin from *Azotobacter vinelandii* contains two centres with different redox properties characterized by high and low redox potentials. Mössbauer measurements on this protein indicate that while the high-potential centre is of the four-iron type, the low potential centre is of a novel type, which appears to contain three iron atoms.[725, 726] The interpretation of the data is difficult because of the two overlapping contributions but good fits to the applied magnetic field spectra can be obtained on the assumption that the high-potential centre is similar to reduced *Chromatium* HiPIP, together with a contribution from the low-potential centre which corresponds to the presence of the three iron atoms with two spin-up (*i.e.* with their magnetic moments parallel to the applied field) and one spin-down (*i.e.* with its magnetic moment antiparallel to the applied field). The interpretation of the data is assisted by the subtraction of spectra taken in magnetic fields applied parallel and perpendicular to the direction of the gamma ray beam.

Iron–Sulphur Enzymes. Now that considerable Mössbauer data on the simpler types of iron–sulphur centres exist it is possible to use these data to identify the centres in enzyme systems and also to help in characterizing the more complex centres that occur in enzymes.

Mössbauer measurements on the oxidized and reduced states of the mono-oxygenase putidamono-oxin from the multi-enzyme system 4-methoxybenzoate

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[725] M. H. Emptage, T. A. Kent, B. H. Huynh, J .Rawlings, W. H. Orme-Johnson, and E. Münck, *J. Biol. Chem.*, 1980, **255**, 1793.
[726] M. H. Emptage, T. A. Kent, B. H. Huynh, W. H. Orme-Johnson, and E. Münck, *Cienc. Biol.*, 1980, **5**, 203.

o-demethylase from *Pseudomonas putida* indicate that it contains two-iron centres similar to those found in the plant-type ferredoxins.[727, 728] Further Mössbauer measurements may prove helpful in elucidating the role of the additional iron atom required by this enzyme for activity.

Metapyrocatechase from *Pseudomonas arvilla* is an enzyme that catalyses the conversion of catechol into α-hydroxymutonic ε-semialdehyde with the insertion of two atoms of molecular oxygen. It is a dioxygenase and contains four iron atoms in a molecule with four subunits. The oxidation state of the iron is thought to be Fe^{2+}, but it has not yet been completely determined. Mössbauer measurements by Tatsuno *et al.*[729] show that at 4.2 K the spectra exhibit the large chemical shift ($1.31\,mm\,s^{-1}$) and quadrupole splitting ($3.28\,mm\,s^{-1}$) associated with the high-spin ferrous state. The spectra of the enzyme with a ten-fold excess of the catechol substrate are essentially identical, which indicates that the substrate changes neither the co-ordination environment nor the valence state of the iron atoms.

Averill *et al.*[730] have investigated the enzyme glutamine phosphoribosyl pyrophosphate amidotransferase from the bacterium *Bacillus subtilis*. The spectra indicate that this enzyme contains a four-iron four-sulphur centre.

The involvement of iron–sulphur centres in Photosystem-I from photosynthetic reaction centres of cyanobacteria (blue-green algae) is of considerable interest. Mössbauer measurements by Rush *et al.*[731] indicate that Photosystem-I contains four-iron centres like those found in bacterial ferredoxins.

Nitrogenase is a complex enzyme system which contains a number of iron–sulphur proteins. One of these contains molybdenum as well as iron and there has been considerable interest in trying to determine the precise number of the molybdenum, iron, and labile sulphur atoms as well as their structural arrangement. These investigations have involved many experimental techniques including Mössbauer spectroscopy. Huynh *et al.*[732] have carried out a Mössbauer study of the Mo–Fe protein of the nitrogenase from *Clostridium pasteurianum* in the temperature range 1.5 to 200 K and in applied magnetic fields of up to 5.5 T. The protein contains two identical co-factor centres labelled M, probably containing six iron atoms and one molybdeum atom, four P centres containing four iron atoms, and about two iron atoms in an environment called S. The co-factor centres can exist in a diagmagnetic $S = 0$ oxidated state, a paramagnetic e.p.r.-active $S = \frac{3}{2}$ native state, and a reduced state. Mössbauer spectra obtained under nitrogen fixing conditions indicate that the reduced state is paramagnetic with an integer electronic spin $S \geqslant 1$. Computer analysis of the high applied magnetic field spectra of the P centres in the oxidized state shows them to be paramagnetic with antiferromagnetic coupling between the iron atoms, with three iron atoms having their magnetic moments directed parallel to the applied field and one with its magnetic moment directed antiparallel to the applied field.

[727] E. Bill, F. H. Bernhardt, V. R. Marathe, and A. Trautwein, *J. Phys. Colloq.*, 1980, **41**, C1—485.
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Synthetic Iron–Sulphur Analogues. One approach to elucidating the nature of the molybdenum–iron–sulphur centres of nitrogenase is to synthesize model compounds with various types of molybdenum–iron–sulphur centre and then compare their spectroscopic properties with those of the enzyme proteins and co-factors.

Tieckelmann *et al.*[733] have synthesized a number of compounds containing one iron atom and one molybdenum atom with a disulphide bridge between them. Mössbauer spectra of $[S_2MoS_2Fe(SC_6H_5)_2]^{2-}$ and $[S_2MoS_2FeCl_2]^{2-}$ in frozen *NN*-dimethylformamide solution show a single quadrupole-split doublet. At 4.2 K the observed chemical shifts (0.47 and 0.60 mm s^{-1}) lie between those for high-spin Fe^{2+} and Fe^{3+} in tetrahedral sulphur environments, although closer to those of Fe^{2+}. Mössbauer spectra taken at low temperatures in applied magnetic fields show that $[S_2MoS_2Fe(SC_6H_5)_2]^{2-}$ has an easy axis of magnetization. The authors conclude that the chemical shift data, taken with other data, indicate that the dimetallic unit is stabilized by an interaction involving net transfer of electron density from Fe^{II} to Mo^{VI}.

Coucouvanis *et al.*[734, 735] have synthesized both a dimetallic cluster containing FeS_2M and a trimetallic cluster containing FeS_2MS_2Fe where M is Mo or W. The Mössbauer chemical shift and quadrupole splitting suggest that in the dimetallic cluster the iron has a valence intermediate between ferrous and ferric and that this is rather insensitive to the change from Mo to W. In the trimetallic cluster the chemical shift and quadrupole splitting are different and by comparison with the values found in reduced rubredoxin indicate that the iron is in a high-spin ferrous state.

Another type of cluster that has been used as a possible model for the molybdenum–iron centre of nitrogenase has been a cubane centre with three iron atoms and one molybdenum atom. Two types of model compound containing two of these clusters have been synthesized, one with a bridge involving an extra iron atom. Mössbauer measurements on these compounds[736] indicate that, although there is some degree of inequivalence between the iron atoms at low temperatures, the chemical shifts and quadrupole splittings are very similar. The chemical shift data indicate an oxidation state of $Fe^{2.67+}$ for the cubane iron atoms, equivalent to a formal $2Fe^{III} + Fe^{II}$ configuration. In the two compounds investigated, in which an iron atom is involved in the bridging, it appears to be low-spin ferric in one compound and high-spin ferrous in the other. Measurements in an applied magnetic field indicate that, of the three cubane iron atoms, two have their magnetic moments aligned parallel to the applied field and one has its magnetic moment aligned antiparallel to the applied field.

Mössbauer measurements have been carried out by Christou *et al.*[737] between 4.2 and 293 K on compounds containing the anions $[Fe_6W_2S_8(OMe)_3(SPh)_6]^{3-}$ and $[Fe_6M_2S_8(SR)_9]^{3-}$ where M = Mo or W. These contain two cubane clusters

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[737] G. Christou, C. D. Garner, R. M. Miller, C. E. Johnson, and J. D. Rush, *J. Chem. Soc., Dalton Trans.*, 1980, **12**, 2354.

similar to those described above. The parameters obtained are similar to those of $[Fe_4S_4(SR)_4]^{2-}$ complexes and on the basis of the Mössbauer chemical shift each iron atom appears to have a net oxidation of about 2.5+, which implies that each molybdenum or tungsten atom has an oxidation state of 3+ or 4+.

Iron-storage Proteins.—The iron-storage protein ferritin is found in fungi, plants, and animals. It consists of a small core of inorganic ferric oxyhydroxide phosphate polymer in a protein shell. The related insoluble material haemosiderin appears to be a denaturation product of ferritin with very similar cores. Because of the small size of the ferritin cores they exhibit the phenomenon of superparamagnetism, which leads to very characteristic Mössbauer spectra enabling ferritin to be readily detected and quantified in various systems.

While considerable work has been done on the proteins and mechanisms of iron-storage in higher organisms there is as yet rather little known about iron-storage in bacteria and micro-organisms. Mössbauer spectroscopic data have recently been presented which indicate the presence of a new and apparently universal type of iron-storage material in prokaryotic cells.[738-741] The new material has a lower Mössbauer hyperfine field than ferritin, 43.0 T compared with 49.7 T. More importantly it exhibits a considerable difference in the way in which its Mössbauer spectrum changes as a function of temperature. It shows a magnetic ordering transition at about 3 K, whereas ferritin shows a superparamagnetic blocking temperature of the order of 50 K. Some of this new material appears to be membrane bound and some has been isolated in the form of a soluble protein. The determination of the precise differences between the nature of the iron in the new material and that in ferritin awaits further work.

Kellershohn *et al.*[742] have studied lyophilized samples of horse, beef, and calf liver and spleen at room, liquid nitrogen, and liquid helium temperatures. The lyophilized spleen samples at 4.2 K show the characteristic six-line spectrum of the normal mammalian iron-storage protein ferritin. In the lyophilized samples of liver, however, there is an Fe^{3+} doublet observed in addition to the six-line pattern, even at 4.2 K. The doublet has parameters consistent with the presence of the new type of iron-storage material previously found in bacteria.

9 Protein–Protein Interactions and Ligand Binding

Contributed by L. W. Nichol, P. D. Jeffrey, and D. J. Winzor

In previous coverages of this subject, the arrangement has emphasized experimental methods for the study of interacting systems and theoretical developments in the analysis of results obtained with them. However, during the period covered

[738] E. R. Bauminger, S. G. Cohen, F. Labenski de Kanter, A. Levy, S. Ofer, and S. Rottem, *J. Phys. Colloq.*, 1980, **41**, C1—491.

[739] E. R. Bauminger, S. G. Cohen, D. P. E. Dickson, A. Levy, S. Ofer, and J. Yariv, *Biochim. Biophys. Acta*, 1980, **623**, 237.

[740] E. R. Bauminger, S. G. Cohen, F. Labenski de Kanter, A. Levy, S. Ofer, and S. Rottem, *Cienc. Biol.*, 1980, **5**, 185

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[742] C. Kellershohn, C. Audebert, D. Fortier, J. N. Rimbert, and C. Hubert, *Rev. Phys. Appl.*, 1980, **15**, 1175.

by this report the literature has been largely directed toward the examination of particular protein interaction patterns. Accordingly, the present treatment is arranged in terms of types of interacting systems proceeding, in increasing order of complexity, from intramolecular considerations, through self-associations to mixed associations. In relation to the last category, we have included a section on indefinite cross-linking whose study offers the newest challenge in the field and on the operation of combined effects exemplified by the interplay of ligand binding with protein association. Such topics have also been covered in general articles,[743-745] the last cited being reports of meetings devoted to the discussion of various aspects of macromolecular interactions.

Intramolecular Interactions.—*Conformational Considerations.* A simple, systematic approach to the specification of the quaternary structures of proteins from measurements of the sedimentation coefficients of promoters and oligomers has been formulated by Andrews and Jeffrey.[746] Sedimentation ratios of oligomeric structures containing from two to six oblate, prolate, or spherical protomers in spatially equivalent positions were calculated on the basis of Kirkwood's equations and are presented in the form of graphs. These allow sedimentation velocity measurements for any protein oligomers, up to and including hexamers, comprised of protomers capable of representation as ellipsoids of revolution (ranging from oblate of axial ratio 2:1 up to prolate ellipsoids of axial ratio 5:1), to be analysed readily in terms of quaternary structure. In a companion paper[747] the same authors assessed the validity of the approach by applying it to a selection of specific proteins. It was concluded that the sedimentation analysis will usually yield a reasonably good representation of the mode of assembly of protein molecules in oligomeric structures if accurate experimental data are available and if a low level of resolution is acceptable. Extension of the existing relationships to oligomers containing more than six subunits was illustrated with reference to the arthropod haemocyanins, for which the approach provided (for all known types) a coherent set of assembly modes which are consistent with their images in the electron microscope.

Changes in protein conformation brought about by the binding of small ligands continue to be investigated actively. Nuclear magnetic resonance measurements with a 360 MHz instrument have been used[748] to study the effects of calcium and magnesium on the solution conformation of calmodulin. It was found that the calcium-induced conformational transition occurs in two steps each involving the binding of two calcium ions. There were some differences evident in the binding of calcium and magnesium. Comparisons were made with the metal ion dependent conformations of skeletal troponin-C and it was concluded that the large sequence homology of the two proteins may be reflected in regions of very similar tertiary

[743] 'Methods in Enzymology', ed. C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1979, Vol. 61.
[744] 'The Regulation of Coagulation', ed. K. G. Mann and F. B. Taylor, jun., Elsevier/North Holland, New York, 1980.
[745] 'Biophysical Discussions. Proteins and Nucleoproteins, Structure, Dynamics and Assembly', *Biophys. J.*, 1980, Vol. 32, No. 1.
[746] P. R. Andrews and P. D. Jeffrey, *Biophys. Chem.*, 1980, **11**, 49.
[747] P. D. Jeffrey and P. R. Andrews, *Biophys. Chem.*, 1980, **11**, 61.
[748] K. B. Seamon, *Biochemistry*, 1980, **19**, 207.

structure. An *X*-ray scattering study[749] of the binding of calcium to another protein, prothrombin, at 21 °C, pH 7.4, indicates that the shape of the molecule can be represented by two ellipsoids and that the effect of calcium binding can be represented by a change in the angle between their major axes.

Computer modelling in conjunction with small angle *X*-ray scattering was used[750] to analyse the decrease in the radius of gyration accompanying the binding of the substrates MgATP and 3-phosphoglycerate to yeast phosphoglycerate kinase. It was suggested that the change is a hinge motion of the two lobes of the enzyme, which produces a closure of the cleft between them. The effect was compared with that in hexokinase. A battery of techniques including fluorescence spectroscopy, circular dichroism, equilibrium dialysis, and microcalorimetry was employed by Steiner *et al.*[751] to explain the inhibitory effects of the binding of glucose and caffeine on the dimeric enzyme glycogen phosphorylase *b* in terms of structural changes. It appears that the effects are due to subtle conformational changes, those accompanying the binding of caffeine being more extensive and possibly mimicking the effects of a physiological modifier.

Subunit—Subunit Interactions. Two proteins, haemoglobin and aspartate transcarbamoylase, are the outstanding examples of functional oligomeric proteins constructed from different subunits, and research interest in both remained lively during 1980. Perutz and Imai[752] found that the low oxygen affinity of bovine haemoglobin arises from stronger constraints in the quaternary deoxy-structure. They suggested that the difference in the high and low oxygen affinity haemoglobins arises from the substitution of a hydrophobic residue in the latter for a hydrophilic one in the former group. This hydrophobic residue, pointing into the interior of the molecule, could mimic the stabilizing action of DPG by locking helix A firmly to its neighbouring peptide chain segments. Experiments with stripped haemoglobin[753] confirmed that all of the groups contributing to the alkaline Bohr effect at pH 7.4 had already been identified and showed that in the stripped protein, in 0.1 M KCl at 25 °C, residue HisH21(143)β is responsible for about half the acid Bohr effect. A comparative study of the binding of oxygen and carbon monoxide to haemoglobin using electron paramagnetic resonance[754] showed that carbon monoxide binds preferentially to the β-chains while in the absence of an allosteric effector, oxygen-binding is a random process. The preferential binding of oxygen to the α-subunits of deoxyhaemoglobin in the presence of DPG was explained in terms of a stabilization of the T state of haemoglobin and a concomitant decrease of the oxygen affinity in the β-subunits in the T state.

As a result of his analysis of the energetics of subunit assembly and the binding of oxygen and protons by human haemoglobin, Ackers[755] suggested that a combination of hydrogen bonding and proton ionization may account for the

[749] R. Österberg, B. Sjöberg, P. Österberg, and J. Stenflo, *Biochemistry*, 1980, **19**, 2283.
[750] C. A. Pickover, D. B. McKay, D. M. Engelman, and T. A. Steitz, *J. Biol. Chem.*, 1979, **254**, 11 323.
[751] R. F. Steiner, L. Greer, R. Bhat, and J. Oton, *Biochim. Biophys. Acta*, 1980, **611**, 269.
[752] M. F. Perutz and K. Imai, *J. Mol. Biol.*, 1980, **136**, 183.
[753] M. F. Perutz, J. V. Kilmartin, K. Nishikura, J. H. Fogg, P. J. G. Butler, and H. S. Rollema, *J. Mol. Biol.*, 1980, **138**, 649.
[754] P. W. Lau and T. Asakura, *J. Biol. Chem.*, 1980, **255**, 1617.
[755] G. K. Ackers, *Biophys. J.*, 1980, **32**, 331.

main energetic features of the co-operativity. A different approach was taken by Groome[756] who employed the methodology of statistical physics to analyse the haemoglobin co-operativity in terms of the two-state ($t \rightarrow r$) model essentially as viewed by Perutz. The motion of the iron atom with respect to the haem plane is taken to be the important feature of the binding process and its displacement is assumed to be related linearly to the internal tension of the molecule. Agreement of the equations derived on this basis with experimental data for oxygen and carbon monoxide binding to haemoglobin was satisfactory.

The assembly of the catalytic, C, and regulatory, R, subunits of aspartate transcarbamoylase to form the oligomeric enzyme, C_2R_3, has been investigated.[757, 758] A kinetic model consisting of three classes and a total of ten reactions was proposed. Reactions in the first class, C + R, CR + R, and CR_2 + R, are reversible; those in the second class, C_2R + R and C_2R_2 + R, are irreversible; and of the remaining class, C + CR is reversible, and the other four, CR + CR, CR + CR_2, C + CR_2, and C + CR_3, are irreversible. The assembly data were fitted by assigning three different equilibrium constants to the first class of three reactions, and second order rate constants of $3 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ and $1.3 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ to the second and third classes, respectively. Conformational changes resulting from ligand binding to the catalytic chains of a form of the enzyme in which Ni^{II} had been substituted for Zn^{II} on the regulatory chains have also been studied.[759] It was found that the conformational changes were fully propagated to the regulatory chains in agreement with other evidence that the allosteric transition in aspartate transcarbamoylase is concerted. The results of an investigation by spectral techniques of the effect of pH (range 6.0—8.5) on local (tertiary structure) and gross (quaternary structure) conformational changes in this enzyme led Lauritzen and Lipscomb[760] to the 'cautious' suggestion that the crystal structures near pH 6 may represent those in solution at higher pH. Further evidence for the essential correctness of the present view of the structure of aspartate transcarbamoylase may be adduced from the interesting paper by Marchi and Horas.[761] They used current ideas about the structures as the basis for statistical mechanical calculations employing the 'decorated' Ising model to compute the reaction-rate curve of the enzyme at pH 7.0, in the presence and absence of the inhibitor cytosine triphosphate, and succeeded in obtaining good agreement with experimental curves.

Self-associating Systems.—*Discrete Polymer Formation.* The forces involved in protein association reactions were assessed by analysis of a selection of protein complexes of known structure.[762] Particular attention was directed toward the relative contribution of hydrophobic forces and some interesting conclusions emerged. It was noted that hydrophobic interactions, although constituting the driving force for association reactions in aqueous media, may only compensate

[756] L. J. Groome, *J. Theor. Biol.*, 1980, **83**, 477.
[757] M. A. Bothwell and H. K. Schachman, *J. Biol. Chem.*, 1980, **255**, 1962.
[758] M. A. Bothwell and H. K. Schachman, *J. Biol. Chem.*, 1980, **255**, 1971.
[759] R. S. Johnson and H. K. Schachman, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1995.
[760] A. M. Lauritzen and W. N. Lipscomb, *Biochem. Biophys. Commun.*, 1980, **95**, 1425.
[761] E. Marchi and J. Horas, *J. Theor. Biol.*, 1980, **85**, 413.
[762] P. D. Ross and S. Subramanian, *Biophys. J.*, 1980, **32**, 79.

marginally for entropy losses incurred in macromolecular associations by loss of translational and rotational degrees of freedom. The major contributions to the observed negative free-energy changes accompanying macromolecular associations resulted from large negative enthalpy changes associated with strengthening of hydrogen bonds in the interior of macromolecules, incidental protonation, and van der Waals interactions brought about directly by the hydrophobic interactions. Large positive entropy and enthalpy changes were found to accompany the association of jack bean concanavalin A dimers to tetramers at pH values between 5.5 and 7.5, where only dimers and tetramers are present.[763] The study was carried out by the high speed sedimentation equilibrium technique as a function of pH, temperature, and $CaCl_2$ concentration. Analysis was carried out in terms of Wyman linked functions so that the participation of hydrogen ions and preferential interactions with solvent components could be taken into account. It was concluded that the association of concanavalin A in $CaCl_2$ is accompanied by the loss of four calcium ions and 0—8 water molecules per tetramer. Histidine-51 or -121 was identified as the ionizable group involved in the self-association.

The contribution of the excluded volumes of proteins to the standard-state free-energy change accompanying the association of protein molecules was assessed by model calculations utilizing a rectangular parallelopiped approximation for the shapes of the particles.[764] It was concluded that such contributions could be significant at concentrations relevant to certain physiological environments. The calculations also showed that as protein concentration (volume occupancy) in solution increases, compact quasi-spherical conformations and, indeed, self-association leading to oligomers with such characteristics, are increasingly favoured.

A combination of electron microscopy and equilibrium sedimentation was used to study the self-association of the protein filamin isolated from smooth muscle, and its proteolytic fragment, heavy merofilamin.[765] Heavy merofilamin does not aggregate under conditions of high ionic strength, as was evident both in the electron microscope and the ultracentrifuge. However, filamin associates reversibly to dimers and subsequent aggregation is irreversible. Under the conditions investigated the tetramer was always present to some extent and higher aggregates formed upon storage or warming solutions. It was suggested that filamin binds to F-actin and produces gelation *via* self-association which cross-links the actin filaments.

The effect of pressure on deactivation of lactate dehydrogenase from pig muscle showed that high pressure deactivation corresponds to high pressure dissociation, the ultimate product of the process being the monomer.[766] Specific solvent conditions were found to be of critical importance. The binding of the co-enzyme NADH shifted the dissociation to higher pressures while binding of phosphate ion stabilized the native tetramer even more, preventing dissociation completely at pressures $\leqslant 2$ kbar.

[763] D. F. Senear and D. C. Teller, *Biophys. J.*, 1980, **32**, 433.
[764] A. P. Minton, *Biophys. J.*, 1980, **32**, 77.
[765] P. J. A. Davies, D. Wallach, M. Willingham, I. Pastan, and M. S. Lewis, *Biochemistry*, 1980, **19**, 1366.
[766] B. C. Schade, R. Rudolph, H.-D. Lüdemann, and R. Jaenicke, *Biochemistry*, 1980, **19**, 1121.

Indefinite Self-association. Further elaboration of analytical techniques for analysing indefinite self-associations of proteins has been proposed.[767] Four types of indefinite self-association were considered of the so-called 'attenuated equilibrium constant' type. These are analogues of the model reaction schemes considered previously, which were framed in terms of an isodesmic association, or some variation of it. The present publication extended a previous treatment, involving progressive decreases in equilibrium constants, by including non-ideality and by making provision for including experimental techniques giving weight-average, rather than number-average, molecular weights. This utilized the 'Adams–Fujita' assumption concerning the second virial coefficient in protein self-association and deployed the Steiner method for determining the monomer concentration as a function of total concentration from experimental data. Highly detailed descriptions of the methodology were supplied and the approach was tested successfully with two simulated examples of one of the system types considered.

A different approach to the analysis of an indefinite self-association was taken by Wills *et al.*[768] These authors showed how the composition-dependence of the activity coefficients of all oligomeric species in an indefinite self-association could be included in the analysis of experimental results by calculating virial coefficients on a co-volume and charge basis, utilizing existing statistical-mechanical and electrostatic theory. The reaction analysed was the association of the protein hen egg-white lysozyme at pH 8.0, ionic strength 0.15, and 15 °C. Sedimentation equilibrium experiments were used up to a concentration of 19.7 $g l^{-1}$ and the 'Ω analysis' rather than Steiner's method applied to obtain the monomer concentration. The concentration range investigated was extended to 56.6 $g l^{-1}$ by frontal gel chromatography on Sephadex G-50 and inclusion of third virial coefficients was required in the analysis. An excellent fit to the experimental results over this entire concentration range was provided by modelling the reaction as an isodesmic indefinite self-association with an association constant of 460 $l mole^{-1}$. The requirement for this great concentration range to be studied to provide reasonable discrimination between competing models highlights the necessity for providing a realistic physical basis for the estimation of virial coefficients.

Formation of Rod-like Structures. Lauffer and Shalaby[769] studied the polymerization of tobacco mosaic virus protein as a function of pH and ionic strength between pH 5.9 and 6.8. They were able to evaluate enthalpy and entropy changes, the salting-out constant, and the electrical work contribution to the association. Their experimental results were in good agreement with theoretical calculations from models for the reaction. Hirth *et al.*[770] discussed the self-assembly of tobacco mosaic virus in terms of the influence of the viral RNA and protein components upon the process. They noted that heterologous reconstitution experiments show that specificity of interaction between RNA and proteins from different strains of virus is not complete. It is suggested that diminished specificity of U2 coat protein is a result either of amino-acid substitution close to the RNA binding site, or of

[767] J. M. Beckerdite, C. C. Wan, and E. T. Adams, jun., *Biophys. Chem.*, 1980, **12**, 199.
[768] P. R. Wills, L. W. Nichol, and R. J. Siezen, *Biophys. Chem.*, 1980, **11**, 71.
[769] M. A. Lauffer and R. A. Shalaby, *Arch. Biochem. Biophys.*, 1980, **201**, 224.
[770] L. Hirth, G. Lebeurier, A. Nicolaieff, and K. E. Richards, *Biophys. J.*, 1980, **32**, 460.

changes at distant parts of the subunit which are transmitted as configurational changes to the binding region.

During 1980, investigations of many aspects of the formation of microtubules, ranging from the thermodynamics of the process [771] to the assembly of tubulin into membranes,[772] were reported. In the first paper, it was reported that curvature of the van't Hoff plot for microtubule assembly is solely a function of the microtubule disassembly reaction. The apparent rate constant for disassembly is biphasic, having different values above and below 20 °C. The result was explained in terms of the effect of the dissociation of microtubule associated proteins (MAPs) in increasing the rate of microtubule disassembly. Sternlicht *et al.*[773] studied the effect of MAPs on the inhibition of microtubule assembly by tubulin–colchicine complex (TC). Their results were not consistent with impaired MAP function, but suggested, rather, that the large percent inhibition values observed at low TC concentrations are a consequence of the small concentrations of tubulin available for microtubule assembly in these experiments. The mechanism of microtubule polymerization was studied by kinetic methods [774] and data supporting a two-step process were obtained for the growth phase. Microtubules were formed when the oligomer fraction alone was employed but not from dimer alone. It was thought that the oligomer probably consists of tubulin and MAPs with a molecular weight of a million or more. The kinetics of tubulin ring formation were studied [775] by relaxation techniques at pH 6.5, I 0.1, in the presence of 1 mM GDP. Under these conditions only one type of ring is present and microtubule assembly is prevented at all temperatures. The results indicated three steps in the ring formation process and the concentration-dependence of the overall reaction suggested the presence of a substantial number of intermediates and/or microheterogeneity of association constants. Zeeberg *et al.*[776] used radioactively labelled rat and porcine tubulins to compare assembly and disassembly mechanisms of microtubules. These processes are not mirror images. In the assembly, tubulin dimer equilibrates with tubulin in rings, but in the disassembly it does not. Apparently, intact rings are formed directly by a cleavage from the microtubule.

Caseins. The association of purified bovine α_{S2}-casein at pH 6.7 was studied [777] by viscosity and light scattering over the ionic strength range 0.02 to 1.2 and it was found that the light scattering results could be fitted by an isodesmic indefinite self-association with a standard free-energy change of about $-38\,\mathrm{kJ\,mol^{-1}}$ at 20 °C over this entire range. The viscosity results suggested that the shape of the particle and its association products is close to spherical. The light scattering measurements were consistent with this observation. The voluminosity (volume of solution occupied per g of protein) calculated on this basis is about $5\,\mathrm{ml\,g^{-1}}$ and is of the same order of magnitude as that previously deduced for α_{S1}-casein, casein micelles,

[771] K. A. Johnson, *Biophys. J.*, 1980, **32**, 443.
[772] H. Feit and J. W. Shay, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 324.
[773] H. Sternlicht, I. Ringel, and J. Szasz, *J. Biol. Chem.*, 1980, **255**, 9138.
[774] J. S. Barton and G. H. Riazi, *Biochim. Biophys. Acta*, 1980, **630**, 392.
[775] Y. Engelborghs, J. Robinson, and G. Ide, *Biophys. J.*, 1980, **32**, 440.
[776] B. Zeeberg, J. Cheek, and M. Caplow, *Biochemistry*, 1980, **19**, 5078.
[777] T. H. M. Snoeren, B. van Markwijk, and R. van Montfort, *Biochim. Biophys. Acta*, 1980, **622**, 268.

and association products of κ-casein. Application of simple electrostatic theory to the effect of calcium ions on the precipitation of α_{s1}-casein aggregates successfully explains the observations as a modification of the negative charge of the protein particles by the binding of positively charged calcium ions.[778] A more rigorous treatment based on electrical double layer theory also predicted the observed linear relationship between the logarithm of the coagulation time and the square of the residual charge on the casein molecule. Moreover, the parameters required in the equations were meaningful in terms of the physical properties of the system.

Equilibrium sedimentation was used [779] to study the effect of temperature on the association of β-casein at pH 6.7. The data were fitted by a monomer–*n*-mer association scheme and it was found that the value of *n* as well as the equilibrium constant was affected by temperature. The predominantly hydrophobic nature of the interaction was indicated by the enhancement of the association with increasing temperature. The effect of low concentrations of sodium dodecyl sulphate (SDS) on this equilibrium was reinvestigated[780] at neutral pH by a range of techniques including analytical ultracentrifugation and fluorescence spectroscopy. It appears that the interaction of the detergent with the protein actually displaces the equilibrium towards polymer formation, despite the increase in net negative charge accompanying the binding. Presumably, this effect is outweighed by a favourable change in the hydrophobic interactions accompanying association. The result accounts, at least qualitatively, for the anomalous electrophoretic mobilities of caseins in polyacrylamide gels containing SDS and indicates the need for caution in the interpretation of such experiments with any protein.

An assessment of the distribution of the casein components in casein micelles was presented [781] in a study that used chromatography on controlled pore glass columns to prepare the fractions, whose size distributions were determined by electron microscopy on freeze-fractured specimens. A linear relationship between κ-casein content and the micelle surface-to-volume ratio was obtained for all of the fractions examined. Recalculation of results previously published gave the same result. It was concluded that casein micelles have the same fundamental structure whether natural or artificially prepared, that is, the κ-casein is predominantly located at the surface, in accord with the coat-core model of Waugh.

Mixed Associations.—*Protein–Ligand Binding.* There have been numerous studies on the binding of low molecular weight compounds (ligands) to protein acceptors. With some systems the structures of the resultant complexes have been elucidated by crystallographic studies at high resolution, notable examples being provided by the interaction of carbohydrate ligands with wheat germ agglutinin [782] and with yeast hexokinase A.[783] In the latter study, conformational differences induced on the binding of glucose were observed in the relative orientations of the two distinct lobes comprising the folded polypeptide backbone of the enzyme.

[778] D. S. Horne and D. G. Dalgleish, *Int. J. Biol. Macromol.*, 1980, **2**, 154.
[779] K. Takase, R. Niki, and S. Arima, *Biochim. Biophys. Acta*, 1980, **622**, 1.
[780] L. K. Creamer, *Arch. Biochem. Biophys.*, 1980, **199**, 172.
[781] T. C. A. McGann, W. J. Donnelly, R. D. Kearney, and W. Buchheim, *Biochim. Biophys. Acta*, 1980, **630**, 261.
[782] C. S. Wright, *J. Mol. Biol.*, 1980, **141**, 267.
[783] W. S. Bennett and T. A. Steitz, *J. Mol. Biol.*, 1980, **140**, 211.

Structural information has also been obtained by proton magnetic resonance on group involvement in the active sites of enzymes, such as ribonuclease [784] and α-chymotrypsin,[785] in hapten binding to myeloma protein,[786] and on the lack of perturbation of the haem cavity of met-aquo myoglobin following intercalation of the protein with a variety of ligands.[787] In other applications of the nuclear magnetic resonance technique, ^{113}Cd and ^{23}Na nuclei have been used to explore the binding properties of the calcium-regulating protein calmodulin,[788, 789] while ^{19}F and ^{31}P n.m.r. resonances have been employed to study, respectively, inhibitor binding to thymidylate synthetase [790] and co-enzyme attachment to dihydrofolate reductase.[791] Other spectral studies on binding have included electron paramagnetic resonance determination of the binding of Cu^{II} to conalbumin,[792] fluorescence work on ligand equilibria with lumazine protein [793] and parvalbumin,[794] and changes in the circular dichroism spectrum of lactate dehydrogenase induced by dye binding.[795]

In the area of thermodynamic characterization of binding processes, several calorimetric studies have been performed to determine enthalpy changes in protein–ligand interactions,[796–798] and there has been the usual intensive investigation of binding of drugs, steroids, amino-acids, and dyes to serum proteins.[799–804] Among the interesting findings in the latter studies was the observation that the binding of L-tryptophan and of 2-(4′-hydroxybenzeneazo)benzoic acid to human albumin is dependent on acceptor concentration.[804] This is particularly noteworthy since it is atypical of binding responses to acceptors which do not self-interact; the authors suggested that a ligand-initiated association may be involved. In addition to conventional binding studies, we find avant garde work on the binding of *N*-acetylglucosamine and of its polymers to lysozyme studied in the solid state at high temperatures [805] and at subzero (−100 °C) temperatures.[806] Non-aqueous solvents were necessarily used

[784] G. I. Yakovlev, M. Y. Karpeisky, S. I. Bezborodova, O. P. Beletskaja, and V. G. Sakharovsky, *Eur. J. Biochem.*, 1980, **109**, 75.
[785] P. Wyeth, R. P. Sharma, and M. Akhtar, *Eur. J. Biochem.*, 1980, **105**, 581.
[786] A. T. Morris, D. Lancet, I. Pecht, D. Givol, and R. A. Dwek, *Int. J. Biol. Macromol.*, 1980, **2**, 39.
[787] G. N. La Mar and D. L. Budd, *Biochim. Biophys. Acta*, 1979, **581**, 201.
[788] A. Delville, J. Grandjean, P. Lazzlo, C. Gerday, H. Brzeska, and W. Drabikowski, *Eur. J. Biochem.*, 1980, **109**, 515.
[789] S. Forsén, E. Thulin, T. Drakenberg, J. Krebs, and K. Seamon, *FEBS Lett.*, 1980, **117**, 189.
[790] C. A. Lewis, jun., P. D. Ellis, and R. B. Dunlap, *Biochemistry*, 1980, **19**, 116.
[791] P. J. Cayley, J. Feeney, and B. J. Kimber, *Int. J. Biol. Macromol.*, 1980, **2**, 251.
[792] J. L. Zweier, *J. Biol. Chem.*, 1980, **255**, 2782.
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[798] N. V. Beaudette, N. L. Langerman, and R. L. Kisliuk, *Arch. Biochem. Biophys.*, 1980, **200**, 410.
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[800] G. F. Lata, H.-K. Hu, G. Bagshaw, and R. F. Tucker, *Arch. Biochem. Biophys.*, 1980, **199**, 220.
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[802] J. Wilting, W. F. van der Geisen, L. H. M. Janssen, M. M. Weideman, M. Otagiri, and J. H. Perrin, *J. Biol. Chem.*, 1980, **255**, 3032
[803] H. Bruderlein and J. Bernstein, *J. Biol. Chem.*, 1979, **254**, 11570.
[804] C. J. Bowmer and W. E. Lindup, *Biochem. Biophys. Acta*, 1980, **624**, 260.
[805] J. Berthou, A. Lifchitz, J. Saint-Blancard, and P. Jolles, *FEBS Lett.*, 1979, **108**, 10.
[806] A. L. Fink, R. Homer, and J. P. Weber, *Biochemistry*, 1980, **19**, 811.

in the latter study, as they were in the investigation of the interactions between lecithins and insulin by dielectric constant and spectral measurements.[807]

Theoretical work has also continued on acceptor–ligand binding primarily directed toward the interpretation of equilibrium binding results presented as Hill plots in terms of co-operativity effects;[808-811] the analyses were therefore restricted to systems which do not exhibit acceptor concentration-dependence. Moreover, a series of theoretical papers has dealt with kinetic considerations in irreversible enzyme inhibition,[812] in reversible ligand binding to a multivalent acceptor (including consideration of negatively co-operative effects),[813] and in relation to systems such as oxygen–haemoglobin[814] where positive co-operativity operates. In the latter connection, further experimental comment has been made on the role of diffusion in limiting the rate of gaseous ligand binding.[815]

Dissimilar Protein Interactions. In this area, a familiar group of interactions involves the specific complex formation between proteinases and proteinase inhibitors. Studies of such systems have ranged from the use of the Bowman–Birk inhibitor (mol. wt. 8000) as the immobilized reactant in the affinity chromatographic purification of trypsin, chymotrypsin, and kallikrein[816] to comparative kinetic studies on native and oxidized α_1-proteinase inhibitor with serine proteinases,[817] where it was noted that oxidation could decrease the effectiveness of the inhibitor in controlling proteolysis. Stopped-flow kinetic studies monitored with fluorescence techniques were conducted with systems of this type involving both pepsin[818] and subtilisin[819] inhibitors, while a thermodynamic study by calorimetry[820] revealed that conversion of the active-site serine side-chains of trypsin to dehydroalanine affected the pH-dependence of its heat of reaction with soybean inhibitor. Kahn *et al.*[821] measured by dilatometry the volume change accompanying the association of bovine trypsin with bovine pancreatic trypsin inhibitor: since no significant conformational changes were involved, the large and positive volume change was attributed to expulsion of water into the bulk solvent resulting from the burial of hydrophobic surfaces upon association. Also noteworthy in this area were the elegant crystallographic determinations[822, 823] of the structure of the potato inhibitor complex of carboxypeptidase A.

Heterogeneous associations leading to discrete complex formation have also been explored in relation to the operation and control of other diverse biochemical

[807] L. Galzigna, L. Garbin, F. Rigoni, and N. Siliprandi, *Mol. Cell. Biochem.*, 1980, **30**, 3.
[808] W. G. Bardsley, R. Woolfson, and R. M. W. Wood, *J. Theor. Biol.*, 1980, **85**, 45.
[809] W. G. Bardsley, R. Woolfson, and J.-P. Mazat, *J. Theor. Biol.*, 1980, **85**, 247.
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[814] P. E. Phillipson and J. Wyman, *Biopolymers*, 1980, **19**, 857.
[815] R. J. Morris and Q. H. Gibson, *J. Biol. Chem.*, 1980, **255**, 8050.
[816] K. D. Lin, D. L. Hwang, and D. E. Foard, *J. Chromatogr.*, 1980, **195**, 385.
[817] K. Beatty, J. Bieth, and J. Travis, *J. Biol. Chem.*, 1980, **255**, 3931.
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[820] B. Y. K. Yung and C. G. Trowbridge, *J. Biol. Chem.*, 1980, **255**, 9724.
[821] P. C. Kahn, J. M. Schwanwede, A. M. Ippolito, and B. Mihalyfi, *Biophys. J.*, 1980, **32**, 86.
[822] D. C. Rees and W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 277.
[823] D. C. Rees and W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4633.

processes, as the following systems exemplify: (a) enzyme–enzyme interactions in coupled metabolic systems have been detected between rabbit liver aldolase and fructose 1,6-bisphosphatase,[824] pyridoxal kinase and pyridoxine 5-P-oxidase,[825] and flavin reductase and immobilized bacterial luciferase;[826] (b) in the area of blood coagulation, interactions have been studied between thrombin and fibrin,[827] thrombin and antithrombin,[828] and between proteinase–HSA complexes and fibrinogen or fibrin;[829] (c) in relation to electron transport, we find accounts of mixed association between cytochromes *c* and b_5,[830] cytochrome *c* peroxidase and ferricytochrome *c*,[831] cytochrome b_5 and haemoglobin,[832] and cytochromes *c* of *Pseudomonas* AM 1 and methanol dehydrogenase;[833] (d) in connection with microtubule assembly, the effects of the enzymes lactoperoxidase[834] and tyrosine hydroxylase[835] on the assembly process have been elucidated, together with a quantitative analysis of tubulin–colchicine binding to microtubules,[836] the latter under conditions where co-polymerization is negligible. Interactions of particular proteins, such as concanavalin A[837–840] and calmodulin,[841] with a variety of other proteins have also continued to claim attention.

It is not possible to be encyclopaedic concerning the work on protein–protein interactions, as reference to other examples[842–846] will illustrate; but three other systems merit particular mention. First, sedimentation equilibrium studies[847] on mixtures of the calf thymus core histones in 2 M NaCl, pH 7.5, have revealed the operation of two reversible reactions governed by identical association constants,

$$\mathrm{H2AH2B} + (\mathrm{H3H4})_2 \leftrightarrows \mathrm{H2AH2B(H3H4)_2}$$
$$\mathrm{H2AH2B} + \mathrm{H2AH2B(H3H4)_2} \leftrightarrows (\mathrm{H2AH2BH3H4})_2$$

This example provides an excellent illustration of the formation of discrete species (ultimately, the symmetrical octamer) of finite size by mixed protein association.

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[843] C. A. Sayers and A. J. Barrett, *Biochem. J.*, 1980, **189**, 255.
[844] H. G. Mannherz, R. S. Goody, M. Konrad, and E. Nowak, *Eur. J. Biochem.*, 1980, **140**, 367.
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[847] J. E. Godfrey, T. H. Eickbush, and E. N. Moudrianakis, *Biochemistry*, 1980, **19**, 1339.

The second system leads us into greater complexity by detailing the mixed association in aqueous solution between two self-associating proteins, human apolipoprotein A-I and A-II; but again the claim was made that specific mixed oligomers are formed.[848] Thirdly, mention is made of a study[849] in which proteins bearing opposite net charge electrostatically interacted at low ionic strength. As detailed later, this situation parallels those mixed (cross-linking) associations which are termed indefinite in that an infinite array of complexes may coexist in equilibrium. The perturbation of such systems by adding polyethylene glycol,[849] leading to precipitation, is an important observation in the understanding of the interplay of excluded volume interactions and chemical equilibria.

Histone–Histone Interactions. Interactions in this important group of proteins have been selected for more detailed comment because this area of research is clearly expanding. Ring and Cole[850] reported on chemical cross-linking studies of H1 histone to the nucleosomal histones, while Carter *et al.*[851] used cross-linking techniques to form histone dimers and discussed their observations in terms of histone packing models. Allan *et al.*[852] described procedures for the dissociation of histones H1 and H5 from the native core complex in chicken reticulocyte chromatin and studied the properties of the native and depleted chromatin. Reconstitution of chromatin by reassociation of 'stripped' material with histone H1 was also reported.[853] In another cross-linking study,[854] it was demonstrated that histone H1 can be linked quantitatively to the octamer of mononucleosomes to yield a histone nonamer. Kawashima and Imahori[855] found that at pH 5 and low ionic strength heterotype histone tetramers (H2AH2BH3H4) could be formed, while at high ionic strength oligomers of this unit were produced. The pH-dependence of oligomer formation was found to be such that at pH 4—6 heterotype oligomers were formed, while homotype oligomers were found in the range pH 7—9. Stein and Page,[856] employing osmotic pressure and sedimentation methods, found that the situation at pH 8, 25 °C, in a solution of total core histones could be well described as a dimer + hexamer ⇆ octamer equilibrium. The dimer has the composition H2AH2B and the hexamer $H2AH2B(H3H4)_2$; this work, therefore, correlates with the reaction scheme presented above in relation to the studies by Godfrey *et al.*[847]

Cross-linking Interactions.—*Chemically Induced Cross-linking.* The use of bifunctional chemical reagents has been particularly prevalent in the study of membrane proteins, as exemplified by work on the cross-linking of bacteriorhodopsin by the probe *p*-azidophenylisothiocyanate.[857] This reagent interacted, *via* the phenylisothiocyanate moiety, specifically with a nucleophilic group of the protein and the cross-link was then induced by utilizing the property of photo-induced reactivity

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[857] H. Sigrist and P. Zahler, *FEBS Lett.*, 1980, **113**, 307.

of the arylazide moiety. Since both reactions occur with protein from within the lipid bilayer, the probe offers general promise as a hetero-bifunctional reagent.[857] In other examples of the use of chemical cross-linking reagents, we find the demonstration of polymer formation of the ATPase protein of rabbit skeletal sarcoplasmic reticulum induced by 1,5-difluoro-2,4-dinitrobenzene[858] and the formation of a mixed covalent complex between yeast cytochrome *c* peroxidase (a haemoprotein from the intermembranous space of mitochondria) with cytochrome *c* from horse heart.[859] The studies mentioned illustrate the diverse use of chemical cross-linking in providing models for enzyme–substrate systems,[859] in comparing properties of enzymes extracted from membranes and in the bound state,[858] and in attempting to identify the nearest neighbours of certain membrane proteins.[860] Moreover, information has been sought from this technique on subunit interactions, such as the influence of physiological ligands on the cross-linking of the α subunits of the Na^+,K^+–ATPase by *o*-phenanthroline and Cu^{II}.[861,862] Also noteworthy in the continuing elucidation of the role of α-lactalbumin in the lactose synthetase system are the resonance energy transfer measurements performed with a fluorescent derivative of α-lactalbumin covalently cross-linked to galactosyltransferase using dimethyl pimelimidate,[863] and the modification studies of Richardson and Brew.[864]

Self-association Leading to Networks. Cross-linking reactions arise with certain multifunctional protein systems in the absence of any artificial chemical modification. First considering reactions of this type between similar protein molecules, the indefinite self-association of lysozyme[768] provides a simple example of a bifunctional protein cross-linking in a head-to-tail fashion. More extensive three-dimensional polymer arrays arise when the associating monomer unit is multifunctional. Light-scattering work[865,866] on fibrin formation and assembly provides an example when viewed in conjunction with the affinity chromatography studies of Olexa and Budzynski.[867] Fibrinogen on activation with thrombin forms fibrin monomer, which associates to form protofibrils and by their lateral association the fibrin network is ultimately formed. Four different polymerization sites are involved, one available on fibrinogen without thrombin action, a second revealed by thrombin activation, a third formed by the alignment of two fibrin molecules, which is complementary to the fourth site also revealed by thrombin activation. Recent additional work on this subject includes the demonstration[868] that fibrin polymers produced in the earliest phase of the fibrinogen–fibrin conversion can be isolated and studied by gel chromatography at pH 7.4 and high molarity NaCl, and the theoretical work of Burchard and Müller[869] on the

[858] G. Bailin, *Biochim. Biophys. Acta*, 1980, **624**, 511.
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[867] S. A. Olexa and A. Z. Budzynski, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1374.
[868] G. F. Smith, *Biochem. J.*, 1980, **185**, 1.
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statistics of branched polymers composed of rod substructures, which accounts for a rod-like association of fibrin monomers, a lateral aggregation of rod-like entities and branching.

Collagen is of similar interest, both with regard to fibril-forming potential by intermolecular edge association[870] and its ability to undergo extensive cross-linking to give insoluble matrices in the lung[871] and in a range of mature tissues.[872]

Mixed Association in Network Formation. This type of interaction, where bi- or multi-functional proteins of dissimilar kind heterogeneously associate to form a network, is typified by the hypothesis that spectrin and actin are so involved in the formation of the cytoskeletal network believed to be the determinant of erythrocyte shape. Advances in this area have included the experimental demonstrations[873] that, regardless of their states of phosphorylation, spectrin dimer probably has one binding site for F-actin while spectrin tetramer has two, and that no interaction was detectable between spectrin and G-actin. It does appear then that the spectrin tetramer and polymerized form of actin participate in this formation of the erythrocyte cytoskeleton; but the reconstitution work of Fowler and Taylor[874] also implicates band 4.1 (a membrane component) in the optimum formation of an *in vitro* gel. Purified band 4.1 (and band 2.1) has been shown to associate with spectrin.[875]

Fibronectin, a high molecular weight glycoprotein, probably functions as an adhesive in its multiple interactions with collagen and other components in the formation of an extracellular fibrillar matrix present in layers of cultured cells. The cross-linking of fibronectin and fragments of it to collagen[876] and to *Staphylococcus aureus*[877] mediated by Factor XIIIa (plasma transglutaminase) has been explored to show, *inter alia*, that the glutaminyl residues of fibronectin involved in the cross-linking are distinct from the site which mediates strong binding to collagen.

Theoretical work has also proceeded on antigen–antibody induced particulate aggregation.[878] Cross-linking interactions between a bivalent antibody and a multivalent antigen form a set of reversible reactions leading to an array of complexes in solution comprising alternating reactant molecules. Analysis of these equilibria has led to binding equations that permit definitive comment on the forms of the family of intersecting binding curves, which arises when a series of antigen concentrations are explored.[879] The work commented on the fitting of binding results obtained with such systems by radioimmunoassay. The theory[879] has been extended and utilized to show how reversibly cross-linking systems may be quantitatively elucidated from sedimentation equilibrium, binding, and pre-

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cipitin studies.[880] The model system chosen, ovalbumin and lysozyme bearing opposite net charge at low ionic strength, was interpreted in terms of the operation of a spectrum of forces leading to a large array of complexes, formally similar to those encountered in more specific cross-linking interactions.[880]

Interplay of Ligand Binding with Protein Association.—*Theoretical Developments.* The problems of analysing quantitatively systems that involve a significant mutual interplay between ligand binding and protein self-association has been reviewed by Steiner.[881] Particular emphasis was placed on evaluation of the pertinent equilibrium constants from concentration profiles obtained in mass migration experiments, experimental studies with haemoglobin, phosphorylase *b*, and tubulin being used to illustrate the approach. In another theoretical investigation, Kurganov[882] has considered the effect of preferential, competitive binding of a substrate analogue on the rate of enzymic reaction observed with a reversibly dimerizing enzyme system. A plot of reaction velocity *versus* concentration of substrate analogue can exhibit a pronounced intermediate plateau, a maximum with preceding S-shape, or a maximum and a minimum, the precise form being a consequence of the values of the equilibrium constants as well as the enzyme and substrate concentrations used to generate the plot.

Whereas Woolfson and Bardsley[883] were concerned with the problem of ligand-linked changes in the association state of a single phase system, a situation treated earlier,[884] Wyman and co-workers[885, 886] have addressed the case in which a change of phase is involved. Application of the resultant polyphasic linkage relationships to experimental measurements of the dependence of the solubility of sickle-cell haemoglobin on oxygen partial pressure suggests the occurrence of some reversible oxygen binding to the gel state of the protein.

The use of the dependence of catalytic activity on enzyme concentration has been explored to characterize the reversible association of the enzyme in the special situation where the rate of interconversion between the monomeric and polymeric states is sufficiently slow that their proportions remain unperturbed by substrate binding.[887] Results obtained with two eukaryotic aminoacyl-tRNA synthetases were used to illustrate the approach.

Examples. A link between self-association and catalytic activity has been established[888] for *E. coli* carbamyl phosphate synthetase, which exists as a monomer–dimer system in the presence of phosphate. Addition of positively co-operative effectors (purine nucleotides) favours further association to form a tetrameric enzyme species, whereas addition of negative effectors (pyrimidine nucleotides) promotes the conversion into dimer. Phosphate-induced dimerization has also been reported[889] for bovine neurophysin, a similar displacement of the

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[881] R. F. Steiner, *Mol. Cell. Biochem.*, 1980, **31**, 5.
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[886] J. Wyman and S. J. Gill, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5239.
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[889] R. Tellam and D. J. Winzor, *Arch. Biochem. Biophys.*, 1980, **201**, 20.

monomer–dimer equilibrium being effected by oxytocin and vasopressin[890] because of their preferential interaction with the dimeric state of this hormone-carrier protein. Another reported example of preferential binding to a dimer is the co-enzyme-induced association of the flavoenzyme D-amino-acid oxidase.[891]

The substrate-induced dissociation of glycerol 3-phosphate dehydrogenase[892] and of glyceraldehyde 3-phosphate dehydrogenase[893] are recently reported examples of self-associating systems with preferential ligand binding to the smaller macromolecular state. In this connection it is also of interest to note two cases where ligand binding causes dissociation of a complex between dissimilar macromolecular reactants. Danishefsky and Pixley[894] have reported on the interference of heparin in the inhibition of thrombin by α_1-proteinase inhibitor, while Greene and Eisenberg[895] have investigated the dissociation of the actin–myosin subfragment-1 complex by compounds such as ADP and pyrophosphate.

In a study of the vinblastine-induced aggregation of calf brain tubulin[896] the dependence of the weight-average sedimentation coefficient upon total protein concentration was best described by invoking an isodesmic, indefinite self-association of a 1 : 1 complex of tubulin monomer and the antimitotic drug. The association of tubulin into microtubules has also been investigated with particular emphasis on the inhibitory effect of GDP.[897, 898]

Sickle-cell Haemoglobin. The association of deoxy-HbS continues to attract attention, the inhibitory effects of alkylureas and alkylamides,[899, 900] peptides,[901] and aliphatic alcohols[900] on the gelling process having been studied in the quest for antisickling agents. Of particular chemical interest in this respect was the development of a class of compounds based on bis(3,5-dibromosalicyl) fumarate, which is believed to interfere with the sickling process by virtue of specific HbS modification within the DPG-binding site.[902] From studies of mixtures of HbS and either normal (HbA) or foetal (HbF) haemoglobin it has been found that the gel phase contains both types of haemoglobin.[903, 904] However, the solubility of HbS is increased substantially by the presence of the second haemoglobin, especially HbF, an observation in keeping with its antisickling effect.[904] A report of acceleration of the rate of deoxy-HbS polymerization by erythrocyte membrane is of potential clinical interest in that it focuses attention on the need to consider

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[891] H. Tojo, K. Horiike, K. Shiga, Y. Nishina, H. Watari, and T. Tamano, *FEBS Lett.*, 1980, **114**, 4.
[892] J. Batke, G. Asboth, S. Lakatos, B. Schmitt, and R. Cohen, *Eur. J. Biochem.*, 1980, **107**, 389.
[893] M. Kálmán, M. Nuridsány, and J. Ovádi, *Biochim. Biophys. Acta*, 1980, **614**, 285.
[894] I. Danishefsky and R. Pixley, *Biochem. Biophys. Res. Commun.*, 1979, **91**, 862.
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[904] R. E. Benesch, R. Edalji, R. Benesch, and S. Kwong, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5130.

effects of this red blood cell component in the search for antisickling agents that might be of therapeutic value.[905]

Muscle Protein Interactions. The muscle protein system abounds with interactions between different protein species, and we select for comment the thin filament (actin)–thick filament (myosin) interaction. Greene and Eisenberg[895,906] have studied the binding to F-actin filaments of two soluble myosin fragments, namely subfragment S-1 and heavy meromyosin, which contain one and two globular heads respectively. On the basis of the stoicheiometry and strength of the interactions it was concluded that both myosin fragments bind to actin *via* the globular head, and that heavy meromyosin binds 600-fold more strongly because both of the heads can bind to the F-actin strand. By means of an enzymic technique to measure the number of myosin heads bound to actin, Cooke and Franks[907] also concluded that 94—100% of the myosin heads were bound to actin in rigor rabbit skeletal muscle. In an investigation concerned with the possible competitition between myosin and the tropomyosin–troponin complex for F-actin sites,[908] data were presented that suggest that in contracting muscle the tropomyosin–troponin may be capable of competing with myosin for actin sites, but that the interaction is relatively weak. This finding presumably relates to the observation[909] of positive co-operativity in the binding of S-1 to the F-actin–tropomyosin–troponin system (but not to F-actin), the effect of Ca^{II} (the stimulator of muscle contraction) being to decrease the degree of co-operativity; a theoretical model has been proposed[910] to account for these observations. From the physiological viewpoint this concept of interplay between myosin, tropomyosin–troponin, and F-actin to achieve the thin filament–thick filament overlap in muscle contraction may have to be modified further to incorporate the additional interaction of the tropomyosin–troponin complex with aldolase, for which physicochemical[911] and electron microscopic[912] evidence has been presented.

Acknowledgement.—The authors are indebted to Elisabeth A. Owen for assistance with the literature search.

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[909] L. E. Greene and E. Eisenberg, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2616.
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3
Peptide Synthesis

BY I. J. GALPIN

1 Introduction

The highlight of the year's literature has undoubtedly been the heroic two-man solution synthesis of ribonuclease A.[1] This achievement is particularly timely, insofar as the continued advances in molecular biology may stifle further striving towards the synthesis of natural proteins by the methods of organic chemistry. Peptide chemists with such ambitions may wish to consider turning their attention to oligonucleotide synthesis! On the other hand, synthesis of smaller peptides, and particularly of peptide and protein analogues, will more likely remain the province of the peptide chemist. The year's literature reveals continued activity, particularly in the hormone and neuropeptide fields. In many cases an interdisciplinary approach has allowed structure activity relationships to be investigated in considerable depth. Both solution synthesis and the solid phase method have been widely used, and in the latter considerable efforts have been made to improve the nature of the solid support and the character of the linkage to the growing peptide chain.

The Proceedings of the Sixteenth Japanese Peptide Symposium[2] have been published but no attempt is made in the present report to cover the contents in detail. The second volume of 'The Peptides, Analysis, Synthesis and Biology' has appeared.[3] This volume deals with special methods of peptide synthesis and approximately half the book is devoted to a masterly account of solid phase peptide synthesis by Barany and Merrifield. The liquid phase method, partial synthesis, and the four-component condensation are also described in this text. The use of organophosphorus reagents in peptide synthesis is described by Ramage in a more general text on the use of organophosphorus reagents in organic synthesis.[4] In the hormonal proteins and peptides series, Volume 7 describing hypothalamic hormones,[5] Volume 8 describing prolactin,[6] and Volume 9 describing techniques of protein chemistry[7] have appeared.

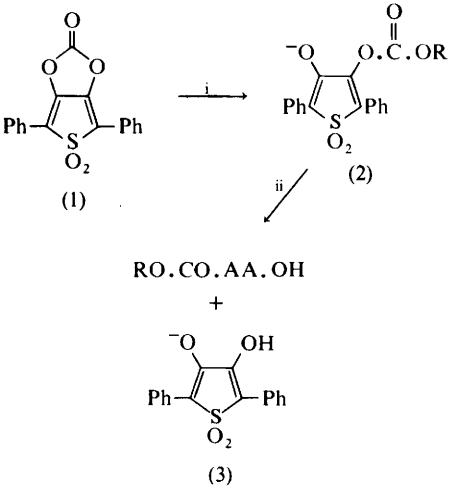
[1] H. Yajima and N. Fujii, *J. Chem. Soc., Chem. Commun.*, 1980, 115
[2] 'Peptide Chemistry 1978, proceedings of the 16th symposium on peptide chemistry, Kyushu', ed. N. Izumiya, Protein Research Foundation, Osaka, 1979.
[3] 'The Peptides, Analysis, Synthesis and Biology. Vol. 2, Special Methods of Peptide Synthesis', ed. E. Gross and J. Meienhofer, Academic Press, New York, 1980.
[4] 'Organophosphorus Reagents in Organic Synthesis', ed. J. I. G. Cadogan, 'Organophosphorus Reagents in the Synthesis of Peptides', R. Ramage, Academic Press, London, 1980.
[5] 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, London, 1979, Vol. 7.
[6] 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, London, 1980, Vol. 8.
[7] 'Hormonal Proteins and Peptides', ed. C. H. Li, Academic Press, London, 1980, Vol. 9.

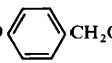
The overall layout of this chapter follows the established pattern of previous years' reports, although description of the syntheses achieved in Section 3 has been reduced. However, the majority of synthetic peptides and useful derivatives published are to be found in the appendices in Sections 4 and 5.

2 Methods

Protective Groups.—*Established Methods of Amino-group Protection.* Two reviews covering protecting groups have been published. The first discusses the use of a wide range of protecting groups that may be removed by photolysis.[8] The application of these groups in carbohydrate and nucleotide synthesis in addition to peptide synthesis is described. The second review[9] covers the use of halogenated protecting groups such as the trichloro-t-butoxycarbonyl (TCBOC) group which may be removed by the action of the supernucleophile cobalt(I) phthalocyanine.

The compound (1) (see Scheme 1) has been used to prepare the activated intermediates (2), which may be used to prepare the Boc- or Z-derivatives of an amino-acid.[10] In addition, coupling of the enolic component (3) with the *N*-protected amino-acid gave the corresponding active esters in high yield.



Reagents: i, Bu^{t}OH or Me-O-  -CH_2OH–pyridine; ii, amino-acid (AA)–tetramethylguanidine

Scheme 1

An improved method for the preparation of *N*-phenylisopropoxycarbonyl (Ppoc) amino-acids which utilizes the corresponding fluoroformate has been reported.[11] The fluoroformate, which is prepared by a reaction of fluorophosgene with 2-phenylpropan-2-ol, is rather labile at 0 °C undergoing total decomposition in approximately three days. Using dioxan as the preferred solvent, the fluoro-

[8] V. N. R. Pillai, *Synthesis*, 1981, 1.
[9] H. Eckert, W. Breuer, J. Geller, I. Lagerlund, M. Listl, D. Marguarding, S. Steuber, I. Ugi, S. Zahr, and H. V. Zychlinski, *Pure Appl. Chem.*, 1979, **51**, 1219.
[10] G. Schnorrenberg and W. Steglich, *Angew. Chem. Int. Ed. Engl.*, 1979, **18**, 307.
[11] H. Franzen and U. Ragnarsson, *Acta Chem. Scand., Ser. B*, 1980, **33**, 690

formate is treated with the amino-acid in the presence of tetramethylguanidine, the pH being maintained between 9.2 and 9.5. This method gave between 70 and 90% yield and is thus a considerable improvement over the use of the corresponding mixed phenyl carbonate. An improved procedure for the preparation of the Nefkens reagent (*N*-ethoxycarbonylphthalimide), which is used for the introduction of the phthaloyl *N*-protecting group,[12] claims to give a yield of 95% in contrast to the 43% described in the original work.

A detailed kinetic and mechanistic investigation of the thiolytic removal of the dithiosuccinoyl (Dts)[13] protecting group has been carried out.[14] The findings indicate that the fastest overall deprotection is observed for thiols of intermediate acidity (pK_a 8—9.5) using polar aprotic media having a high dielectric constant. A very wide variety of mono- and di-thiols were investigated for the reductive deprotection and in all cases removal of the Dts group and reaction of the intermediate carbamoyl disulphide were much more facile than was reduction of any acyclic aliphatic disulphides.

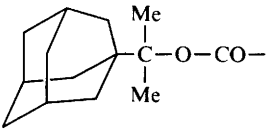
The use of the base labile 9-fluorenylmethoxyloxycarbonyl (Fmoc) protecting group has continued to increase. Fmoc derivatives are generally prepared by a reaction of the corresponding chloroformate with the amino-acid in alkaline solution,[15] and direct acid catalysed reaction with isobutylene has also now been used to prepare side-chain butylated derivatives.[15] This group, which is seeing increasing usage, is cleaved rapidly by the action of cyclic secondary amines such as piperidine, the rate limiting step being proton removal, which is dependent both on basic strength and steric interaction between the incoming base and the proton being removed. Recent applications include high yield solid phase syntheses of several gastrins[16] (the basic conditions used throughout the assembly minimize problems with acid-sensitive tryptophan residues) and a 39-residue fragment of cytochrome-c (see ref. 37). Polyamide supports were used in both the foregoing. Recently some concern has been shown that the incoming Fmoc amino-acid may be de-blocked by a resin bound amino-component.[17a] Removal of the Fmoc function from Fmoc.Leu.OH was studied using a glycyl polystyrene resin. In this experiment a $t_{\frac{1}{2}}$ of 300 h was found using 1.8 equivalents of di-isopropylethylamine. This increased to 1500 h in the presence of 1.2 equivalents of the base. These values are much greater than those observed in solution,[17a] where a $t_{\frac{1}{2}}$ of approximately 16 h has been found. The authors conclude that in solid phase synthesis using DCCI in the presence of hydroxybenzotriazole during the first 25 min of coupling the concentration of amine reduces very rapidly and thus should not cause a problem. The use of the Fmoc group in solution synthesis has now been adequately demonstrated by the synthesis of a VIP fragment.[17b] The synthesis, which used Fmoc amino-acid nitrophenyl esters for stepwise elongation of the fragment, used the addition of hydroxybenzotriazole to increase the reaction

[12] P. M. Worster, C. C. Leznoff, and C. R. McArthur, *J. Org. Chem.*, 1980, **45**, 174.
[13] G. Barany and R. B. Merrifield, *J. Am. Chem. Soc.*, 1977, **99**, 7363.
[14] G. Barany and R. B. Merrifield, *J. Am. Chem. Soc.*, 1980, **102**, 3084.
[15] C.-D. Chang, M. Waki, M. Ahmad, J. Meienhofer, E. O. Lundell, and J. D. Haug, *Int. J. Pept. Protein Res.*, 1980, **15**, 59.
[16] E. Brown, B. J. Williams, and R. C. Sheppard, *J. Chem. Soc., Chem. Comm.* 1980, 1093.
[17] (*a*) M. Bodanszky, S. S. Deshamane, and J. Martinez, *J. Org. Chem.*, 1979, **44**, 1622. (*b*) A. Bodanszky, M. Bodanszky, N. Chandramouli, J. Z. Kwei, J. Martinez, and J. C. Tolle, *J. Org. Chem.*, 1980, **45**, 72.

rate and therefore circumvent any cleavage of the Fmoc group by the incoming amino-component. The HOBt also served to minimize racemization. In contrast to the solid phase usage of this protecting group, diethylamine was preferred as the base for the removal of the protecting group rather than piperidine.

New Methods of Amino-group Protection. The simplest of all urethane protecting groups, the methoxycarbonyl (Moc) group, has been used in a synthesis of MSH release inhibiting factor.[18] Methanesulphonic acid at 5 °C for 5 h in the presence of dimethylsulphide was used for removal of the protecting group.[18] Although the Moc group has been successfully used in this synthesis it is not clear what real advantages the group has over the established methods of protection, although its stability to trifluoroacetic acid may be of some value.

1-(1-Adamantanyl)-1-methylethoxycarbonyl fluoride (Adpoc.F) has been used to introduce the Adpoc protecting group[19] (4). The fluoroformate, which is



(4)

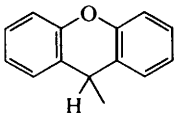
prepared from fluorophosgene in dichloromethane in the presence of triethylamine, is stable yet highly reactive. It may easily be used to introduce amino-protection and, similarly to Ppoc fluoroformate,[11] is considerably superior to the corresponding phenyl mixed carbonate, which in general gives rise to more by-products with lower yields. The group is cleaved by acids 10^3 times faster than the corresponding Boc derivative; derivatives have a shelf life of months at room temperature. The new derivatives are claimed to be particularly useful in the synthesis of tryptophanyl peptides.

The use of 4-methyl-thiophenoxycarbonyl as an *N*-protecting group in depsipeptide synthesis has been reported.[20] The group that is introduced using the corresponding chloroformate is unaffected by trifluoroacetic acid and is stable to base (pH 12); however, after oxidation with peracetic acid the group becomes base labile. Trimethylsilyloxycarbonyl derivatives of amino-acids have been prepared by treating amino-acid esters in polar solvents at between 60 and 80 °C with hexamethyldisilazane and carbon dioxide.[21] Thermolysis of these derivatives gives the TMS derivatives and treatment with thionyl chloride gives the isothiocyanato amino-acid. Although the derivatives appear to be stable, distillable liquids, their application to synthesis is not described.

The 9-xanthyl (5) protecting group has been utilized for the blocking of the nitrogen function of *N*-carboxyanhydrides.[22] These derivatives are prepared by a reaction of the amino-acid NCA with xanthydrol using hot toluene or acetic

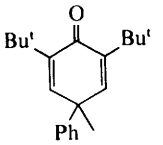
[18] H. Irie, H. Nakanishi, N. Fujii, Y. Mizuno, T. Fushima, S. Funakoshi, and H. Yajima, *Chem. Lett.*, 1980, 705.
[19] H. Kalbacher and W. Voelter, *J. Chem. Soc., Chem. Commun.*, 1980, 1265.
[20] H. Kunz and K. Lorenz, *Angew. Chem. Int. Ed. Engl.*, 1980, **19**, 932.
[21] V. P. Kozyukov, N. V. Mironova, and V. F. Mironov, *J. Gen. Chem. USSR*, 1980, **49**, 2246.
[22] J. Halstroem, K. Brunfeldt, and K. Kovacs, *Acta Chem. Scand, Ser. B*, 1980, **33**, 685.

anhydride as solvent. The derivatives, which are claimed to be isolated in high yield, are optically pure, giving low racemization during coupling. Protection is removed by treatment with ethanol giving the free amino-group and the corresponding xanthyl ether, the method being potentially useful as no alkylating cations are apparently generated on deprotection.



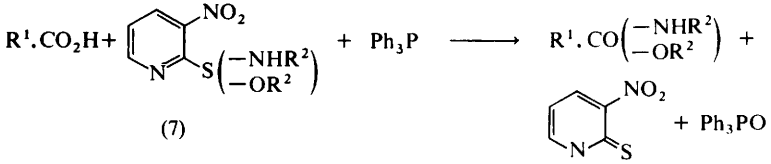
(5)

Anodic oxidation of 3,5-di-t-butyl-4-hydroxybiphenyl in the presence of amino-acid esters has been used to introduce the 3,5-di-t-butyl-4-oxo-1-phenyl-2,5-hexadienyl protecting group (6).[23] The group, which is removed by treatment with 50% trifluoroacetic acid in dichloromethane for 15 min at 25 °C or by hydrogenolysis over palladium, had been used for the synthesis of a few small peptides.



(6)

The 3-nitro-2-pyridinesulphenyl group (Npys) has been shown to be useful both for amino-protection and activation.[24] The group is introduced by the sulphenyl chloride giving the Npys derivatives as crystalline solids. The group may be simply removed by treatment with 0.1—0.2M HCl in dioxan, being resistant to both TFA and 88% formic acid. In addition, selective removal may be effected by treatment with triphenylphosphine or 2-pyridinethiol-1-oxide. Neither of these conditions of removal was found to affect any of the standard protecting groups. When used as an activated intermediate, the sulphenamide or sulphenate (7) may be condensed



(7)

Scheme 2

with a carboxylic acid in the presence of triphenylphosphine, the resulting amide or ester being produced by an oxidation–reduction mechanism (see Scheme 2). The group has been used in both solution and solid phase peptide synthesis, being cleanly removable in the presence of both Boc and Bpoc protecting groups. It is interesting that soft bases such as chloride are effective for the removal of the

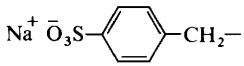
[23] M. H. Khalifa, G. Jung, and A. Rieker, *Angew. Chem. Int. Ed. Engl.*, 1980, **19**, 712.
[24] R. Matsueda and R. Walter, *Int. J. Pept. Protein Res.*, 1980, **16**, 392.

protecting group, but that the trifluoroacetate anion is ineffective; protonation of the pyridine nitrogen also enhances the stability of the derivative. In DCCI coupling of the Npys amino-acids 2-pyridinethiol-l-oxide was found to be particularly efficient as a catalyst, being superior to both HOBt and HONSu. In the former case less than 0.8% racemization was observed on isoleucine to glycine coupling. Use of the group has also been extended to side-chain protection.

Protection of Terminal Carboxy-groups. Very few papers describing carboxy protection have appeared. A general review covering a wide range of carboxyl protecting groups has been published [25] as have methods of improving ester synthesis when using dicyclohexylcarbodi-imide.[26] The improvements hinge on the use of pyridine or *p*-toluenesulphonic acid as catalysts in the esterification reaction.

The use of 2,2,2-trichloroethyl esters for carboxyl protection in the synthesis of several methionine-containing peptides has been demonstrated.[27] This protecting group was used in conjunction with trifluoroacetyl for *N*-α-amino-protection, the ester function being removed at intermediate stages by treatment with zinc. Final deprotection using sodium borohydride removed both the trifluoroacetyl and the 2,2,2-trichloroethyl protecting groups.

The use of the 4-sulphobenzyl protecting group (8) [28] has been proposed. The group is more stable to acid that simple benzylic esters and may be removed by



(8)

catalytic hydrogenolysis or by alkaline hydrolysis. The stability of this group to HBr in acetic acid allows it to be used in conjunction with benzyloxycarbonyl for *N*-α-amino-protection. The sulphonic acid moiety permits purification of intermediates by ion exchange chromatography whilst maintaining protection of the *C*-terminus.

Side-chain Protection. The utility of the piperidino-oxycarbonyl protecting group for protection of the N^{ε}-amino-function of lysine has been demonstrated in a synthesis of the 23–40 portion of the insulin B chain.[29] The synthesis, which utilized the picolyl ester for *C*-terminal carboxyl protection, used the Pipoc group for lysine side-chain protection, these two groups facilitating purification based on the 'basic handle' approach. A preferred route to the lysine derivative is described that used piperidine-2,4,5-trichlorophenylcarbonate for the introduction of this basic protecting group. The group was selectively removed at the end of the synthesis by treatment with dithionite.

Protection of hydroxy- and amino-groups based on the 4-dimethylcarbamoylbenzyl group has been described.[30] Serine and tyrosine hydroxy-functions were

[25] E. Haslam, *Tetrahedron*, 1980, **36**, 2409.
[26] K. Holmberg and B. Hansen, *Acta Chem. Scand., Ser. B*, 1979, **33**, 410.
[27] J. F. Carson, *Synthesis*, 1980, 730.
[28] A. Hubbuch, R. Bindewald, J. Foehles, V. Naithani, and H. Zahn, *Angew. Chem. Int. Ed. Engl.*, 1980, **92**, 394.
[29] J. G. Warnke and G. T. Young, *J. Chem. Soc., Perkin 1*, 1980, 2797.
[30] V. S. Chauhan, S. J. Ratcliffe, and G. T. Young, *Int. J. Pept. Protein Res.*, 1980, **15**, 96.

both protected as the 4-substituted benzyl derivative, and the lysine ε-amino-group was protected as the 4-substituted benzyloxycarbonyl derivative. The 4-dimethylcarbamoylbenzyl based protection is more stable to trifluoroacetic acid than the corresponding benzyl-substituted derivatives. This increased acid stability is due to the fact that the amide becomes protonated, thus hindering reaction with a second proton. The group is stable to hydroxide ion and hydrazine but it is slowly cleaved by treatment with HBr in acetic acid. Clean removal is best achieved by hydrogenolysis using a palladium catalyst. It was hoped that in this work the introduction of this 4-substituent would lead to increased solubility; however, no such increase in solubility was observed. The 3-nitro-2-pyridine sulphenyl group (Npys)[24] has also been used for the protection of serine, threonine, lysine, and cysteine (see p. 253).

The *in situ* sulphonation of hydroxy-amino-acids in peptides has been examined.[31] Treatment of peptides with chlorosulphonic acid in trifluoroacetic acid *O*-sulphonates serine and theonine but at the same time sulphonates the aromatic rings of tyrosine and tryptophan. The use of sulphuric acid in trifluoroacetic acid gives a slower reaction, but satisfactory *O*-sulphonation of serine, threonine, and tyrosine occurs without modification of tryptophan or ring substitution in tyrosine. Under the conditions described all other amino-acids are stable.

A detailed investigation of the iodine oxidation of *S*-trityl cysteine- and *S*-acetamidomethyl cysteine-containing peptides has been made.[32] It was found that the rates of iodine oxidation of these two groups are highly solvent dependent and that using suitable conditions the *S*-trityl group may be selectively oxidized to the disulphide in the presence of *S*-acetamidomethyl cysteine. Two groups of solvents have emerged: group 1 contains methanol, acetic acid, dioxan, and mixtures of these with water and group 2 contains chloroform, dichloromethane, trifluoroethanol, and hexafluoropropan-2-ol. In solvents of the first group simultaneous oxidation of *S*-Acm and *S*-trityl groups takes place, whereas in solvents of group 2 *S*-trityl is selectively converted into the disulphide and *S*-Acm is unaffected. In solvents of group 1 the rate difference of iodine oxidation between the two groups in question is relatively small, whereas in group 2 solvents the rate difference is very large indeed, with the oxidation of *S*-trityl always being the most rapid. DMF appears to be an exceptional solvent in which the rates of iodine oxidation of these two groups are approximately equal. Strangely, occasionally Acm-derivatives may be found to oxidize more rapidly than trityl, particularly in the presence of added water. The iodine oxidation gives rise to side products with hydroxy-free tyrosine, but with t-*butyl*-tyrosine no side-reactions are observed. Similarly, histidine is iodinated when present as the free base but not in its protonated form. Tryptophan will only survive short reaction times otherwise unstable iodinated products are obtained. In addition, electrophilic substitution *via* the sulphenyl iodide may occur.[33] This rigorous study of iodine oxidation used model peptides, 1—14

[31] A. Previero, J.-C. Cavadore, J. Torreilles, and M.-A. Coletti-Previero, *Biochem. Biophys. Acta*, 1979, **581**, 276.

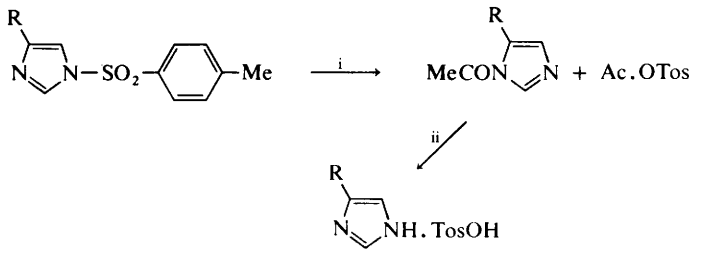
[32] B. Kamber, A. Hartman, K. Eisler, B. Riniker, H. Rink, P. Sieber, and W. Rittel, *Helv. Chim. Acta*, 1980, **63**, 899.

[33] P. Sieber, B. Kamber, B. Riniker, and W. Rittel, *Helv. Chim. Acta*, 1980, **63**, 2358.

somatostatin and various fragments of insulin, as test cases. In many cases hexafluoropropan-2-ol mixed with dichloromethane was found to be the best solvent. In the somatostatin synthesis [33] it was found that ring closure between the cysteine residue and the tryptophan residue may be achieved, giving a thioether linkage *via* the sulphenyl iodide. The largest amount of this product was observed when using 90% aqueous trifluoroethanol as the solvent. Model peptides showed that in this cyclization process the ring size was crucial and under normal conditions, in which the disulphide is very rapidly formed, no thioether formation takes place.

For some time the oxidation of *S*-acetamidomethylcysteine to the corresponding sulphoxide has given cause for concern and a detailed study of the corresponding sulphoxide has now been made.[34] The sulphoxide was obtained by sodium perborate oxidation of Boc.Cys(Acm).OH. The resulting water soluble sulphoxide was obtained as a mixture of diastereoisomers. Attempted deprotection with hydrogen fluoride or methane sulphonic acid in the presence of anisole lead to the production of *p*-methoxyphenylcysteine. Treatment of the sulphoxide with mercuric acetate or iodine did not remove the Acm protecting group. The presence of the sulphoxide in a peptide may be readily detected using amino-acid analysis in the presence of phenol. It appears to be very difficult to convert the sulphoxide back into the free thiol once it has been formed. The xanhydryl protecting group (5)[22] (p. 252) has also been used as a protecting group for cysteine and the amide function of asparagine and glutamine.

Protection of the imidazole function of histidine has again received attention. It has recently been shown [35] that the N^{im}-tosyl protecting group may be introduced by the action of *p*-toluenesulphonyl chloride in the presence of sodium carbonate in acetonitrile, giving a 90% yield of the derivative. This group is known to be removed by treatment with hydroxybenzotriazole, hydrogen fluoride, or sodium in liquid ammonia. However, it has recently been found [35] that it is also easily removed by the action of carboxylic acid anhydrides in the presence of pyridine. Acetic anhydride removed the protection in 3—5 h and acetic–formic mixed anhydride in 2 h, trifluoroacetic anhydride being the most effective, removing the protecting group in approximately 30 min. The cleavage proceeds by the route shown in Scheme 3, where it may be seen that the N^{im}-tosyl group is replaced by an



Reagents: i, Ac_2O–pyridine; ii, H_2O

Scheme 3

[34] H. Yajima, K. Akaji, S. Funakoshi, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, 1980, **28**, 1942.
[35] J. M. Van Der Eijk, R. J. M. Nolte, and J. W. Zwikker, *J. Org. Chem.*, 1980, **45**, 547.

acetyl group, which is subsequently hydrolysed. A related study examining both the *p*-toluenesulphonyl and the *p*-methoxybenzenesulphonyl (MBS) protecting groups for the N^{im} function of histidine has been made.[36] It was found that the MBS function was more stable to acid than the corresponding tosyl function. The MBS group is stable to trifluoroacetic acid in the presence of anisole; however, it is readily cleaved in 1 h by the action of trifluoroacetic acid and dimethylsulphide. Other thiols such as mercaptoethanol and ethane dithiol also appear to be effective. Usage of the group was exemplified by the synthesis of LHRH.

A variety of sulphonyl-based protecting groups have been used for the masking of the arginine–guanidine function. The MBS group was used in a trial solid phase assembly of a cytochrome-c fragment[37] [better results were later obtained using bis(adamantyloxycarbonyl)arginine together with other changes], and the mesitylene-2-sulphonyl arginine (MPS) group was applied in the synthesis of kyotorphin.[38] It has been demonstrated[39] that the MPS group may be removed by treatment with a mixture of thianisole, trifluoromethanesulphonic acid, and trifluoroacetic acid, removal being complete in 90 min at 25 °C. However, if anisole replaces thioanisole, then the cleavage time increases dramatically to 23 h. The tosyl group may also be removed from arginine under similar conditions as may the 2,6-dichlorobenzyl group from tyrosine; in this case no $O \rightarrow C$ rearrangement is observed. The 4-methoxy-2,6-dimethylbenzenesulphonyl (MDS)[40] protecting group has also been used for guanidino-protection. Deprotection may be achieved with methane sulphonic acid although it gives rise to an increase in succinimide formation from Asp and Asn peptides. The group is completely stable to 1M HCl in dioxan but may be removed by trifluoroacetic acid in the presence of thioanisole, requiring 1—2 h at 50 °C or between 5 and 8 h at room temperature. It appears from several of the foregoing papers that 'push–pull' deprotection using a strong acid in the presence of thioanisole may be widely applicable.

Formation of the Peptide Bond.—Interest in the mechanism of di-imide activation of carboxylic acids has continued. In separate studies, the presence of intermediate *O*-acylisourea has been inferred both kinetically[41] and spectroscopically.[42a] Conclusions drawn in the latter case have been challenged[42b] and withdrawn (J. H. Jones, personal communication). In the kinetic study[41] carried out in aqueous solution using water-soluble carbodi-imide, observation of general acid catalysis is held to exclude a stepwise mechanism for activation (successive protonation and carboxylate addition) in favour of concerted addition.

Owing to the difficulties encountered in the removal of dicyclohexylurea from reaction products, one group[43] has looked at the use of di-imides that produce a soluble urea after reaction. Studies showed that di-imides with structure (9) gave

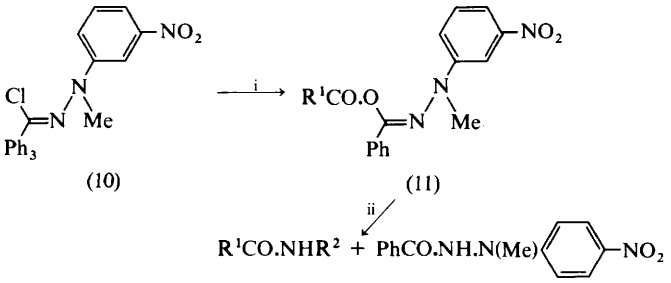
[36] K. Kitagawa, K. Kitade, Y. Kiso, T. Akita, S. Funakoshi, N. Fujii, and H. Yajima, *Chem. Pharm. Bull.*, 1980, **28**, 926.
[37] E. Atherton, V. Wooley, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1980, 970.
[38] H. Yajima, H. Ogawa, H. Ueda, and H. Takagi, *Chem. Pharm. Bull.*, 1980, **28**, 1935.
[39] Y. Kiso, M. Satomi, K. Ukawa, and T. Akita, *J. Chem. Soc., Chem. Commun.*, 1980, 1063.
[40] M. Fujino, O. Nishimura, M. Wakimasu, and C. Kitada, *J. Chem. Soc., Chem. Commun.*, 1980, 668.
[41] I. T. Ibrahim and A. Williams, *J. Chem. Soc., Chem. Commun.*, 1980, 25.
[42] (*a*) M. S. Bates, J. H. Jones, and M. J. Witty, *J. Chem. Soc., Chem. Commun.*, 1980, 773; (*b*) N. L. Benoiton and F. M. F. Chen, *J. Chem. Soc., Chem. Commun.*, 1981, 543.
[43] A. Tartar and J.-C. Gesquiere, *J. Org. Chem.*, 1980, **44**, 5000.

$$R^1-N=C=N-R^2$$

(9) R^1 = Ph, R^2 = Et
Bzl, Pr^i
Bzl, Et

rise to ureas that were much more soluble in dichloromethane; it was also claimed that *N*-acylurea formation and racemization were both reduced.

Imidoylhalides (10) of the type shown in Scheme 4 may be used as coupling reagents.[44] Reaction with the incoming carboxyl component gives the *O*-acylisoamide (11) which then reacts rapidly in the second step with the amino-component. As reaction with carboxylate is much more rapid than with amines no

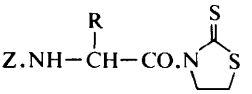


Reagents: i, $R^1CO_2^-$; ii, R^2NH_2

Scheme 4

amidine formation apparently takes place and thus a 'one-pot' coupling may be achieved. Racemization appears to be pH dependent and using the Anderson test it was shown that 21% racemization occurred at pH 9.3; 2.5% racemization was observed at pH 7.8 and this reduced to 1% when the pH was lowered to 7.2. If the phenyl group of the imidoylhalide (10) is replaced by t-butyl no detectable racemization is observed at pH 9.5. The use of iminodithiocarbonates in peptide bond formation has also been investigated,[45] although at present the method does not seem practical as coupling is carried out at 110 °C and gives rise to 9—10% of diastereoisomers.

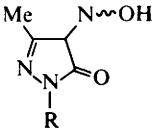
3-Acylthiazoline-2-thione may be used to form activated compounds of the type shown (12) by coupling with the *N*-protected amino-acid using DCCI.[46] These yellow activated intermediates readily undergo reaction with an amino-component to give a peptide bond; the reaction may be monitored by the disappearance of the yellow coloration of the intermediate.



(12)

[44] A. F. Hegarty and D. G. McCarthy, *J. Am. Chem. Soc.*, 1980, **102**, 4537.
[45] H. Berndt, *Tetrahedron Lett.*, 1980, **21**, 3265.
[46] H. Yajima, K. Akaji, Y. Hirota, and N. Fujii, *Chem. Pharm. Bull.*, 1980, **28**, 3140.

4-Oximino-pyrazol-5-ones (13) may be used for the preparation of active esters using the *N*-protected amino-acid and DCCI.[47] The pyrazalones may be used as a mixture of *E* and *Z* isomers and it is claimed that in coupling the active esters do not give rise to racemization in common solvents even when an excess of triethylamine is present. Comparable yields are found with both the methyl and phenyl compounds; however, the methyl case is probably slightly superior.



(13) R = Ph or Me

Hexafluoropropan-2-ol may also be converted to active esters using the DCCI method.[48] These esters are 10^3 times less reactive than the corresponding *p*-nitrophenyl esters. In fact hexafluoropropyl esters may be obtained from more active esters by base catalysed transesterification. With hexafluoropropan-2-ol as solvent, coupling was found to be much slower than in dimethoxyethane or dimethylformamide. The esters are not, however, formed by transesterification of methyl esters, which may be particularly important, as the related alcohol, trifluoroethanol, is frequently used as a solvent in peptide synthesis. A small peptide was assembled by using benzyloxycarbonyl for amino-group protection; the ester grouping was stable under the conditions of hydrogenolysis used to remove the *Z* function. In a more general context, it is also interesting to note that both hexafluoropropan-2-ol and trifluoroethanol react with DCCI giving *NN*-dicyclohexyl-*O*-alkylisoureas, thus confirming the widely held belief that these solvents are not suitable as media for DCCI couplings. A so-called thermal synthesis of a pentapeptide[49] has been carried out by heating a protected dipeptide pentachlorophenyl ester with a tripeptide methyl ester, trifluoroacetate. The reaction was carried out at between 100 °C and 105 °C at a pressure between 10^{-2} and 10^{-3} torr, using no solvents. The reaction appeared to give no racemization, whereas a matrix-mediated reaction using diatomaceous earth gave 5.5% racemization and was incomplete after 37 h.

The influence of the nature of the activating group on the rates of *O*- and *N*-acylation of hydroxyamino-acids has again been studied.[50] The maximum rate of both *O*- and *N*-acylation was observed using pentafluorophenyl active esters, the slowest reaction being achieved with hydroxybenzotriazole esters. Also it was found that the hydroxy-function of tyrosine was acylated more readily than that of serine or threonine. A synthesis of somatostatin, which was carried out in the absence of hydroxy-group protection,[51] produced a satisfactory yield of the

[47] C. B. Vicentini, A. C. Veronese, P. Giori, P. G. Baraldi, and M. Guarneri, *Int. J. Pept. Protein Res.*, 1980, **16**, 48.
[48] L. S. Trzupek, A. Go, and K. D. Kopple, *J. Org. Chem.*, 1980, **44**, 4577.
[49] G. Matoni and H. Berndt, *Tetrahedron Lett.*, 1980, **21**, 37.
[50] S. K. Girin and Y. P. Shvachkin, *J. Gen. Chem. USSR*, 1980, **49**, 395.
[51] Y. P. Shvachkin, S. K. Girin, A. P. Smirnova, A. A. Shishkina, and N. M. Ermak, *J. Gen. Chem. USSR*, 1980, **49**, 1455.

hormone. Threonine-10 and -11 and serine-13 were unprotected and no *O*-acylation was observed with pentafluorophenyl active esters providing that the addition of the base was carefully controlled throughout the coupling.

In a study of the Curtius rearrangement of acylamino-acid and peptide azides,[52] it was shown that the rate of isocyanate formation was highly dependent on the amino-acid involved. The study by infrared spectroscopy showed that the isocyanates were decomposed by triethylamine, triethylamine hydrochloride, or hydroxybenzotriazole and that the half life at 25 °C was very variable (general range 22—100 min, histidine 115 min, proline 420 min, and glycine 610 min).

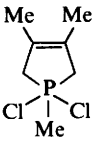
N-Carboxyanhydrides have been used on two occasions in synthesis. The first approach used 9-xanthyl amino-acid *N*-carboxyanhydrides and has been discussed above. The second procedure used the *N*-carboxyanhydride directly,[53] being coupled with an amino-acid in the presence of potassium borate at a pH of 10.2 at 0 °C. With excess NCA polymer is readily produced, but an excess of the amino-acid results in the production of the dipeptide in yields of between 70 and 90%.

A variety of compounds containing phosphorus have been used as coupling reagents. The known diphenylphosphorazidate (DPPA) (14) and diethylphosphorocyanidate (DEPC) (15) have been applied in both solid phase[54] and

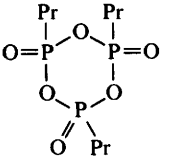
$(PhO)_2.PO.N_3$ (14) $(EtO)_2.PO.CN$ (15)

solution synthesis.[55] It was claimed that, particularly in solid phase synthesis, these compounds showed greater reactivity than DCCI when DMF was used as the solvent.

1,3,4-Trimethyl-Δ^3-phospholene 1,1-dichloride (16) has been applied as a fragment condensing reagent in the liquid phase synthesis of leucine enkephalin.[56] Protection of tyrosine was not required. The use of alkyl phosphonic acid anhydrides (17) as coupling reagents[57] has been demonstrated. These compounds



(16)



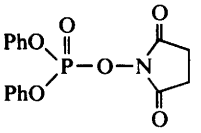
(17)

are simply prepared and are stable; they also show a low tendency to racemization in couplings when a two-fold excess is used in the presence of *N*-ethylmorpholine as base at between 0 °C and 5 °C. The Anderson test showed no racemization and several small peptides have been prepared.

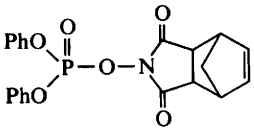
Adducts formed between diphenylphosphochloridate and *N*-hydroxy-compounds in the presence of base have been prepared as coupling reagents.

[52] Y. Okada, Y. Tsuda, and M. Yagyu, *Chem. Pharm. Bull.*, 1980, **28**, 2254.
[53] K. Kircher, H. Berndt, and H. Zahn, *Annalen*, 1980, 275.
[54] N. Ikota, T. Shiori, and S. Yamada, *Chem. Pharm. Bull.*, 1980, **28**, 3064.
[55] N. Ikota, T. Shiori, S. Yamada, and S. Tachibana, *Chem. Pharm. Bull.*, 1980, **28**, 3347.
[56] E. Vilkas, M. Vilkas, and J. Sainton, *Int. J. Pept. Protein Res.*, 1980, **15**, 29.
[57] H. Wissmann and H.-J. Kleiner, *Angew. Chem. Int. Ed. Engl.*, 1980, **92**, 129.

Compound (18) was prepared by reaction of the phosphochloridate with hydroxysuccinimide in 60—80% yield.[58] The reagent may be used for preparation of *N*-hydroxysuccinimide active esters in high yield; alternatively it may be used directly in 'one pot' coupling reactions using triethylamine or *N*-methylmorpholine as base. The compound (19)[59] formed by reaction of the phosphochloridate with *N*-hydroxynorborn-5-ene 2,3-dicarboximide was also prepared. This reagent may be



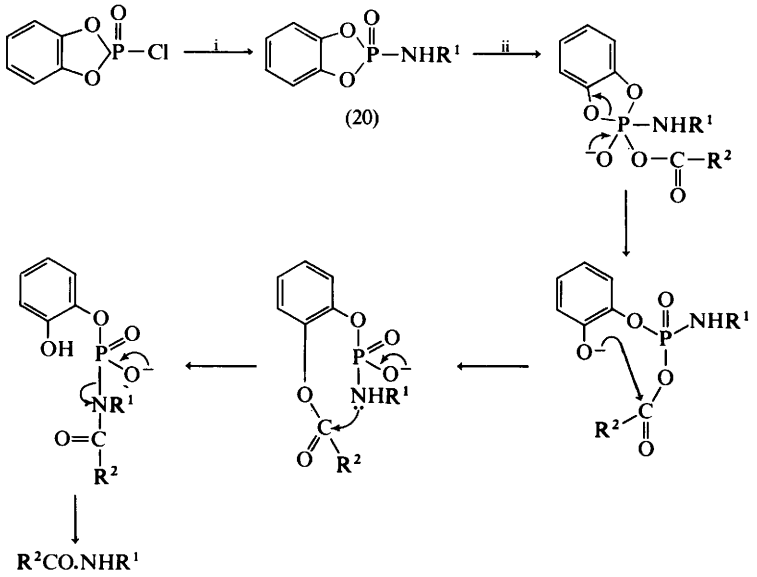
(18)



(19)

used for preactivation, for direct one pot reactions, and for the formation of the corresponding active ester. Racemization, 2—3%, was observed depending on the solvent employed.

The reaction of tetra-alkylammoniumcarboxylates with cyclic phosphoramidates (20) has been studied.[60] It is proposed that in this case the amide bond is formed subsequently to amine capture, the actual amide bond formation being a result of the cascade of intramolecular acyl transfers shown in Scheme 5. The method was used to prepare simple di- and tripeptides. The Anderson test



Reagents: i, R^1NH_2; ii, $R^2CO_2^-$ Bu_4N^+

Scheme 5

[58] H. Ogura, S. Nagai, and K. Takeda, *Tetrahedron Lett.*, 1980, **21**, 1467.

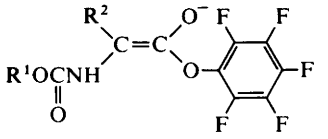
[59] Y. Kiso, T. Miyazaki, M. Satomi, H. Hiraiwa, and T. Akita, *J. Chem. Soc., Chem. Commun.*, 1980, 1029.

[60] M. Wakselman and F. Acher, *Tetrahedron Lett.*, 1980, **21**, 2705.

indicated 0.85% racemization when triethylammonium salts were used, but no racemization with tetrabutylammonium salts.

The four-component condensation method has been used in the preparation of a cyclic eledoisin analogue and *cyclo*(-Gly-)$_6$.[61] A development of the method using 9-formylfluorene as the aldehyde component[62] has been used with the fluorenyl group finally cleaved with ammonia to give the peptide product.

Racemization.—A kinetic study of the racemization associated with pentachlorophenyl, *o*-nitrophenyl, pentafluorophenyl, and hydroxy-succinimide esters has been made.[63] The study utilized Z- and Boc-methionine as the carboxy-component and valine methyl ester hydrochloride as amino-components; THF was used as the solvent with triethylamine as the base. When benzyloxycarbonyl is used for amino-protection, racemization is twice as rapid as when protection is by the t-butoxycarbonyl group. Also racemization of glycylmethionine dipeptides was more rapid than for the protected amino-acid by a factor of approximately 100. In general, racemization parallels the pK_a value of phenol components and proceeds *via* the oxazolone mechanism. Pentafluorophenyl esters, however, seem to be atypical in that they racemize *via* the enolate (21). It is proposed that as the



(21)

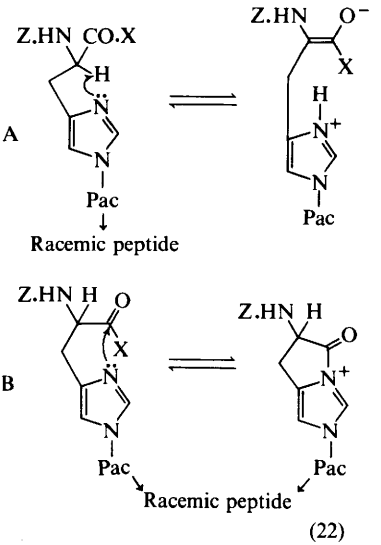
pK_a's of pentafluorophenol and pentachlorophenol are quite similar (5.5 and 5.2 respectively) that solvation is more important than electron withdrawing effects in determining the extent of racemization.

The racemization of histidine derivatives in peptide synthesis has also been investigated.[64] Earlier, it had been found that *Z*-histidine with N^{π}-phenacyl protection of the imidazole ring gave no racemization but that the N^{τ}-substituted phenacyl derivative gave considerable racemization. Two possible mechanisms shown in Scheme 6 were proposed: A, involving base catalysed intramolecular catalysis of racemization, and B, the formation of optically labile heterocyclic intermediate (22). Evidence is now presented favouring mechanism A.

The influence of relative configuration of amino-acids on the degree of racemization has been studied using tritium labelled compounds[65] in DCCI coupling reactions. A new range of additives for the suppression of racemization during DCCI coupling has also been investigated.[66]

In a synthesis of human beta-endorphin[67] the formation of D-*allo*-isoleucine and D-*allo*-threonine was used to check the extent of racemization on fragment

[61] A. Failli, H. Immer, and M. Goetz, *Can. J. Chem.*, 1980, **57**, 3257.
[62] C. F. Hoyng and A. D. Patel, *Tetrahedron Lett.*, 1980, 4795.
[63] J. Kovacs, E. M. Holleran, and K. Y. Hui, *J. Org. Chem.*, 1980, **45**, 1060.
[64] J. H. Jones, W. I. Ramage, and M. J. Witty, *Int. J. Pept. Protein Res.*, 1980, **15**, 301.
[65] A. Arendt, A. M. Kolodziejczyk, and T. Sokolowska, *Pol. J. Chem.*, 1979, **53**, 2209.
[66] J. Przybylski, *Pol. J. Chem.*, 1979, **53**, 2627.
[67] O. Nishimura, S. Shinagawa, and M. Fujino, *J. Chem. Res. (S)*, 1979, 352.



(22)

Scheme 6

coupling. The fragments which were assembled by HONB active esters were coupled by the DCCI–HONB method using 2 or 4 equivalents of the additive. Approximately 3% D-*allo*-isoleucine and between 2 and 4% D-*allo*-threonine were observed.

A convenient test for racemization has been developed [68] in which diastereoisomeric trifluoroacetyltetrapeptide methyl esters formed by 2 + 2 coupling are separated on g.l.c. (Table 1). The values shown are generally in accordance with

Table 1 *Racemization observed on coupling* Tfa.Pro.Val.OH *to* H.Pro.Val.OMe

Method	*Racemization observed*/%
DCCI	23—45
DCCI–HOSu–THF	5.2±0.8
DCCI–HOBt	8.6±3.4
DCCI–HONb	6.3±2.7
Mixed anhydride–TEA	0.9±0.1
Azide	1.0±1.0
DPPA	4.5±0.1
DEPC	2.7±0.5

expectation, apart from the case using a mixed anhydride in the presence of triethylamine; this appears to be rather lower than would normally be anticipated. For other condensations, considerable variation in racemization for a given method is observed when the carboxy-terminal amino-acid of the carboxy-component is varied (Table 2).

Detection of racemization by monitoring the methyl ester signal of a diastereoisomeric pair in the n.m.r. has been used as a laboratory experiment for

[68] I. Tomida and N. Nishimura, *Agric. Biol. Chem.*, 1980, **44**, 1241.

Table 2 *Variation of observed racemization (%) for various dipeptide combinations (Tfa.dipeptide.acid + dipeptide methyl ester)*

	DCCI–HONSu	*DCCI–HOBt*	*Mixed anhydride–TEA*
Pro-Val–Pro-Val	5.2±0.8	8.6±3.4	0.9±0.1
Pro-Ala–Pro-Val	3.5±1.5	5.0±0.0	1.4
Pro-Val–Val-Pro	1.7±0.9	1.4±7.8	8.2±1.8
Pro-Ala–Val-Pro	0.9	1.5	0.7

undergraduates.[69] In these experiments a benzoyl amino-acid was coupled to H.Lys(Z).OMe. From the results it is clear that there is a much greater danger of racemization in DCCI–HOBt coupling than is generally realized.

General Deprotection and Side-reactions During Synthesis.—In the Japanese peptide laboratories much attention has been paid to the use of trifluoroacetic acid–thioanisole as a deprotecting system.[70–74] The use of this system was mentioned earlier with reference to removal of side-chain protecting groups from arginine and histidine. This deprotection procedure depends on thioanisole acting as a soft nucleophile and trifluoroacetic acid as an acid. The promoting effect is strongest for thioanisole but deprotection may also be satisfactorily achieved in the presence of dimethylsulphide and ethane dithiol. Deprotection is much slower in the presence of phenol or anisole, for example the *N*-benzyloxycarbonyl group is cleaved in 3 h at 25 °C with thiophenol but 27 h is required in the presence of phenol.[71] Under these conditions no oxygen to carbon migration is found in the deprotection of *O*-benzyltyrosine.[70, 71] One problem may lie in the fact that *O*-benzylserine and *O*-benzylthreonine are not completely deprotected under conditions which will satisfactorily remove the benzyl group from tyrosine or the benzyloxycarbonyl from lysine. In fact, treatment with boron trifluoride etherate in the presence of a thiol gives better cleavage of aliphatic ethers.[75] The method has been applied in a total deprotection at the end of the synthesis of wasp venom (mastopyran).[72, 73] In this case cleavage was complete in 3—4 h, whereas when the thioanisole was replaced with anisole cleavage required 2—5 days. As well as accelerating the cleavage, thioanisole acted as a good scavenger and no side-reaction due to electrophilic attack on any of the residues was observed. Deprotection at the end of a porcine VIP synthesis[74] was also carried out using the TFA–thioanisole–*m*-cresol procedure. In this synthesis succinimide formation may occur at Asp-Asn or Asp-Ala; however, in both cases the side-reaction is suppressed when using this deprotection procedure. In a total synthesis of chicken VIP,[76] deprotection with either HF or TFA in the presence of *m*-cresol also considerably suppressed imide formation at the Asp-Asn bond. Results contained

[69] N. L. Benoiton, K. Kuroda, and F. M. F. Chen, *Int. J. Pept. Protein Res.*, 1980, **15**, 475.
[70] Y. Kiso, K. Ukawa, S. Nakamura, K. Ito, and T. Akita, *Chem. Pharm. Bull.*, 1980, **28**, 673.
[71] Y. Kiso, K. Ukawa, and T. Akita, *J. Chem. Soc., Chem. Commun.*, 1980, 101.
[72] H. Yajima, J. Kanaki, M. Kitajima, and S. Funakoshi, *Chem. Pharm. Bull.*, 1980, **28**, 1214.
[73] H. Yajima, N. Fujii, Y. Hirota, Y. Nasada, Y. Hirai, and T. Nakajima, *Int. J. Pept. Protein Res.*, 1980, **16**, 426.
[74] M. Takeyama, K. Koyama, K. Inoue, T. Kawano, H. Adachi, T. Tobe, and H. Yajima, *Chem. Pharm. Bull.*, 1980, **28**, 1873.
[75] K. Fuji, K. Ichikawa, M. Node, and E. Fujita, *J. Org. Chem.*, 1979, **44**, 1661.
[76] H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano, and H. Adachi, *Int. J. Pept. Protein Res.*, 1980, **16**, 33.

in these and earlier papers indicate that this method of deprotection may become considerably more important.

It has been found [77] that *O*-acylation of tyrosine may be minimized by the use of pentafluorophenyl active esters in the presence of hydroxybenzotriazole. This approach has successfully been applied to the synthesis of somatostatin using no hydroxylic protection.[51]

Electrophilic attack on tryptophan during acidolytic deprotection [78] has been studied. Both N^{in}-Bu^t and *C*-butylated tryptophan derivatives were observed on deprotection with HF or TFA. However, the presence of ethane dithiol completely inhibited the t-butylation reaction in HF, but this type of additive appeared to be less efficient when TFA was used as the deprotecting acid. When TFA is used a mixture of ethanedithiol and dimethylsulphide appears to be the best scavenger; anisole appeared to be particularly ineffective as a scavenger in trifluoroacetic acid. The butylated side products could be monitored both by n.m.r. and by amino-acid analysis following enzymic digestion.

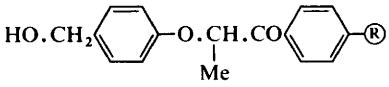
Repetitive Methods of Peptide Synthesis.—*Solid Phase Synthesis.* Development of new resins, new resin linkages, and improved coupling reagents has continued. A short review of current polyamide-based methods has been published [79] following the Sheffield Biochemical Society meeting.

The swelling properties of both polystyrene [80] and polyamide [81] resins have been studied. In a thorough study [80] it was claimed that peptides with a molecular weight up to 6000 could be prepared using polystyrene based resins. However, it was notable that the swelling characteristics varied considerably depending on the nature of the solvent. For example, one gram of resin in dichloromethane occupied 6.2 ml and in DMF 3.3 ml; however, when peptide content rose to 80% the extent of swelling changed considerably becoming 12 ml and 28 ml respectively in the two solvents. At this point there was no indication that the upper limit of swelling had been reached. Interestingly, the space available within the bead is apparently not the critical factor in determining whether or not a synthesis will be successful. Auto-radiography using tritiated valine with a gel loaded to the 70% level with peptide shows that space still remains within the bead after the addition of a 60-residue peptide. The initial loading therefore would become critical only for the synthesis of larger peptides. It is clear that the nature of the resin and the protecting groups used must be carefully considered if the synthesis of a large peptide is being attempted. A comparison between polyamides and polystyrene resins has also been made.[81] Poly-*N*-acrylyldialkylamine based resins in both the protonated and acylated forms swell considerably in polar solvents whereas polystyrene based resins do not. Resins of both types swell extensively in dichloromethane and DMF.

Multi-detachable resins, which allow the possibility of orthogonal cleavage,[82]

[77] S. K. Girin and Y. P. Shvachkin, *J. Gen. Chem. USSR*, 1980, **49**, 606.
[78] Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 464.
[79] R. C. Sheppard, *Biochem. Soc. Trans.* 1980, **8**, 744.
[80] V. K. Sarin, S. B. H. Kent, and R. B. Merrifield, *J. Am. Chem. Soc.*, 1980, **102**, 5463.
[81] G. L. Stahl, C. W. Smith, and R. Walter, *Int. J. Pept. Protein Res.*, 1980, **15**, 331.
[82] J. P. Tam, F. S. Tjoeng, and R. B. Merrifield, *Tetrahedron Lett.*, 1979, 4935.

have been examined in detail.[83, 84] The Pop and Pon resins described in last year's report[82] have now been described in detail.[83] These resins, which avoid the serious side-reactions that can follow from HF cleavage, were used in the synthesis of enkephalin and angiotensin-II. The initial photolytic or cyanolytic cleavage was followed by hydrogenolysis or treatment with trifluoroacetic acid to give the free peptide. The photo-lability of the esters derived from the resin bound alcohols (23) and (24) has been investigated.[84] Photolysis of Bpoc peptides



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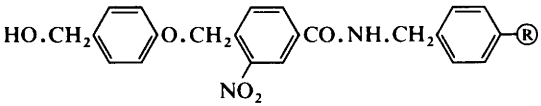
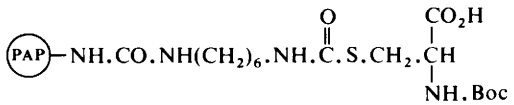


(24)

attached through this linkage gave the Bpoc peptide *p*-hydroxybenzyl esters. The ester function could then be removed by aqueous base or under oxidative conditions. Alternatively, methylation gave the *p*-methoxybenzyl ester which could be cleaved under milder conditions. The resins were used with Bpoc, Fmoc, and Nps protected peptides.

In a synthesis of thymosin-α_1 on a benzhydrylamine polymer[85] side-chain attachment through Boc.Asp.OBzl was used. The paper generally used a standard synthetic protocol except that benzoyl chloride in pyridine (15 min, 0 °C) was used to block the growing chain when incomplete acylation had taken place instead of the more usual acetylation. A bi-direction synthesis on a poly-*N*-acryloyl-pyrrolidine resin[86] has been used to prepare Asp-5 arginine vasopressin. In this synthesis Cys-6 was attached to the polyacryloylpyrrolidine resin by an *S*-carbamoyl linkage (25), the tripeptide fragment Pro-Arg(Tos)-GlyNH_2 was added at the carboxy-terminus, and the amino-terminus was extended stepwise.



(25)

In a model study Boc.Asn was coupled to glycyloxymethylphenoxymethyl-copoly(styrene–DVB) resin.[87] The Asn-Gly was cleaved from the resin with 50% TFA in dichloromethane and the alpha and beta peptides were separated on a sulphonated ion exchange resin. Both DCCI and the symmetrical anhydride

[83] J. P. Tam, F. S. Tjoeng, and R. B. Merrifield, *J. Am. Chem. Soc.*, 1980, **102**, 6117..
[84] J. P. Tam, R. D. Dimarchi, and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 1980, **16**, 412.
[85] S. S. Wang, R. Makofske, A. Bach, and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 1980, **15**, 1.
[86] C. W. Smith, G. Skala, and R. Walter, *Int. J. Pept. Protein Res.*, 1980, **16**, 365.
[87] S. Mojsov, A. R. Mitchell, and R. B. Merrifield, *J. Org. Chem.*, 1980, **45**, 555.

methods gave rise to large amounts of β-cyanoalanine and smaller amounts of aspartamidoacetic acid in addition to α- and β-Asp-Gly and Asn-Gly. The use of DCCI in the presence of HOBt or HONp gave between 98 and 99% of the required Asn-Gly dipeptide. Protection of the amide side-chain as an Mbh derivative immediately eliminated all side-chain nitrile formation. It was found that the dehydration reaction occurred on activation but did not occur after the asparagine residue had been incorporated in the peptide chain. Interestingly, 50% TFA in dichloromethane or HF gradually rehydrates β-cyanoalanine to asparagine. The work showed that the DCCI–HOBt method gave the best results.

The synthesis of RNase *S*-peptide 1—14 analogues by replacement of histidine-12 by β-2 or 4-pyridyl-L-alanine by the solid phase method encountered some difficulties.[88] It was found that Boc-β-2 or 4-pyridylalanine was insoluble and DMF–HMPA has to be used as the solvent; moreover, 17 h was insufficient for complete coupling. Such protracted reaction times produced some racemization at residue-12 in the β-2-pyridylalanine case; however, this could be separated from the bulk of the material by ion exchange chromatography followed by reverse phase h.p.l.c. It was also found in a synthesis of α- and β-MSH[89] that considerably extended coupling times might be required (up to 15 h).

A comparison has been made between two syntheses of human parathyroid hormone (53—84).[90] In the first case, standard DCCI couplings were used, whereas in the second *in situ* formation of the symmetrical anhydride was used, the anhydride being prepared by reaction of carboxy-component with DCCI. Asparagine was incorporated as its nitrophenyl ester in both routes; however, it was found that incomplete acylation was achieved and that acetylation was required at this point. The results showed that fewer repeat couplings were required when using the first approach. Gel filtration followed by ion exchange chromatography gave the required material in both cases although using h.p.l.c. several by-products were isolated which did not contain histidine. This was thought to be due to incomplete deprotection of the residue immediately preceding histidine in the sequence.

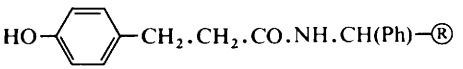
A solid phase synthesis of somatostatin using fluorenylmethyloxycarbonyl for N^{α} protection has been described.[91] The synthesis used a *p*-benzyloxybenzyl alcohol type resin, symmetrical anhydride acylation, and intermediate cleavage through the use of 75% piperidine in DMF. Detachment from the resin and side-chain protecting-group cleavage was brought about with TFA, the final oxidation being with potassium ferricyanide.

Problems involving diketopiperizine formation when extending from a di- to tripeptide on a resin have been observed on several occasions. However, diketopiperizene formation is particularly facile when proline is being added to resin bound prolylproline.[92] The problem may be overcome by coupling Boc prolylproline to the proline resin, the best results being obtained by eliminating the separate neutralization step and adding base (triethylamine) during coupling.

[88] C. Hoes, J. Raap, W. Bloemhoff, and K. E. T. Kerling, *Recl. Trav. Chim.*, 1980, **99**, 99.
[89] Y. C. S. Yang, V. J. Hruby, C. B. Heward, and M. E. Hadley, *Int. J. Pept. Protein Res.*, 1980, **15**, 130.
[90] M. Rosenblatt, G. W. Tregear, G. L. Shepard, G. A. Tyler, M. Veroni, and J. T. Potts, jun., *Arch. Biochem. Biophys.*, 1980, **199**, 286.
[91] C.-D. Chang, A. M. Felix, M. H. Jimenez, and J. Meienhofer, *Int. J. Pept. Protein Res.*, 1980, **15**, 485.
[92] P. R. Ainpour and E. Wickstrom, *Int. J. Pept. Protein Res.*, 1980, **15**, 225.

Peptide synthesis on a phenolic resin support has been demonstrated.[93] Linear or cyclic peptides may be assembled on the phenolic resin and removal may be achieved by treatment with hydrogen peroxide under alkaline conditions or by transesterification with dimethylaminoethanol, followed by hydrolysis of the intermediate ester by sodium bicarbonate. Using radio-labelled Boc glycine comparisons were made between a straightforward Merrifield resin and a phenolic polymer. It was found that with the phenolic polymer the percentage loss was much less than from the Merrifield resin. Transesterification of the Merrifield test peptide in DMF had a $t_{\frac{1}{2}}$ on the Merrifield resin of 20 h, whereas on the phenolic resin it had a $t_{\frac{1}{2}}$ of 5 h. This and other peptides were assembled using Bpoc for *N*-α-amino-protection with t-butyl based side protection. No transpeptidation was observed with either Asn- or Asp(OBu^t).Gly.

An alternative phenolic support (26) has been prepared from a benzhydrylamine resin.[94] Several phenolic resins were examined but this particular one gave the best



(26)

retention of bound glycine in a simulated coupling cycle. This was thought to be due to the fact that the phenolic group was remote from any electron withdrawing group. LHRH was studied as a test peptide, and in this synthesis histidine was not protected. The Bop reagent[95] was used to couple fragments on the resin. Hydrolysis could be achieved using sodium hydroxide but, owing to the risk of racemization, transesterification was used to remove the peptide from the resin.

In a synthesis of calcitonin fragments[92] problems were encountered in the synthesis of the tetrapeptide fragment Val-Gly-Ala-Pro due to diketopiperazine formation. This indicates that a preneutralization procedure should not be used and that neutralization should take place at the same time as adding the activated carboxyl-component. The known transesterification technique using 2-dimethyl-aminoethanol was also applied in the removal of a protected fragment from the resin in a synthesis of the B-chain 1—9 of human insulin.[96]

Sporopollenin, the outer coat of a pollen grain, has been proposed as a support for solid phase peptide synthesis.[97] This natural material is claimed to be stable to chemical reagents and may be chloromethylated with stannic chloride–chloromethyl ether to give a chloromethylated derivative, which may be used for synthesis.

The use of catalytic transfer hydrogenation for cleaving resin bound peptides has again been studied.[98, 99] Both examples use palladium-black with cyclohexene to cleave the benzylic ester, but the second paper[99] applied this to the Sparrow resin[100] rather than to the normal Merrifield polymer.

[93] D. Hudson and G. W. Kenner, *Int. J. Biol. Macromol.*, 1980, **2**, 63.
[94] P. Rivaille, J. P. Gautron, B. Castro, and G. Milhaud, *Tetrahedron*, 1980, **36**, 3413.
[95] B. Castro, J. R. Dormoy, G. Evin, and C. Selve, *Tetrahedron Lett.*, 1975, 1219.
[96] E. M. Salem, O. Schou, and T. Christensen, *Indian J. Chem.*, 1980, **18**, 162.
[97] G. Mackenzie and G. Shaw, *Int. J. Pept. Protein Res.*, 1980, **15**, 298.
[98] N. S. S. Kumari, S. A. Khan, and K. M. Sivanandaiah, *Indian J. Chem.*, 1979, **17**, 152.
[99] R. Colombo, *Chem. Lett.*, 1980, 1119.
[100] J. T. Sparrow, *J. Org. Chem.*, 1976, **41**, 1350.

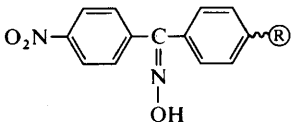
Other Repetitive Methods.—Two papers have appeared [101, 102] describing the use of a soluble chloromethyl polystyrene. The resin, which is prepared by co-polymerization of styrene and chloromethylstyrene (63% *meta*, 28% *para*), gives a controlled reproducible chloromethyl content ranging from 0.096 to 2.43 equivalents per gram. The solubility of the resin depends on the content and composition and added fragments can become important when solubility is to be maintained in fragment coupling. When the coupling of penta- and decapeptides to the resin was examined it appeared that the coupling yield was dependent solely upon the degree of substitution of the resin. In this work after each cycle any remaining free amino-groups were blocked by treatment with DCCI–HOBt–acetic acid. Protected amino and peptide fragments could be coupled to the resin by a variety of techniques [102] using either potassium carbonate or potassium fluoride in the presence of 18-crown-6. Amino-acids with highly branched or hydrophobic side-chains appeared to be more reactive.

The use of polyethyleneglycol as a support was illustrated in the synthesis of an alamethacin analogue,[103] an apamin fragment,[104] and MCD peptide.[105] With both the venom peptides, 4 or 5 fragments were assembled by conventional solution methods and these were then coupled on the resin using the DCCI–HOBt method.

A synthesis of porcine motilin which contains 22 residues by the REMA method has been reported.[106] Fragments 1—8 and 9—22 were synthesized by the REMA method, minimum protection being used.

The importance of minimizing hydrophobicity and hence internal aggregation through careful choice of side-chain protecting-groups has been emphasized in syntheses of cytochrome-c residues 66—104.[37] This sequence, which contains eight lysine residues, could not be assembled satisfactorily on a polyamide support using the butoxycarbonyl group for side-chain protection. Replacement by the more polar N^{ε}-trifluoracetyl derivatives together with other changes enabled a satisfactory synthesis to be achieved.[37]

Polymer bound oximes (27) have been used as supports for solid phase peptide synthesis.[107] The first residue is attached by DCCI coupling and the properties in general follow those of the Merrifield resin except that the high degree of activation allows cleavage by aminolysis or hydrazinolysis.



(27)

[101] M. Narita, S. Itsuno, M. Hirata, and K. Kusano, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 1028.
[102] M. Narita, K. Kusano, M. Hirata, and M. Okawara, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2405.
[103] R. Oekonomopulos and G. Jung, *Biopolymers*, 1980, **19**, 203.
[104] P. Hartter, *Hoppe-Seyler's Z. Physiol. Chem.*, 1980, **361**, 515.
[105] P. Hartter, *Hoppe-Seyler's Z. Physiol. Chem.*, 1980, **361**, 503.
[106] E. Izeboud and H. C. Beyerman, *Recl. Trav. Chim.*, 1980, **99**, 124.
[107] W. F. DeGrado and E. T. Kaiser, *J. Org. Chem.*, 1980, **45**, 1295.

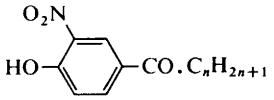
The perchloric acid titration method has been used to monitor solid phase synthesis on two occasions.[96, 108] In the second paper [108] several heptapeptides were prepared and the formation of pyroglutamic acid was followed by potentiometric titration, pyroglutamic acid being formed from *N*-terminal γ-benzyl glutamate.

2- and 4-fluorobenzyloxycarbonyl and fluoro-Ppoc amino-acids have been synthesized and coupled to Merrifield resins.[109] It was found that the ^{19}F n.m.r. signal was highly dependent on the environment and thus solvent effects, coupling reactions, and optical purity may be examined using this model system.

The use of an internal reference amino-acid in synthesis on a solid phase support has been demonstrated in the synthesis of a 71—118 fragment of an immunoglobulin.[110] This work used either hydroxymethylphenylacetamidomethyl or *N*-acetyl-α-aminobutyramidomethyl groups to provide the internal reference.

A qualitative test for monitoring the completeness of solid phase couplings using chloranil has been described.[111] Beads containing free amino-functions show a greenish-blue colour under the test conditions. The test is sensitive to 5—8 micromoles per gram for amino-acids and 2—5 micromoles per gram for proline when 1 mg of resin is used. The chloranil method is claimed to be superior to the ninhydrin test and gives good results when compared with the results obtained by perchloric acid titration. The presence of histidine and occasionally the Bpoc protecting group can give rise to slight problems due to the blurring of the colour, although all other amino-acids seem to be satisfactory.

Polymeric Peptides.—The synthesis of polypeptides from dipeptide-4-acyl-2-nitrophenyl esters of the alcohol (28) has been examined.[112] Reaction is brought



(28) n = 1, 5, or 11

about by addition of triethylamine to the dipeptide hydrobromide 4-acyl-2-nitrophenyl ester. Various oligomers and diketopiperazine are produced, with polymers predominating in nonpolar solvents for longer alkyl chains and diketopiperazine predominating in polar solvents.

Sequential co-polypeptides containing L-3,4-dihydroxyphenylalanine (DOPA) and L-glutamic acid have been prepared by the nitrophenyl ester polymerization method.[113] The dimethyl ether of DOPA and the methyl ester of glutamic acid were used for protection, and these monomers were combined in a stepwise manner using DCCI. The protecting groups ultimately were removed by treatment with boron tribromide in chloroform.

[108] P. Soerup, H. Braae, P. Villemoes, and T. Christensen, *Acta Chem. Scand., Ser. B*, 1979, **33**, 653.
[109] S. L. Manatt, C. F. Amsden, C. A. Bettison, W. T. Frazer, J. T. Gudman, B. E. Lenk, J. F. Lubetich, E. A. McNelly, S. C. Smith, D. J. Templeton, and R. P. Pinnell, *Tetrahedron Lett.*, 1980, **21**, 1397.
[110] G. R. Matsueda and E. Haber, *Anal. Biochem.*, 1980, **104**, 215.
[111] T. Christensen, *Acta Chem. Scand., Ser B*, 1979, **33**, 763.
[112] K. Hanabusa, K. Kondo, and K. Takemoto, *Makromol. Chem.*, 1980, **181**, 635.
[113] H. Yamamoto and T. Hayakawa, *Biopolymers*, 1979, **18**, 3067.

The synthesis of lysine containing polypeptides has been studied.[114-116] The first paper[114] describes the synthesis of poly(ε-L-lysine). The monomeric unit was assembled by conventional methods and the polymerization was brought about using pentachlorophenyl active esters. The synthesis of branched lysine polypeptides[115] has also been investigated; in this case a variety of amino-acids and dipeptides were grafted onto the lysine using the azide, active ester, or NCA methods. Polymers were grafted on using the NCA method alone. The synthesis of poly[Lys(Z)-Val-Lys(Z)] by polymerization of the nitrobenzyl tripeptide monomer has been reported.[116] The tripeptide monomer was prepared using active esters by straightforward solution synthesis. Treatment of the Boc tripeptide nitrobenzyl ester with trifluoroacetic acid followed by neutralization with triethylamine brought about polymerization.

The synthesis of homo- and co-oligopeptides using the liquid phase approach has been studied with a view to investigating the development of conformation as the chain extends from the support.[117] Various oligopeptides were assembled containing up to 20 residues; reactivity of the amino-terminus was reduced when β-sheet or α-helical structure was present. However, on both polyethyleneglycol and methoxypolyethyleneglycol, randomly coiling peptides retained the reactivity of the amino-terminus when up to 20 residues were present. However, with amino-acid residues favouring β-structure, a limit is obtained much earlier and it appears that the solubilizing properties of polyethyleneglycol are not sufficient to prevent aggregation of the growing peptide.

Semisynthesis.—The past year has seen further diversification of the semisynthetic method and an extensive review[118] has been published. The review covers enzymic synthesis, cytochrome-c, and many of the factors which may be found to complicate semisynthesis. [29,59-Lys(Msc)]proinsulin has been prepared as part of a programme directed towards semisynthesis of the prohormone.[119] The reaction sequence involves initial protection at the *N*-terminus with t-butoxycarbonyl-anhydride or citraconic anhydride and then treatment of the N-α_1-derivative with *N*-methylsulphonylethoxycarbonyloxysuccinimide. Glucagon has been treated with cyanogen bromide to give a fragment which could be used in the preparation of analogues.[120]

Eight analogues of cytochrome-c have been prepared[121] by the same general route that has been used previously.[122] Acetylation of lysine-72,73, and 79 was investigated as was the replacement of tyrosine-74 by leucine. The synthetic 66—77 segment was coupled with natural 1—65 and 80—104 fragments giving analogues of the native compound. A biologically active three-fragment complex of horse heart cytochrome-c has also been described.[123] The complex was formed by

[114] D. R. S. Kushwaha, K. B. Mathur, and D. Balasubramanian, *Biopolymers*, 1980, **19**, 219.
[115] F. Hudecz and M. Szekerke, *Collect. Czech. Chem. Commun.*, 1980, **45**, 933.
[116] R. Giaschi and M. D'Alagni, *Makromol. Chem.*, 1979 **180**, 2893.
[117] S. A. El Rahman, H. Anzinger, and M. Mutter, *Biopolymers*, 1980, **19**, 173.
[118] G. I. Tesser and P. J. Boon, *Recl. Trav. Chim.*, 1980, **99**, 289.
[119] E. E. Buellesbach and V. K. Naithani, *Hoppe-Seyler's Z. Physiol. Chem.*, 1980, **361**, 723.
[120] D. E. Wright, V. J. Hruby, and M. Rodbell, *Biochem. Biophys. Acta*, 1980, **631**, 49.
[121] P. J. Boon, A. J. M. Van Raay, G. I. Tesser, and R. J. F. Nivard, *FEBS Lett.*, 1979, **108**, 131.
[122] P. J. Boon, G. I. Tesser, and R. J. F. Nivard, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 61.
[123] M. Juillerat, G. R. Parr, and H. Tanuichi, *J. Biol. Chem.*, 1980, **255**, 845.

non-covalent combination of the haem fragment 1—25 and fragments 28—38 and 56—104. The three-fragment complex showed biological activity and physiochemical properties which were similar to those of native horse heart cytochrome-c.

Many studies in connection with ribonuclease have been made. These include an *X*-ray study of the semisynthetic non-covalent complex formed between the 1—118 and 111—124 fragments of bovine pancreatic ribonuclease.[124] With a view to preparing semisynthetic analogues of ribonuclease A the preparation of 8-lysine(acetimidyl)*S*-protein has been studied,[125-127] and a stable folded intermediate has been characterized at pH 1.7. A Gly-48(6—49) staphylococcal nuclease fragment has been prepared.[128] In this synthesis trypsin was found to catalyse the reformation of the peptide bond between the nuclease-T fragments 6—49 and 49—149. However, during this reformation lysine-49 is excised, thus forming des-Lys-49 nuclease (6—149). Gly-48 nuclease-T fragment 6—49 was synthesized on hydroxymethyl pam resin. When the deprotected material was added to nuclease-T 50—149, between 30 and 50% nuclease activity was regenerated. If however a 10-fold excess of the Gly-48 (6—49) nuclease-T fragment was added and then incubated with trypsin in a 90% glycerol solution, 20% of the 50—149 nuclease-T was converted into nuclease molecular weight material, as shown by gel electrophoresis. This semisynthetic method gave a material which was approximately equal in activity to the original native nuclease; thus the preparation of sequence analogues should be feasible using the same semisynthetic approach.

Enzyme Mediated Synthesis.—Papain catalysed oligomerization of amino-acids has been studied.[129] In this paper the polymerization of methionine-methyl ester using papain to catalyse the reaction was studied in citrate buffer at pH 5.5. Under carefully defined conditions mainly octamer was produced along with a little hexamer and heptamer. It was found that most chains terminated at an ethyl ester but that a few terminated as the free acid.

The use of trypsin in cytochrome-c semisynthesis has been mentioned above.[121] The enzyme has also been used to catalyse the reaction between 1—38(Msc-7) horse heart cytochrome-c and the protected dipeptide H.Orn(Msc)-Trp.NH.Me.[130] For this reaction to be successful the now established procedure of using water with organic co-solvents must be employed.

A serine protease has been used to cleave on the carboxy-side of lysine-29 in porcine insulin.[131] The product des-Ala^{B30} insulin was then coupled with threonine t-butylester using the same enzyme for the formation of the amide bond. A large excess of the ester was present and the pH was controlled at 6.5, the reaction being carried out in the presence of organic co-solvents. The coupled product was isolated in 85% yield after 20 h at 37 °C

[124] D. M. Sasaki, P. D. Martin, M. S. Doscher, and D. Tserhoglou, *J. Mol. Biol.*, 1979, **135**, 301.
[125] P. Hoogerhout, W. Bloemhoff, and K. E. T. Kerling, *Recl. Trav. Chim.*, 1979, **98**, 515.
[126] A. M. Labhardt and R. L. Baldwin, *J. Mol. Biol.*, 1979, **135**, 245.
[127] A. M. Labhardt and R. L. Baldwin, *J. Mol. Biol.*, 1979, **135**, 231.
[128] A. Komoriya, G. A. Homandberg, and I. M. Chaiken, *Int. J. Pept. Protein Res.*, 1980, **16**, 433.
[129] R. Jost, E. Brambilla, J. C. Monti, and P. L. Luisi, *Helv. Chim. Acta*, 1980, **63**, 375.
[130] L. W. Westerhuis, G. I. Tesser, and R. J. F. Nivard, *Recl. Trav. Chim.*, 1980, **99**, 400.
[131] K. Morihara, T. Oka, H. Tsuzuki, Y. Tochino, and T. Kanaya, *Biochem. Biophys. Res. Commun.*, 1980, **92**, 396.

The damage that may be done to a protease by water miscible organic solvents has been considered,[132] and an investigation into the use of immiscible solvents has been carried out. Acetyl-leucylphenylalanine methyl ester was coupled to leucine amide by treatment with α-chymotrypsin. Various immiscible organic solvents *e.g.* dichloromethane or ethyl acetate gave yields in the 70—80% region. However, miscible organic solvents such as dimethylformamide only gave a 40% yield. The best results were obtained using trichloroethylene (75%) at a pH of 10. Under these conditions Boc protected dipeptides could be satisfactorily coupled whereas frequently under similar conditions such peptides have not coupled satisfactorily. It was also found that papain gave much higher yields when immiscible solvents such as ethyl acetate or carbon tetrachloride were used in preference to methanol. The alternative of using an immobilized enzyme was also studied, but with immobilized chymotrypsin, and frequently the yields were in the 30% region. The application of immobilized chymotrypsin to the synthesis of acetyl tyrosine ethyl ester[133] has been investigated. The immobilized enzyme in this case was used in a biphasic system containing aqueous ethanol and chloroform.

Purification Methods.—A variety of chromatographic techniques have been used in the analysis and purification of peptides. A wide range of references have been encountered and these are collected together in Table 3.

Table 3 *Purification methods*

Application	*Ref.*
Ion exchange	
Chromatography of *S*-sulphocysteine and related compounds	134
G.l.c.	
Separation of pentafluoropropionyl amino-acid derivatives	135
Separation of trifluoroacetyl amino-acid trimethylsilyl esters	136, 137
Separation of 3-methyl histidine	138
Analysis of cysteic and cysteine sulphinic acid	139
Separation of enantiomers of proline and secondary amines	140
H.p.l.c.	
Separation of underivatized dipeptides on a weak anion exchange bonded phase	141
Dynamic cation exchange separations of tyrosinyl peptides	142
Prediction of peptide retention times from composition	143
Effects of pH and ion-pair formation on retention times	144

[132] P. Kuhl, A. Koennecke, G. Doering, H. Daeumer, and H.-D. Jakubke, *Tetrahedron Lett.*, 1980, **21**, 893.
[133] D. Tarquis, P. Monsan, and G. Durand, *Bull. Soc. Chim. Fr. II*, 1980, 76.
[134] T. Ubuka, M. Kinuta, R. Akagi, and S. Kiguchi, *J. Chromatogr.*, 1980, **188**, 442.
[135] H. Kawa and N. Ishikawa, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 1467.
[136] G. Michael, *J. Chromatogr.*, 1980, **196**, 160.
[137] G. Michael, *J. Chromatogr.*, 1980, **188**, 251.
[138] H. Vielma and J. Mendez, *J. Chromatogr.*, 1980, **196**, 166.
[139] S. L. MacKenzie and A. J. Finlayson, *J. Chromatogr.*, 1980, **187**, 239.
[140] T. Saeed, P. Sandra, and M. Verzele, *J. High Resoln. Chromatogr.*, 1980, **3**, 35.
[141] M. Dizdaroglu and M. G. Simic, *J. Chromatogr.*, 1980, **195**, 119.
[142] R. A. Wall, *J. Chromatogr.*, 1980, **194**, 353.
[143] J. L. Meek, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1632.
[144] M. T. W. Hearn, B. Grego, and W. S. Hancock, *J. Chromatogr.*, 1980, **185**, 429.

Table 3 (*cont.*)

Application	*Ref.*
Separation of optical isomers of tryptophan, 5-hydroxytryptophan, and DOPA on cellulose columns	145
Separation of enantiomers using chiral eluants	146
Separation of vasopressin analogues	147
H.p.l.c. of enkephalin and endorphin analogues	148
H.p.l.c. of proinsulin fragments	149
H.p.l.c. of somatostatin	150
Analysis of insulin related peptides by reverse phase h.p.l.c.	151
H.p.l.c. of proctolin and some analogues	152
Use of TFA as eluant in reverse phase h.p.l.c. of secretin	153
Miscellaneous	
Ligand-exchange chromatography for enantiomer separation	154
Reverse phase separation of optical isomers of Dns-amino-acids	155
Macroporous silicas with an activated thiol group for covalent chromatography of peptides and proteins	156

3 Syntheses Achieved

Several interesting syntheses have been reported in the last year. However, due to the limitations of space only a small number will be considered in this section, the remainder being listed in the appendix. A particularly large number of papers on the synthesis of insulin and proinsulin have appeared this year, many being published as tributes to the late Vincent du Vigneaud. Two groups of workers are progressing towards the synthesis of proinsulin. In the first series of papers, the 53—81,[157] 41—81,[158] and 31—81[159] portions of bovine proinsulin have been synthesized. Boc.Arg.(Tos)ONSu and a protected 53—59 fragment were coupled to the insulin A chain *S*-sulphonate.[157] The product was purified and coupled to the protected 41—52 fragment by the azide method.[158] After purification on QAE Sephadex A25 in 7M urea the fragment was coupled to the 31—40 protected peptide azide,[159] again being purified on QAE Sephadex A25 in 7M urea.

In the second synthesis, which was mentioned briefly above,[119] a total fragment condensation approach to the synthesis of the prohormone is envisaged.[160-163]

[145] G. Guebitz, W. Jellenz, and D. Schoenleber, *J. High Resoln. Chromatogr.*, 1980, **3**, 31.
[146] E. Oelrich, H. Preusch, and E. Wilhelm, *J. High Resoln. Chromatogr.*, 1980, **3**, 269.
[147] G. Lindeberg, *J. Chromatogr.*, 1980, **193**, 427.
[148] B. L. Currie, J.-K. Chang, and R. Cooley, *J. Liq. Chromatogr.*, 1980, **3**, 513.
[149] J. G. R. Hurrell, R. J. Fleming, and M. T. W. Hearn, *J. Liq. Chromatogr.*, 1980, **3**, 473.
[150] M. Abrahamsson and K. Groeningsson, *J. Liq. Chromatogr.*, 1980, **3**, 495.
[151] M. T. W. Hearn, W. S. Hancock, J. G. R. Hurrell, R. J. Fleming, and B. Kemp, *J. Liq. Chromatogr.*, 1979, **2**, 919.
[152] A. N. Starratt and M. E. Stevens, *J. Chromatogr.*, 1980, **194**, 421.
[153] D. Voskamp, C. Olieman, and H. C. Beyerman, *Recl. Trav. Chim.*, 1980, **99**, 105.
[154] A. Foucault, M. Caude, and L. Oliveros, *J. Chromatogr.*, 1979, **185**, 345.
[155] W. Lindner, J. N. LePage, G. Davies, D. E. Seitz, and B. L. Karger, *J. Chromatogr.*, 1979, **185**, 323.
[156] V. I. Lozinskii, I. G. Tsoi, Y. A. Davidovich, and S. V. Rogozhin, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1979, 1358.
[157] H. Aiba and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 192.
[158] H. Aiba and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 197.
[159] H. Aiba and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 201.
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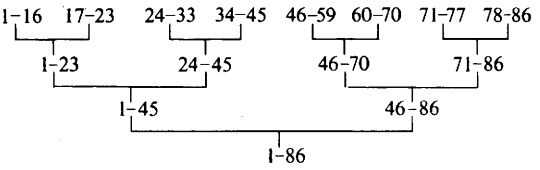
The overall projected route to the prohormone is shown in Scheme 7. The general approach was to use benzyloxycarbonyl for N^{α}-protection, or trityl or Bpoc if sulphur was present. Side-chain t-butyl esters and ethers were used along with N^{ε}-*Z*. lysine. The cysteine residues were protected as the *S*-trityl derivative. In this synthesis arginine and histidine remained unprotected, which was found to be of some advantage when counter-current distribution was used for purification. Such purification by counter-current distribution using either toluene–chloroform–methanol–water or chloroform–methanol–hexane–DMF–water was highly efficient and complemented the alternative use in the synthesis of gel filtration on Sephadex LH20. The subfragments indicated in Scheme 7 were synthesized and



Scheme 7

combined to give the major 1—45 and 46—86 fragments. Coupling of these fragments with DCCI at glycine-45 appeared to be successful and the product was found to be very similar to the hexa-*S*-sulphonate of native bovine proinsulin after deprotection and sulphitolysis. End group analysis indicated contamination with the 46—86 fragment; unfortunately limitations in the supply of the protected 1—45 acid have prevented immediate repetition of the synthesis.

Syntheses of many A chain analogues and insulins containing A chain analogues have been published.[164–168] In the synthesis of A chain fragments, cysteine has been protected by a combination of the acetamidomethyl and diphenylmethyl protecting groups.[164–166] This allowed the formation of the A-6,11-disulphide bridge by iodine oxidation of the acetoamidomethyl cysteine residues at positions 6 and 11, whilst leaving the diphenyl cysteine residue at position 7 untouched. In the synthesis of insulin containing A-14-phenylalanine[167] and A-19-phenylalanine[168] the chains were prepared by straightforward solution synthesis using fragment condensation. Before coupling to native B chain the synthetic A chains were purified as their *S*-sulphonates.

A synthesis of a two chain cystine peptide corresponding to the asymmetrical insulin intermediate A1—21–B18—26 has been described.[169] The overall synthesis follows the route shown in Scheme 8. The asymmetrical disulphide (29), which is formed by the sulphenylthiocarbonate method, was coupled to B21—26 bound to the resin giving the resin-bound, asymmetrical disulphide (30). This was extended

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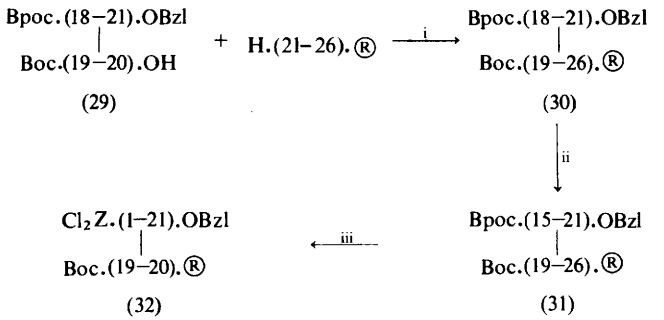
[169] W. Kullmann, *Tetrahedron Lett.*, 1980, **21**, 589.



Reagents*: i, DCCI–HONSu; ii, Boc.(16—17).OH, DCCI–HONSu, Boc.Gln.ONp; iii, Boc.(13—14).OH, DCCI–HONSu, Boc.(5—12).OH, DCCI–HONSu, $Cl_2Z.(1—4).OH$, DCCI–HONSu
*Intermediate deprotections by 0.5% TFA–CH_2Cl_2

Scheme 8

to the intermediate (31) and subsequently to the fully protected A1—21–B19—26 resin-bound disulphide (32). A summary of the coupling yields found in this fragment condensation synthesis is provided in Table 4.

Interest in the enkephalin and endorphin area continues, and two solution syntheses of endorphins have been published. The first [170] describes the synthesis of

Table 4 *Fragment coupling yields in partial solid phase synthesis of insulin*

Fragment added (equivalents)		*Yield*/%
A(18—21)–B(19—20)	(1.4)	70
A(16—17)	(4.2)	95
A15	(2 × 4.3)	99
A(13—14)	(2 × 4.3)	78
A(5—12)	(2 × 2)	69
A(1—4)	(2 × 3.5)	96

human-β-endorphin using acid labile N^{α} protection with benzyl-based side-chain protection. Five fragments were assembled and coupled by either the azide or the DCCI–HOBt method. Coupling of the 1—9 to the 22-residue fragment by the DCCI–HOBt method was complicated by solubility problems. These were eventually overcome, however, by using DMF–phenol as a solvent. At the end of the synthesis protecting groups were best removed by treatment with sodium in liquid ammonia rather than treatment with anhydrous hydrogen fluoride. The synthesis of salmon endorphin has also been published.[171] In this synthesis 1—7, 8—15, 16—18, 19—24, and 25—29 fragments were assembled by solution synthesis using acid labile protecting groups on the side-chains. Fragment combination using the DCCI–HOBt method gave the final fully protected peptide which was eventually deprotected by treatment with trifluoroacetic acid.

The synthesis of non-reducible bicyclic analogues of somatostatin using a combination of solid phase peptide synthesis and solution methods has been examined,[172] using selectively protected amino-suberic or diamino-suberic acids.

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Cyclization of the larger 20-membered ring proceeded satisfactorily but cyclization of the 16-membered ring could only be achieved using the azide method with the addition of hydroxybenzotriazole.

The originally proposed sequence of human big gastrin [173] has been synthesized.[174-177] This elegant synthesis described three routes of combinaton of fragments utilizing the 1—8, 9—14, 15—20, 21—22, 23—27, and 28—34 fragments. The resulting peptide was deblocked with anhydrous TFA in the presence of anisole and ethane dithiol, and partition chromatography on Sephadex G25 allowed separation of partially t-butylated tryptophan containing products. Ultimately, material of high purity was isolated, but it is now known[178] that this material has the incorrect sequence. A synthesis of the corrected 1—19 fragment has now been published.[179] This 1—19 fragment was assembled by condensation of the 1—6, 7—12, and 13—19 portions, each of these being obtained by solution synthesis. Deprotection of this material with 90% trifluoroacetic acid in the presence of scavengers gave the free 1—19 portion of human big gastrin. After h.p.l.c. purification the material was shown to be identical with the amino-terminal half of natural human big gastrin by radioimmunoassay.

A remarkable communication [180] describing the total chemical synthesis of bovine pancreatic ribonuclease A has appeared from the Department of Pharmaceutical Science at Kyoto University. The enzyme was assembled by a combination of 20 sub-fragments using the azide method, purification being achieved by the use of Sephacryl-S200 eluting with DMF–DMSO–HMPA as the mixed solvent system. Arg(Mbs), Lys(Z), Cys(MBzl), Glu(OBzl), and Asp(OBzl) protecting groups were removed at the end of the synthesis by methane sulphonic acid deprotection in the presence of metacresol. Aerial oxidation followed by chromatography on Sephadex G75 gave a product that had 12% of the activity of yeast RNA's. Further purification by affinity chromatography on Sephadex 4B–5′-(4-aminophenyl)phosphoryluridine 2′(3′)-phosphate increased the activity to 82%. Ion exchange chromatography on carboxymethyl cellulose then gave the final product which had activity equivalent to that of the natural enzyme. The identity of the product was confirmed by disc gel electrophoresis at pH 4.3.

4 Appendix I: A List of Syntheses Reported During 1980

Natural Peptides, Proteins, Analogues, and Partial Sequences.—The syntheses are listed under the name of the peptide or protein to which they relate, as in previous years.

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[177] E. Jaeger, M. Gemeiner, W. Goehring, S. Knof, R. Scharf, P. Thamm, G. Wendlberger, and E. Wünsch, *Monatsh. Chem.*, 1980, **111**, 125.
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Peptide	*Ref.*
Adrenocorticotrophin (ACTH)	
Tritiated human corticotrophin	181
[5-Bromotryptophan^{9}]-β-corticotrophin (1—24)-tetracosapeptides	182
Human CLIP (ACTH 18—39)	183
Adrenodoxin bovine (90—103)	184
Alamethicin analogue	103
Angiotensin	
Angiotensin II	83
Two angiotensin II analogues	185
[Sar^{1}, Val^{5}, (4′-azido-3′,5′ditritio)Phe^{8}]angiotensin II	186
Apamin fragments	104
Bombesin-like gastrin releasing peptide	187
Bradykinin	
Bradykinin	98
Bradykinin like peptide	188
5,8-Car bradykinin	189
^{125}I-Bradykinin analogue	190
Bradykinin analogues containing *N*-methyl amino-acids	191
Bradykinin fragment analogues	192
α-Bungarotoxin (38—74)	193
Caerulein	194
Calcitonin human (1—10) carba-analogue	195
Chemotactic peptide analogues	196
Cholecystokinin	
Six sulphated *C*-terminal octapeptide analogues of CCK	197
Desamino-CCK-7	198
C-Terminal octapeptide of cholecystokinin	194
Acetyl *C*-terminal heptapeptide of CCK	199
Chorionic gonadotrophin	
C-Terminal fragments of β-subunits of human chorionic gonadotrophin	200

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200 H. Rolli, K. Blaser, C. Pfeuti, and C. H. Schneider, *Int. J. Pept. Protein Res.*, 1980, **15**, 399.

Peptide	*Ref.*
β-Subunit (116—145) human chorionic gonadotrophin	201
β-Subunit (130—145) human chorionic gonadotrophin	202
β-Subunit (132—147) human chorionic gonadotrophin (h. CG)	203
β-Subunit (116—147) human chorionic gonadotrophin	204
(116—145) and (116—147) human chorionic gonadotrophin	205
Cytochrome-c	
(66—104) fragment of cytochrome-c	37
Cytochrome-c analogues	121
Cytochrome-c analogue	130
S-Methylcysteine and ethionine-80 cytochrome-c	206
Eledoisin	
Eledoisin analogue	61
Eledoisin (6—11) derivatives	207
Endorphin	
Human β-endorphin and related peptides	67
Human β-endorphin	170
Human β-endorphin	208
Salmon endorphin	171
Turkey β-endorphin	209
Des-acylated salmon β-endorphin	209
Endorphin analogues	148
Ala$^{17,18,\text{or }19}$ [Phe^{27}, Gly^{31}]human-β-endorphin	210
Analogues of human β-endorphin extended at *C*-terminus	211
Omission analogues of human-β-endorphin	212
Des-1-Tyr-γ-endorphin, β-(LPH 62—77)	213
Human β-endorphin tritiated at Tyr-1 and/or -27	214
Enkephalin	
Enkephalin	83
Leu-enkephalin	215
Leu-enkephalin	56
Met-enkephalin	71
Enkephalin analogues	148
Enkephalin analogues	216

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[216] M. Kubota, O. Nagase, H. Amano, H. Takagi, and Y. Yajima, *Chem. Pharm. Bull.*, 1980, **28**, 2580.

Peptide	*Ref.*
Enkephalin analogues	217
Olefinic enkephalin isosters	218
5-Pro-enkephalin analogues	219
2-D-Ala,5-Bug-enkephalin amide	220
Tri- and tetra-peptide analogues of enkephalin	221
Hexahydro derivative of [D-Met^2, Pro.NH$_2$^{5}] enkephalin	222
[4-Tryptophyl] enkephalin analogues	223
Tyr modified enkephalin analogues	224
Tyr(SO$_3$) enkephalin	225
N_α-Lysergyl enkephalin analogues	226
Acylhydrazide analogues of enkephalin	227
Enkephalin analogues with a photo-affinity label	228
Enkephalin analogues containing piperazine	229
Fibrinogen	
Fibrinogen binding peptides	230
Fibronopeptide human A (8—16)	231
Gastrin	
Human big gastrin fragments	174, 175, 176
Human big gastrin 1 analogue	177
Human big gastrin (1—19)	179
15-Leucine human little gastrin	16
15-Leucine human minigastrin	16
Glucagon	
Glucagon analogues	120
Iodinated glucagon analogues	234
Glucagon (22—29)	235
Glycopeptide	232
Granuliberin R and related peptides	233
Histone	
Histone models	236
Histone fragments	237
Hydrophobic peptides	238

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Peptide	*Ref.*
Immunoadjuvant peptides	
Immunoadjuvant peptide	239
Immunoadjuvant peptide	240
Immunoadjuvant peptides	241
Derivatives of 3-*O*-(2-acetamido-2-deoxy-α-D-galacto-pyranosyl-L-serine and -L-threonine	242
N-Acetyl-6-amino- and 6-(acylamino)-6-deoxymuramoyl dipeptides	243
Muramyl peptides	244
Muramyl dipeptides	245
Insulin	
Insulin	246
Human insulin	131
Insulin analogues with D-amino-acids at A1	247
Iodinated insulin analogues	234
Monoiodoinsulin substituted at Tyr-A14 or Tyr-A19	248
Insulin related peptides	151
Phe^{A14} porcine insulin	167
Phe^{A19} porcine insulin	168
Porcine insulin with a hybrid B chain	249
(B24- and B25-Leu) insulin	250
(B24- and B25-Leu) insulin	251
$A^{1\,1\,21}/B^{18\,1\,26}$ insulin	169
Insulin A chain$^{(14-21)}$	164
Ala^{12} insulin A chain (6—13)	165
Sheep insulin A chain (6—13)	166
Sheep Ala^{12} A chain of insulin	252
(14—21) A chain of insulin	253
Peptides related to bovine insulin B chain 23—30	29
Human insulin B chain (1—8)	96
Insulin B chain (23—30)	254
B-Chain analogues of insulin	255

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Peptide	*Ref.*
Human insulin B chain	256
B-Chain N^{α}-$(Lys)_n$-insulin (n = 1—4)	257
29,59-Lys(Msc)proinsulin	119
Proinsulin fragments	149
53—81 bovine proinsulin	157
41—81 bovine proinsulin	158
31—81 bovine proinsulin	159
1—23 of human proinsulin	160
24—45 of human proinsulin	161
(1—45)/(46—86) of human proinsulin	162
S-Trityl human proinsulin	163
Kyotorphin and analogues	38
Lipotropin	
Fragments of human β-lipotropin	262
β-LPH (62—77)	263
Luteinizing hormone releasing hormone (LHRH)	
LHRH	94
LHRH analogue	40
LHRH analogues	258
LHRH analogues	259
LHRH analogues	260
Iodinated LHRH derivatives	261
Lysozyme	
Lysozyme analogue (38—49)	264
Lysozyme analogue (50—67)	265
Lysozyme analogue (68—75)	266
Lysozyme analogue (38—75)	267
Mastopyran	
Mastopyran wasp venom	73
Mastopyran wasp venom	72
Mastopyran X (a wasp venom)	268
MCD peptide	105
Melanocyte stimulating hormone (MSH)	
α- and β-MSH	89
MSH analogues modified at positions 2 and 4 (7)	278
γ-MSH	279

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Peptide	*Ref.*
Bovine γ-MSH	280
MSH-release inhibiting factor	18
Analogues of MSH-release inhibiting factor	281
Motilin	
Porcine motilin	55
Porcine motilin	106
Neurotensin 3,11-D-Phe analogue	269
Oxytocin	
[1-Penicillamine, 4-Thr]oxytocin	270
[1-Penicillamine, 2-Phe, 4-Thr]oxytoxin	270
[8-Trp]oxytocin	271
4-Dialkyl glutamine oxytocin	272
Analogues of deamino-6-carba-oxytocin with Ile, Tyr(Me), Met, or Met(O) at position 2	273
Analogues of deamino-1-carba-oxytocin with Val, Ile, or Leu at position 4	274
(1-α-Mercaptoacetic acid, 5-isoasparagine)oxytocin	275
8-α-Hydroxyisocaproic acid oxytocin	276
Desamino[8-α-hydroxyisocaproic acid]oxytocin	276
Six antagonists of oxytocin	277
Pancreatic polypeptide (human)	282
Parathyroid hormone (53—84) human	90
Pepstatin analogues	283
Proctolin analogues	152
Protamine fragments	284
Prothrombin bovine (1—10) fragment	285
Ribonuclease	
RNase *S*-peptide (1—14) analogues	88
RNase (1—118) (111—124)	124
[His^{119}] and [homo-His^{119}] (111—124)RNase	286
8-Lys(acetimidyl) *S*-protein	125
[Gly^{48}] (6—149) nuclease T	128
Bovine pancreatic ribonuclease A	180
Secretin	
Secretin	153

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Peptide	*Ref.*
Analogues of porcine secretin, hybridized with VIP, GIP, and glucagon	287
Secretin (16—27)	288
Somatostatin	
Somatostatin	32
Somatostatin	51
Somatostatin	91
Somatostatin	150
Somatostatin	289
Somatostatin	290
(Phe^4)somatostatin	291
Somatostatin and [D-Trp^8]somatostatin	292
(3,14-Selenocysteine, 8-D-Trp)somatostatin	293
Thioether containing somatostatin analogue	33
Somatostatin non-reducible bi-cyclic analogues	172
Glycosylated analogues of somatostatin	294
Somatotropin	
[Nle^{170}, Ala$^{165,\ 182,\ 189}$]human somatotropin (150—191)	295
[Nle^{170}, Ala$^{165,\ 182,\ 189}$]human somatotropin (154—191)	295
Substance P	
Substance P	40
Substance P analogue	296
Substance P analogues	297
Substance P analogues	298
7,8-Car substance P	189
Substance P fragment	299
5-Gln(Me$_2$)-substance P (5—11)	300
^{3}H labelled (Nle^{11})-substance P	301
Thymic Peptides	
Thymosin-α_1	85
Thymosin-α_1	302
Thymosin-α_1	303

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Peptide	*Ref.*
Fragments and analogues of thymopoietin II	304
Nine analogues of FTS	305
Three fragments of serum factor thymique	306
Thyrotropin releasing hormone (TRH)	
TRH analogues	307
Thyrotropin releasing factor analogues	308
^{3}H-labelled thyroliberin	309
Tropoelastin repeat peptide	310
Tuftsin analogues containing D-amino-acids	311
Vasointestinal peptide (VIP)	
Porcine VIP	312
Porcine vasoactive intestinal polypeptide	74
Porcine (Glu^8)-vasoactive intestinal polypeptide	313
Chicken vasoactive intestinal polypeptide	76
C-Terminal peptide of fragment of chicken VIP	17
Vasopressin	
Arginine vasopressin analogues	314
Vasopressin analogues	147
[Asp^5]arginine vasopressin	86
[3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin	315
[5-N^4,N^4-Dimethylasparagine,8-lysine]vasopressin	316
4-Dialkyl glutamine lysine vasopressin	272
(D-Phe^3,α,γ-diaminobutyric^8)vasopressin	317
[7-Gly, 8-Orn]vasopressin and two analogues	318
Arginine vasopressin antagonists	319
Sequential Oligo- and Poly-peptides	
Block co-polymer (Ala, Lys)	320
Boc[Cys(Me)]$_7$-OMe	321
Polypeptides containing DOPA and glutamic acid	113
Poly-Glu(OMe), Poly-Nva, Poly-Nle	322
Z[Glu (OEt)]$_n$-OEt (n = 2 and 3)	323

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Peptide	*Ref.*
Electron donors and acceptors attached to poly(γ-benzy)glutamate	324
Branched lysine polypeptides	115
Poly[Lys(Z)-Val-Lys(Z)]	116
Poly(ε-lysine)	114
Nucleotide derivatives of poly(oligo)lysine	325
Methionine oligomers	129
Poly(L-lysyl-L-valyl-L-lysine)	326
Poly(L-valyl-L-valyl-L-lysine)	327
Boc(D-Val)$_m$(Val-D-Val)$_n$OMe	
$m = 0$, $n = 1, 2, 3, 4, 6$, and 8	
$m = 1$, $n = 1, 2, 3, 4$	328
Homo and co-oligopeptides	117
Poly-amino-acids	329
Amphipatic saccharide–peptide co-polymers	330
Enzyme Substrates and Inhibitors	
Peptide substrates of angiotensin converting enzyme	331
Bovine carboxy peptidase B substrate	332
Chymotrypsin substrates	333
A ^{14}C-labelled substrate for collagenase	334
Substrate for elastase	335
Substrate for enteropeptidase	336
Gln containing substrates for human plasma factor XIIIa	337
Substrate for vitamin K epoxidase	338
Pentadecapeptide with inhibitory activity toward actomyosin adenosine triphosphatase	339
Inhibitor of *S*-adenosyl-L-methionine decarboxylase	340
Tripeptide inhibitor of angiotensin converting enzyme	341
Bowman–Birk inhibitor (14—22)/(41—49)	342
Oligopeptide inhibitor of cathepsin D	343
Serine protease inhibitor	344
Thiol proteinase inhibitor	345
Thrombin inhibitors	346

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Peptide	*Ref.*
Thrombin inhibitors	347
Trypsin inhibitor ^{13}C-labelled derivative	348
Miscellaneous Peptides	
Peptides containing 3-(9-acridinyl)-D,L-alanine	349
Peptides of aminoethylphosphonic acids	350
Peptides containing β-aminophenylalanine	351
Aspartyl and glutamyl peptides	352
Bitter heptapeptide obtained from casein	353
Peptide with delicious taste	354
Diazomethyl ketone and chloromethyl ketone analogues	355
Di- and tripeptides of Gla	356
γ-Carboxyglutamic acid derivatives	357
Glycyl-peptide amides	358
H.Glu-Asp-Gly.OH	359
Non-symmetrical lanthionyl peptides	360
Z. Met-Gly.OBzl	361
Peptides containing nucleo-amino-acids	362
Nucleo-peptides	363
Nucleo-amino-acids and peptides	364
Peptides containing nucleo-amino-acids	365
Peptide sweeteners	366
Peptide sweeteners	367
Peptide sweeteners	368

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. We have adhered to the recent practice of dividing the derivatives into two groups: those of the coded amino-acids and those of other amino-acids.

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Coded Amino-acids

Compound	*M.p.*/°C	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Alanine					
Adpoc.Ala.OH DCHA	159—160	+6.22	0.65	EtOH	19
Fmoc.Ala.OH	143—144	−18.6	1	DMF	15
Fmoc.Ala.ONp	166—167	−41.5	1—2	DMF + 1% HOAc	17
H.Ala.OPic.2HBr	165—167	+2	1	DMF	29
Moc.Ala.OH	118—119	−3.1*[5]	1.2	MeOH	18
Npys.Ala.OH	144—145	−36.8	1	MeOH	24
Xan.NCA.Ala	118—119	+64.0[a]	1	benzene	22
Z.Ala.OHFP	oil	—	—	—	48
Z.Ala.OTAT	167—169	−121.5	—	$CHCl_3$	46
Z(OMe)Ala.OTAT	139—141	−108.5	—	$CHCl_3$	46
Ppoc.Ala.OH.DCHA	114.5—115.5	−3.9	1	MeOH	11
Arginine					
Adpoc.Arg(NO_2).OH.$\frac{1}{2}H_2O$	206	−3.6	0.8	DMF	19
Boc.Arg(Mds).OH	175—176	+3.5*[4]	0.5	MeOH	40
Fmoc.Arg(Boc).OH	170—171	−11.5	1	DMF	15
H.Arg(Mds).OH	120—121	−7.8	0.7	MeOH	40
Ppoc.Arg(NO_2).OH.DCHA	105(dec.)	+4.0	1	MeOH	11
Z.Arg.(Adpoc).OH	158—160	+15.2	1.2	$CHCl_3$	19
Z.Arg.(Mds).OH	140—141	+5.7	0.5	MeOH	40
Asparagine					
Fmoc.Asn.OH	185—186	−11.4	1	DMF	15
Fmoc.Asn.ONp	170—172	−37.8	1—2	DMF + 1% HOAc	17
Npys.Asn.OH	175—176	−11.0	1	DMF	24
Aspartic acid					
Adpoc.Asp.(OBu^t).OH	178	+10.3	0.8	MeOH	19
Fmoc.Asp.(OBu^t).OH	148—149	−20.3	1	DMF	15
Fmoc.Asp.(OBu^t).ONp	45—50 (amorph.)	−34.2	1—2	DMF + 1% HOAc	17
Z(OMe)Asp(OBzl).OTAT	110—112	−46.8	—	$CHCl_3$	46
Cysteine					
Boc.Cys(Acm)(O).OH	159.5—161	−46.3*[2]	1.1	MeOH	34
Boc.Cys(Npys).OH.DCHA	150—152	−86.5	1	MeOH	24
Fmoc.Cys(Bu^t).OH	135—136	−23.2	1	DMF	15
Fmoc.Cys(SBu^t).OH	74—76	−84.6	1	EtOAc	15
H.Cys(Acm)(O).OH	168	−9.4*[2]	0.9	H_2O	34
H.Cys(MBzl)OBzl.TosOH	138—142	−14.4	2	MeOH	342
Xan.NCA.Cys(Xan)	145—146	+272.1[a]	1	benzene	22
Glutamine					
Bpoc.Gln(Mbh)OTcp	93—95	−4.0	0.25	EtOAc	299
Fmoc.Gln.ONp	182—185	−40.3	1—2	DMF + 1% HOAc	17
Npys.Gln.OH	137—139	+33.5	1	MeOH	24
Z.Gln(Mbh).OTcp	177—178	−8.0	1	DMF	299
Glutamic acid					
Fmoc.Glu(OBu^t).OH	76—77	+0.8	1	EtOAc	15
Fmoc.Glu(OBu^t).ONp	oil	−14.0	1—2	DMF + 1% HOAc	17
Fmoc.Glu.OH	221—223	−17.0	1	DMF	15
Nps.Glu(OMe)OH.DCHA	192	−30.4	1	MeOH	113

*[1] $[\alpha]_D$ recorded at 589 nm, unless indicated by superscript [a] 578 nm, and measured at 20—25 °C. *[2] $[\alpha]_D$ measured at 18 °C. *[3] $[\alpha]_D$ measured at 19 °C. *[4] $[\alpha]_D$ measured at 26 °C. *[5] $[\alpha]_D$ no specified temperature.

Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Xan.NCA.Glu(OMe)	99—100	+86.7[a]	1	benzene	22
Xan.NCA.Glu(ONb)	143	+99.4[a]	1	benzene	22
Glycine					
Adpoc.Gly.OH	181	—	—	—	19
Boc.Gly.OBt	131—132	—	—	—	50
Fmoc.Gly.OH	173—174	—	—	—	15
Fmoc.Gly.ONp	133—136	—	—	—	17
Moc.Gly.OH	97—98	—	—	—	18
Npys.Gly.OEt	70—71	—	—	—	24
Npys.Gly.OH	143—144	—	—	—	24
Ppoc.Gly.OH	73—74	—	—	—	11
Trt.Gly.ONp	155—157	—	—	—	195
Z.Gly.OHFP	oil	—	—	—	48
Z(OMe)Gly.OTAT	115—116	—	—	—	46
Histidine					
Adpoc.His(Adpoc).OH	113	+9.3	0.5	DMF	19
Boc.His(MBS).OH	128—131	+14.2	1.1	MeOH	36
Boc.His.(MBS)OH.DCHA	155—158	+22.7	0.7	MeOH	36
Boc.His(MBS).ONp	138—143	−2.0	2.3	DMF	36
Fmoc.His(Boc.Tf).OH	143—145	+14.7	1	EtOAc	15
H.His(MBS).OH	155—158	+10.9	2.4	10% HOAc	36
Z.His(MBS).OH	64—65	+7.2	0.1	MeOH	36
Z.His(MBS).OH.DCHA	147—150	+29.9	2.5	MeOH	36
Z.His(MBS).ONp	65—68	−4.5	2.0	DMF	36
Z(OMe)His.(MBS).OH	79—81	+9.4	1.3	MeOH	36
Z(OMe).His(MBS)OH.DCHA	155—158	+20.9	0.6	MeOH	36
Z(OMe).His(MBS).ONp	82—84	+2.0	2.3	DMF	36
Isoleucine					
Fmoc.Ile.OH	145—147	−11.9	1	DMF	15
Fmoc.Ile.ONo	101—103	−45.4	1—2	DMF+1% HOAc	17
Moc.Ile.OH	71—73	−0.4*[5]	1.2	MeOH	18
Npys.Ile.OH	114—116	+45.8	1	MeOH	24
Xan.NCA.Ile	172—173	+71.3[a]	1	benzene	22
Leucine					
Adpoc.Leu.OH	176	−12.8	1	MeOH	19
Fmoc.Leu.OH	153—154	−24.1	1	DMF	15
Fmoc.Leu.ONp	115—116	−50.0	1—2	DMF+1% HOAc	17
Moc.Leu.OH	155—157	−11.8*[5]	1.2	MeOH	18
Npys.Leu.OH	104—106	−15.3	1	MeOH	24
Ppoc.Leu.OH	112 (dec.)	−21.0	1	MeOH	11
Xan.NCA.Leu	131—132	+62.5[a]	1	benzene	22
Z(OMe)Leu.NH_2	134—136	−7.8	0.8	MeOH	73
Lysine					
Adpoc.Lys(Z)OH.DCHA	75	−4.5	0.9	MeOH	19
Boc.Lys.(DMCZ)OH.DCHA	168—169	+3	0.4	AcOH	30
Boc.Lys(Npys).OH	107—109	+6.1	1	MeOH	24
Boc.Lys(Npys)OH.DCHA	122—124	−23.7	1	MeOH	24
Boc.Lys(Pipoc)OH.DCHA	135—136	+8	1.07	MeOH	29
Fmoc.Lys(Boc).OH	123—124	−11.7	1	DMF	15
Fmoc.Lys(Boc).ONp	105—107	−35.3	1—2	DMF+1% HOAc	17
H.Lys(DMCZ).OH	163—164	+4	0.5	AcOH	30

Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Ppoc.Lys(Z).DCHA	89—91	+12.7	1	MeOH	11
Z(OMe).Lys(Moc).OH	124—126	−2.1*[5]	1.2	MeOH	18
Methionine					
Adpoc.Met.OH.DCHA	150	−22.8	0.8	MeOH	19
Fmoc.Met.OH	129—132	−28.3	1	DMF	15
Fmoc.Met.ONp	125—127	−51.6	1—2	DMF+1% HOAc	17
H.Met.ONp.HCl	147.5—148.5	+1.75	2	DMF	63
H.Met.OPcp.HCl	168—169	+14.5	2	DMF	63
H.Met.OPfp.HCl	130—130.5	+11.25	2	DMF	63
Npys.Met.OH.DCHA	173—175	+50.1	1	MeOH	24
Ppoc.Met.OH	76—77.5	−16.7	1	MeOH	11
Z.Met.ONp	97—98	−30.75	2	THF	63
Z.Met.OPfp	69.5—71	−13.75	2	THF	63
Z.Met.OTAT	94—96	−107.1	—	$CHCl_3$	46
Phenylalanine					
Adpoc.Phe.OH.DCHA	124	+21.2	1.2	MeOH	19
Fmoc.Phe.OH	181—183	−37.6	1	DMF	15
Fmoc.Phe.ONp	207—209	−43.0	1—2	DMF+1% HOAc	17
Moc.Phe.OH	153—154	+34.7*[5]	1.2	MeOH	18
Ppoc.Phe.OH.DCHA	151—152.5	+37.6	1	MeOH	11
Xan.NCA.Phe	121—122	+112.3[a]	1	benzene	22
Z.Phe.OHFP	80—81.5	—	—	—	48
Z.Phe.OTAT	127—129	−41.5	—	$CHCl_3$	46
Proline					
Fmoc.Pro.OH	114—115	−33.9	1	DMF	15
Fmoc.Pro.ONp	112—113	−62.7	1—2	DMF+1% HOAc	17
Moc.Pro.OH	54	−65.0*[5]	1.2	MeOH	18
Npys.Pro.OH	133—135	−135.9	1	MeOH	24
Ppoc.Pro.OH	105.5—106.5	−34.8	1	MeOH	11
Serine					
Adpoc.Ser(Bu^t).OH	188—189	+15.7	0.8	MeOH	19
Fmoc.Ser(Bu^t).OBzl	70—71	−5.2	1	EtOAc	15
Fmoc.Ser(Bu^t).OH	126—129	−1.5	1	DMF	15
Fmoc.Ser(Bu^t).ONp	54—58 (amorph.)	−29.7	1—2	DMF+1% HOAc	17
Fmoc.Ser.OBzl	97—98	+1.4	1	EtOAc	15
Fmoc.Ser.OH	86—88	+14.9	1	EtOAc	15
H.Ser.(SO_3H).OH.HCl	230	+9.8	3.4	lM HCl	31
Npys.Ser.OH	132—134	+8.1	1	MeOH	24
Z.Ser.(DMC Bzl)OH.DCHA	126—128	+19	0.8	$CHCl_3$	30
Z.Ser.(Npys)OH.DCHA	151—153	−17.4	1	MeOH	24
Threonine					
Fmoc.Thr(Bu^t).OH	129—132	−4.5	1	DMF	15
Fmoc.Thr(Bu^t).OBzl	69—70	+6.65	1	EtOAc	15
Fmoc.Thr(Bu^t).ONp	49—53 (amorph.)	−23.9	1—2	DMF+1% HOAc	17
Fmoc.Thr.OBzl	112—113	−6.25	1	EtOAc	15
Trt.Thr.OMe	oil	+5.4	0.94	$CHCl_3$	369
Trt.Thr(Tos)OMe	120—122	+87.7	0.9	$CHCl_3$	369
Z.Thr.OBu^t	63	−21.8*[3]	1	MeOH	67
Z.Thr(For)OH.DCHA	147—148	+21.6	0.5	DMF	67
Z.Thr(Npys)OH.DCHA	92—94	−47.9	1	MeOH	24

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Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Tryptophan					
Adpoc.Trp.OH	116	−6.8	1	MeOH	19
Boc.Trp(For).OH.DCHA	155—156	+135	3.41	EtOH	89
Fmoc.Tyr.(Bu^t).OH	165—166	−26.6	1	DMF	15
Fmoc.Tyr.(Bu^t).ONp	213—215	−19.6	1—2	DMF + 1% HOAc	17
Npys.Trp.OH.DCHA	128—131	−55.9	1	MeOH	24
Z.Trp.OTAT	129—130	−24.4	—	$CHCl_3$	46
Tyrosine					
Boc.Tyr.(DMC Bzl).OH	81—83	+31	1.3	$CHCl_3$	30
Boc.Tyr.(DMC Bzl).OH. DCHA	145—146	+23	0.96	MeOH	30
Boc.Tyr.(Bzl).OPic	78—80	−18.5	1.1	DMF	29
Fmoc.Tyr.(Bzl).OPcp	182—184	−49.6	1—2	DMF + 1% HOAc	17
Fmoc.Tyr.(Bu^t).OH	150—151	−27.6	1	DMF	15
Fmoc.Tyr.(Bu^t).ONp	90—91	−21.1	1—2	DMF + 1% HOAc	17
H.Tyr.(DMC Bzl).OH	199—200	−6	1.1	HOAc	30
H.Tyr.OBzl	110—114	—	—	—	370
Nps.Tyr.(Bu^t).OH	96—98	+99.2	1	MeOH	24
Xan.NCA.Tyr(Ac)	113—114	+64.5[a]	1	benzene	22
Valine					
Adpoc.Val.OH.DCHA	105	+7.8	0.9	MeOH	19
Boc.Val.OHFP	89—90	—	—	—	48
Fmoc.Val.OH	143—144	−16.1	1	DMF	15
Fmoc.Val.ONo	111—112	−58.4	1—2	DMF + 1% HOAc	17
Moc.Val.OH	110—111	−8.4*[5]	1.2	MeOH	18
Npys.Val.OH	151—152	+35.4	1	MeOH	24
Xan.NCA.Val	115—116	+81.5[a]	1	benzene	22
Z(OMe)Val.OTAT	124—126	−39.5	—	$CHCl_3$	46
Other Amino-acids					
Alanine related					
β-(Adeninyl)-alanine (Aal)					
Boc.Aal.NH.NH_2	226	—	—	—	363
Boc.Aal.OH	155	—	—	—	363
Boc.Aal.OMe	178	—	—	—	363
Phth.Aal.OH	268	—	—	—	363
Tos.Aal.OH	195	—	—	—	363
β-Pyridylalanine					
Ac-β-(2-pyridyl)-D-Ala.OEt	oil	+5.5	1.05	MeOH	88
Ac-β-(2-pyridyl)-L-Ala.OH	154—155	+12.6	1.3	MeOH	88
Boc-β-(2-pyridyl)-L-Ala.OH	106—107	−17.4	1.06	MeOH	88
Boc-β-(4-pyridyl)-L-Ala.OH	217	−3.7	0.63	HOAc	88
β-(Uracilyl)-alanine (Ual)					
Boc.Ual.NH.NH_2	252	—	—	—	363
Boc.Ual.OEt	190	—	—	—	363
Boc.Ual.OH	198	—	—	—	363
Boc.Ual.OMe	186	—	—	—	363

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Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Boc.Ual.ONp	191	—	—	—	363
H.Ual.OMe.2HCl	178	—	—	—	363
D-Arginine					
D-H.Arg(Mts).OH	152—156	+5.0	0.5	MeOH	216
Z(OMe).D-Arg.OH	191—194	+1.6	0.6	AcOH	216
Z(OMe).D-Arg(Mts) OH.CHA	150—153	−6.9	0.2	MeOH	216
t-Butylglycine (Bug)					
Boc.Bug.OH.DCHA	165	−7.2	1	MeOH	220
H.Bug.OMe	170	+17.0	1	MeOH	220
Cystathionine (Cst)					
N^{α}Boc.Cst.α′-OMe	185—186	+37.2	0.5	MeOH	371
$N^{\alpha'}$Boc.Cst.α′O-Me	160—165	−43.6	0.5	MeOH	371
N^{α}Boc,$N^{\alpha'}$-Z.Cst. α′-OMe,α-OH,DCHA	100—104	−14.5	0.5	MeOH	371
$N^{\alpha'}$Nps,N^{α}Boc.Cst. α′-OMe,α-OH,DCHA	58—60	−17.6	0.2	MeOH	371
$N^{\alpha'}$Z.Cst.α-OBu^t	84—87	−4.9	0.2	MeOH	371
$N^{\alpha'}$Z.Cst.α-OBu^t,α′-OMe	oil	+2.4	0.5	benzene	371
$N^{\alpha'}$Z(OMe).Cst.α′-OMe	158—162	−48.7	0.2	MeOH	371
3,4-Dihydroxyphenylalanine (DOPA)					
L-H.DOPA(OMe)$_2$ONp.HBr	219	+20.5	1	MeOH	113
L-Z.DOPA(OMe)$_2$OH	117	+13.4	1	EtOH	113
L-Z.DOPA(OMe)$_2$ONp	138	−10.0	1	EtOAc	113
γ-Carboxyglutamic acid (Gla)					
D,L-For.Gla(OBu^t)$_2$.OH	139—141	—	—	—	372
D,L-H.Gla(OBu^t)$_2$.OEt	131—132	—	—	—	372
D,L-Z.Gla (OBu^t)$_2$.OH. DCHA	135—137	—	—	—	372
N^5-Dimethylglutamine					
Boc.Gln(N^5-Me$_2$).OH	124—125	+2.2	1	EtOH	300
2-Methylalanine					
H.(2Me.Ala)O.CH$_2$CN.HCl	202—205	—	—	—	373
H.(2Me.Ala).ONp.HCl	177—180	—	—	—	373
H.(2Me.Ala).OTmb.HCl	145.5—146.5	—	—	—	373
Nps(2Me.Ala).OH.DCHA	175—178	—	—	—	373
Nps.(2Me.Ala).OH	116.5—121	—	—	—	373
Nps.(2Me.Ala).ONp	132—133	—	—	—	373
Nps.(2Me.Ala).OTmb	133.5—135	—	—	—	373
Z.(2Me.Ala).OTmb	118—119	—	—	—	373
N-Methylphenylalanine					
H(Me)Phe.OMe.HCl	85—87	+59	1	$CHCl_3$	29
Ornithine (Orn)					
H.Orn(Msc).OH	197—198	+2.0	1	H_2O	130
Z.Orn(Msc).ONp	127	−15.3	1	Dioxan	130
Selenocysteine (Sec)					
Boc.Sec(Bzl).OH	130—132	−6.7	1	DMF	293

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4

Peptides with Structural Features not Typical of Proteins

BY P. M. HARDY

1 Introduction

Subject headings in this chapter have in some cases been changed from former reports, reflecting alterations in patterns of published papers rather than overall area of coverage. The only major alteration is the omission of work on the synthesis of the β-lactam antibiotics. The relatively coherent nature of this topic in what is essentially a more fragmentary collection of diverse structural types ensures its regular review in other publications, and in the space available it is impossible to do the subject justice. A few synthetic analogues of hormones have been covered where the type of molecular change is directly pertinent to the topic under review, but simple substitutions with *e.g.* D-amino-acids are, as usual, not covered in this section. No explicit references to earlier work are given; in general these are contained in the 1980 references cited.

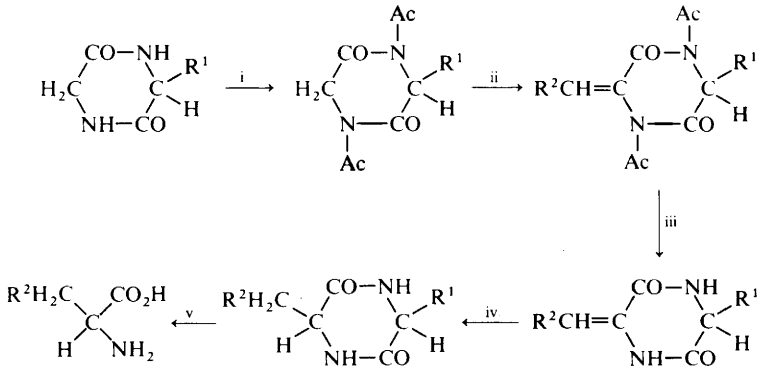
2 Cyclic Peptides

2,5-Dioxopiperazines (Cyclic Dipeptides).—*Cyclo*(-L-His-L-Pro-), found in 1975 to be a metabolite of thyrotropin releasing hormone and having a variety of biological activities, has now been shown to be an inhibitor of dopamine uptake in nerve endings. The mechanism involves a specific inhibition of the $(Na^+ + K^+)$-ATPase.[1] *Cyclo*(-L-Trp-L-Leu-) has been patented for use as the bitter principle in the preparation of lemonade,[2] but the other reports this year of synthetic 2,5-dioxopiperazines composed solely of the amino-acids found in proteins concern stereochemical matters. An n.m.r. study of ^{13}C-enriched *cyclo*(-Ala-Ala-), *cyclo*(-Ala-Phe-), *cyclo*(-Val-Val-), and *cyclo*(-Leu-Leu), both L-L and L-D diastereoisomers, has been made. A combination of ^{13}C–^{13}C and ^{1}H–^{1}H coupling constants enabled the side-chain conformations of valyl and leucyl residues to be established.[3] Empirical conformational energy calculations for *cyclo*(-L-Asp-L-His-) and *cyclo*(-L-Glu-L-His-) suggest that distortions of the dioxopiperazine ring can modify the stability of some conformations through peptide backbone–side-chain or side-chain–side-chain interactions.[4]

The conformations of six *N*-methylated 2,5-dioxopiperazines have been determined by n.m.r. and c.d. and compared to earlier *X*-ray crystallography

[1] F. Battaini and A. Peterkofsky, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 240.
[2] F. Esser and D. Essig, *Ger. P.*, 2 840 442 (*Chem. Abstr.*, 1980, **93**, 186 798*q*).
[3] J. Vicar, F. Pirion, P. Fromageot, K. Bláha, and S. Fermandjian, *Collect. Czech. Chem. Commun.*, 1980, **45**, 435.
[4] M. Genest and M. Ptak, *Int. J. Pept. Protein Res.*, 1980, **15**, 5.

results. Of the six compounds, *cyclo*(-L-*N*MeVal-)$_2$, *cyclo*(-L-*N*MePhe-)$_2$, and *cyclo*(-L-*N*MePhe-D-*N*MePhe-) maintain similar ring shapes in solution and the crystalline state, but *cyclo*(-L-*N*MeAla)$_2$, *cyclo*(-L-*N*MeAla-D-*N*MeAla-), and *cyclo*(-L-*N*MeVal-D-*N*MeVal-) differ substantially in conformation. Strongly protonating solvents were not found to affect the angle of fold of the dioxopiperazine ring.[5] Further work on the asymmetric synthesis of amino-acids by the catalytic hydrogenation of cyclic dipeptides containing an L-amino-acid and an $\alpha\beta$-dehydroamino-acid residue (Scheme 1) more clearly delineates the dependency of

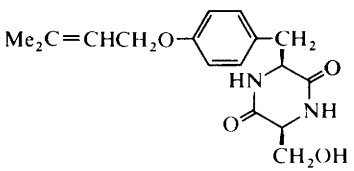


Reagents: i, Ac_2O at 130 °C; ii, R^2CHO, $KOBu^t$; iii, N_2H_4; iv, H_2–Pd, MeOH; v, HCl

Scheme 1

the degree of chiral induction on the amino-acid side-chains. Alkyl side-chains on the initial chiral centre confer high stereospecificity (96.2—99.5%) even with a small side-chain as in L-alanine. However, if the $\alpha\beta$-dehydro-residue is an aromatic one, results are less satisfactory, *e.g.* in *cyclo*(-L-Phe-L-Ala-) and *cyclo*(-L-Trp-L-Ala-) preparations only 88.2% and 71.0%, respectively, of aromatic L-amino-acid were obtained. These results are ascribed to steric hindrance between the aromatic groups and the amide protons in the unsaturated dioxopiperazine preventing coplanarity of the dioxopiperazine and aromatic rings.[6]

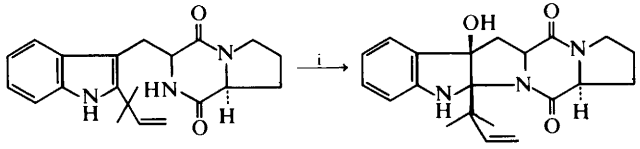
The c.d. spectra of the LL- and DL-isomers of *cyclo*(-3-ferrocenyl-Ala-Pro-) have been compared with the corresponding diastereoisomeric cyclodipeptides containing phenylalanine instead of its analogue,[7] and the metabolite phomamide (1) from *Phoma lingam*, which is thought to be an intermediate in the biosynthesis of



(1)

[5] W. Radding, B. Donzel, N. Ueyama, and M. Goodman, *J. Am. Chem. Soc.*, 1980, **102**, 5999.
[6] T. Kanmera, S. Lee, H. Aoyagi, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1980, **16**, 280.
[7] J. Pospisek, S. Toma, I. Fric, and K. Bláha, *Collect. Czech. Chem. Commun.*, 1980, **45**, 435.

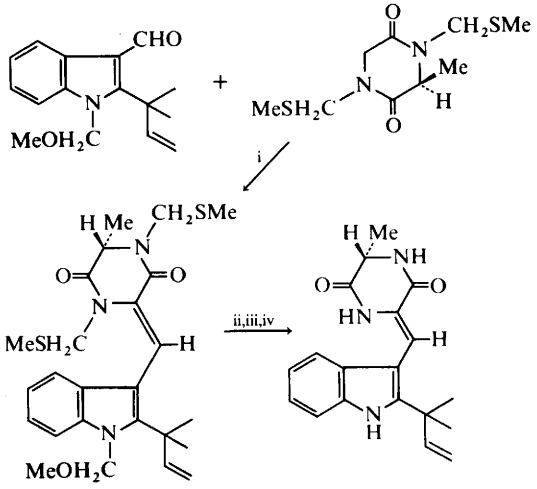
the sirodesmin group of antibiotics, has been synthesized from *cyclo*(-Tyr-Ser-).[8] Natural dioxopiperazines containing modified tryptophan residues have been the subject of two successful synthetic studies. The first synthesis of optically active brevianamide E, isolated from *Penicillium brevicompactum*, by an oxidative photocyclization of deoxybrevianamide E (Scheme 2) has been reported. Since



Reagent: i, *hν*, MeOH, −10 °C, Rose Bengal, O_2

Scheme 2

deoxybrevianamide E has been earlier synthesized, this constitutes a total synthesis, and confirms the relative stereochemistry and the absolute configuration.[9] A second total synthesis of neoechinulin A (2; Scheme 3) involves a



(2)

Reagents: i, $LiNPr^i_2$, THF, −78 °C; ii, MeI, $NaHCO_3$; iii, dioxan, 100 °C; iv, HCO_2H, H_2O, 20 °C

Scheme 3

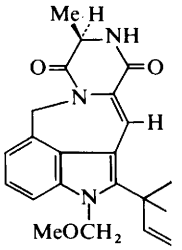
regiospecific aldol condensation of an *N*-alkylated *cyclo*(-Ala-Gly-). A 55% yield of the desired *Z*-isomer was obtained together with 5% of the *E*-isomer. After deprotection, in addition to neoechinulin A a byproduct (3) was obtained that contains an extra ring system and is related to the natural product clavicipitic acid (4).[10]

The feeding of (2*S*,3*S*)- and (2*S*,3*R*)-[3-^{3}H]histidine to cultures of *Penicillium roqueforti* and *P. oxalicum* has led to good incorporation into the metabolites

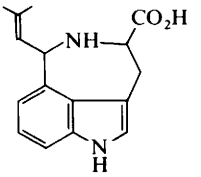
[8] J. P. Ferezou, A. Quesneau-Thierry, M. Barbier, A. Kollman, and J. F. Bousquet, *J. Chem. Soc., Perkin Trans. 1*, 1980, 113.

[9] T. Kametani, N. Kanaya, and M. Ihara, *J. Am. Chem. Soc.*, 1980, **102**, 3974.

[10] S.-I. Nakatsuka, H. Mizayama, and T. Goto, *Tetrahedron Lett.*, 1980, 2817.

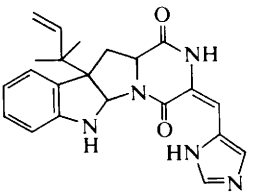


(3)

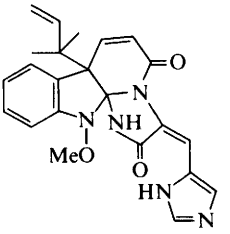


(4)

roquefortine (5) and oxaline (6) respectively. The radioactivity of the products indicates that the 3-*pro-S* hydrogen of (2*S*)-histidine is stereospecifically eliminated while tritium from the 3-*pro-R* position is retained, indicating a *syn*



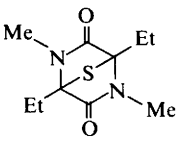
(5)



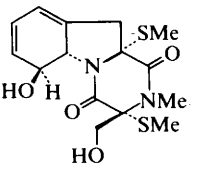
(6)

elimination of the *pro-S* hydrogen at C-3 and H-2. The dehydrohistidine unit of roquefortine is also assigned the *E*-configuration on spectral evidence, according with the previously known stereochemistry of this unit in oxaline.[11]

Sulphur bridged dioxopiperazine ring systems continue to be a focus of interest. *X*-Ray examination of the 3,6-epithio-2,5-dioxopiperazine (7) shows that the C—S bonds are longer than those in L-cysteine. The other intra-ring bond distances are also longer than those found in unbridged boat-shaped dioxopiperazines.[12] Bisdethiobis(methylthio)gliotoxin (8) has been identified as a minor metabolite of



(7)

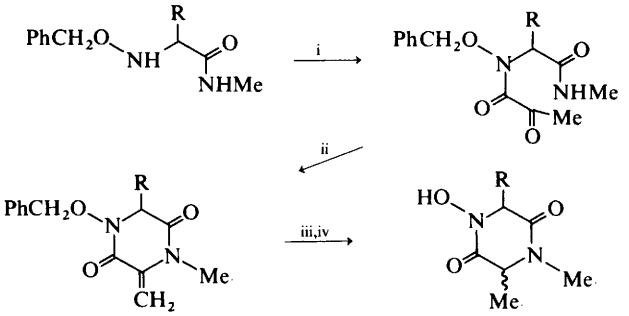


(8)

Gliocladium deliquescens. It can be prepared from gliotoxin by reduction and methylation, and feeding experiments with ^{14}C-labelled gliotoxin have established that it is formed, apparently irreversibly, from gliotoxin.[13] Functionalization of dioxopiperazines at their α-positions has been approached *via* *N*-hydroxy-intermediates. *N*-Methyl-*N'*-hydroxy-2,5-dioxopiperazines can be prepared from

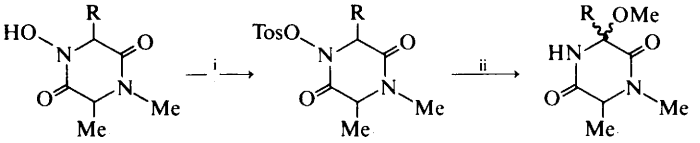
[11] R. Vleggar and P. L. Wessels, *J. Chem. Soc., Chem. Commun.*, 1980, 160.
[12] H. Shimanouchi, Y. Sasada, and K. Koyano, *Acta Crystallogr.*, 1980, **B36**, 475.
[13] G. W. Kirby, D. J. Robins, M. A. Sefton, and R. R. Talekar, *J. Chem. Soc., Perkin Trans. 1*, 1980, 119.

N-pyruvoyl dipeptides (Scheme 4), and α-methoxy-groups introduced after *O*-tosylation (Scheme 5). However, attempts to introduce a methylthio-group in the



Reagents: i, MeCOCOCl; ii, CF_3CO_2H; iii, Pd–C, H_2; iv, $(CF_3CO_2)_3B$

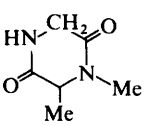
Scheme 4



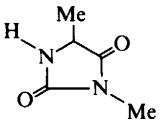
Reagents: i, TosCl, NEt_3; ii, Me_3COK, MeOH

Scheme 5

same way using the sodio-derivative of methanethiol in propan-2-ol led to the reduced dioxopiperazine (9) in one case and a hydantoin (10) in another.[14] Treatment instead with hydrogen sulphide in the presence of zinc chloride



(9)



(10)

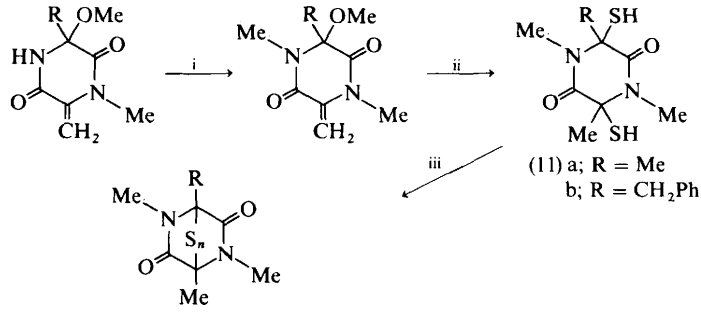
enabled sulphur to be introduced at the α-position; if an αβ-unsaturated amino-acid residue is also present in the ring, two α-thiol groups can be inserted (Scheme 6). However, whereas oxidation of the 3,6-dimethyl derivative (11a) gave a mixture of epithio- and epidithio-dioxopiperazine (12a), the presence of a benzyl side-chain (11b) led only to monosulphide formation (12b).[15]

The other modified cyclodipeptide form which is still being probed involves a cyclol unit. The ergot peptide ergostine (13) prepared using *Claviceps purpurea* from (*R*, *S*)-2-amino-[3-^{13}C, 3$^{2}H_2$]butyric acid (Scheme 7) shows the retention of both atoms of deuterium as well as the ^{13}C-label, ruling out a 2,3-dehydro-intermediate in the formation of the α-hydroxy-α-amino-acid part of the peptide.[16]

[14] J. D. M. Herschied, R. J. F. Nivard, M. W. Tijhuis, H. P. H. Sholten, and H. C. J. Ottenheijm, *J. Org. Chem.*, 1980, **45**, 1880.

[15] J. D. M. Herschied, R. J. F. Nivard, M. W. Tijhuis, and H. C. J. Ottenheijm, *J. Org. Chem.*, 1980, **45**, 1885.

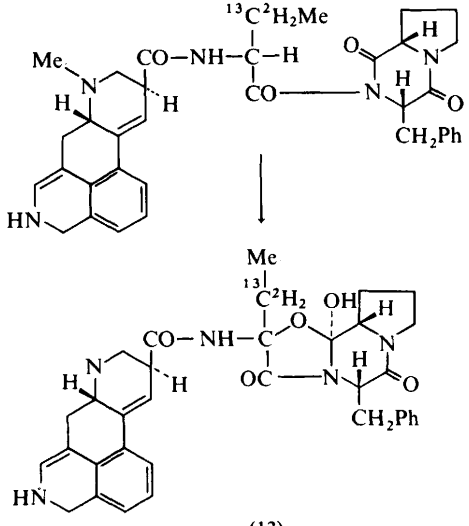
[16] C. M. Belzecki, F. R. Quigley, H. G. Floss, N. Crespi-Perellino, and A. Guicciardi, *J. Org. Chem.*, 1980, **45**, 2215.



(12) a; $n = 1$ or 2
b; $n = 1$

Reagents: i, MeI; ii, $ZnCl_2$, liquid H_2S; iii, I_2

Scheme 6

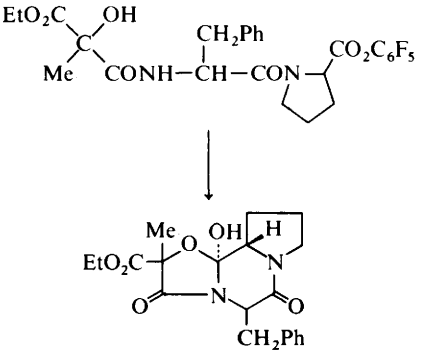


(13)

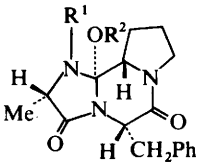
Scheme 7

Oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazines (oxacyclols) have been prepared from dipeptides activated as pentafluorophenyl esters. An example is given in Scheme 8.[17] Final results of the *X*-ray crystallographic examination of azacyclols (14) prepared by the action of mild base on Z- or *p*-Br-Z-L-Ala-L-Phe-L-Pro-ONp indicate that both the five-membered rings assume an envelope conformation, and in the six-membered ring only the carbon atom bearing the OH or OMe group is out of the plane of the other ring atoms.[18] *X*-Ray analysis of the structure of the first peptidic thiacyclol to be prepared is also reported. This compound (15), prepared from *N*-(2-tritylthiopropionyl)-*cyclo*(-D-Phe-L-Pro-) by successive treatments with $Na_2S_2O_3$

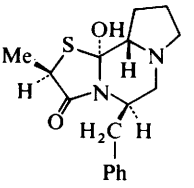
[17] P. Stuetz and P. Stadler, *Swiss P.*, 616 912 (*Chem. Abstr.*, 1980, **93**, 205 033*a*).
[18] G. Lucente, A. Romeo, S. Cervini, W. Fedeli, and F. Mozza, *J. Chem. Soc., Perkin Trans. 1*, 1980, 809.



Scheme 8



(14) R^1 = $PhCH_2OCO$— or *p*-Br-$C_6H_4CH_2OCO$—
R^2 = H or Me



(15)

and $NaBH_4$, shows in its six-membered ring both the C-atom bearing the OH group and the adjacent N-atom out of the plane of the other ring atoms.[19]

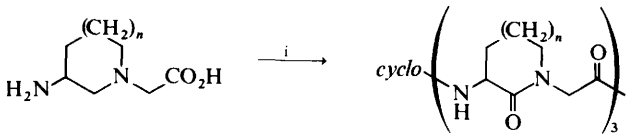
Larger Cyclic Peptides.—This section covers both naturally occurring and synthetic cyclic peptides other than 2,5-dioxopiperazines. The order in which they are considered is based on the number of amino-acid residues in the ring system; pendant amino-acids or peptide chains are ignored for this purpose. Reports relating solely to conformational studies, however, are dealt with separately in the next section.

Ferrichrome has been found to act as an ionophore for Ca^{II} or Mg^{II} ions, but deferri-ferrichrome lacks this ability. The ferrichrome series of siderophores seems to be unique amongst the hydroxamate class in this ability. It has been suggested that ferrichrome complexes to Mg^{II} ions close to the cytoplasmic membrane surface in natural systems, and it is the resulting positively charged complex that is transported across the membrane.[20] Analogues of *cyclo*(-Ala-Sar-)$_3$ have been prepared (Scheme 9) by cyclotrimerization. These compounds contain covalent bridges between the methyl groups of the alanine and sarcosine residues; only the δ-lactam of the *S*-configuration showed the same ability as *cyclo*(-Ala-Sar-)$_3$ to inhibit methane production during fermentation in rumen stomach fluid. During cyclization 2,5-dioxopiperazine formation is inhibited as a result of steric constraints.[21]

[19] G. Lucente, F. Pinner, G. Zanatti, S. Cervini, W. Fedeli, and F. Mazza, *J. Chem. Soc., Perkin 1*, 1980, 1499.

[20] R. C. Hider, A. F. Drake, B. Kuroda, and J. B. Neilands, *Naturwissenschaften*, 1980, **67**, 136.

[21] R. M. Freidinger, D. F. Veber, R. Hirschmann, and L. M. Paege, *Int. J. Pept. Protein Res.*, 1980, **16**, 464.

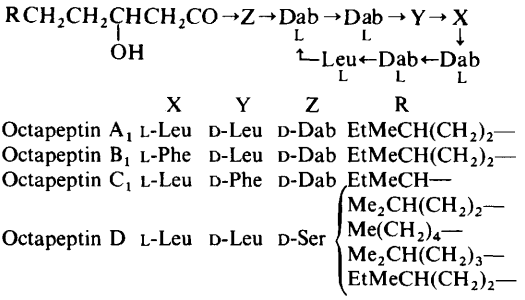


$n = 0$; racemic, *sym* and *asym*
$n = 1$; *SSS* and *RRR*
$n = 2$; *SSS*

Reagent: i, diphenylphosphoryl azide, NEt_3, DMF, $-20\,°C$

Scheme 9

A new member of the octapeptin group of antibiotics from the *Bacillus* strain JP-301, octapeptin D (16),[22] has been characterized, and a ^{13}C-n.m.r. study has confirmed the fatty-acid side-chains and amino-acid differences in octapeptins A_1, B_1, and C_1 (16).[23] The complex of components comprising octapeptin D give the

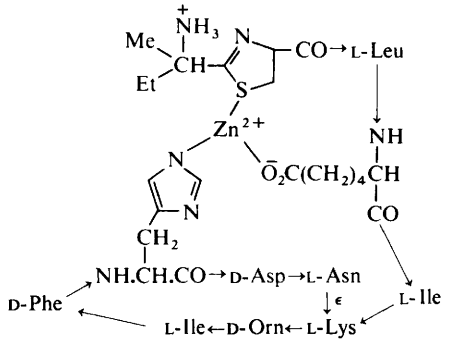


(16)

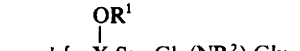
same cyclic peptide on removal of the fatty-acid moiety with the enzyme polymyxin acylase.[22] The antibiotic activity of bacitracin A is dependent on the presence of a divalent metal cation, but the actual binding site has been the source of conflicting reports. The only point hitherto commonly agreed is the lack of involvement of the amino-group of ornithine. A new potentiometric and u.v. study of the pH dependence of metal binding indicates that the metal co-ordinates to the glutamate carboxyl, the histidine imidazole, and the thiazoline ring, as shown in (17).[24] A further ^1H-n.m.r. examination of the Zn^{II} complex of bacitracin A accords with this proposed structure.[25]

Two synthetic octapeptides (18) have been prepared and their abilities to transport ions across the liquid membrane of a Pressman cell (which comprises a U-tube in which an organic liquid such as chloroform separates two aqueous phases) examined. In the case of the cyclic peptide containing glutamic acid with a free α-carboxy-group and *N*-decylglycine, transport was observed to be essentially calcium specific.[26] Gramicidin S can usually be relied on for a few papers each year, and 1980 is no exception. Further exploration of how variation of the amino-acid sequence in a linear pentapeptide precursor affects the composition and yield

[22] T. Kato and J. Shoji, *J. Antibiotics*, 1980, **33**, 186.
[23] M. S. Puar, *J. Antibiotics*, 1980, **33**, 760.
[24] D. A. Scogin, H. I. Mosberg, D. R. Storm, and R. B. Gennis, *Biochemistry*, 1980, **19**, 3348.
[25] H. I. Mosberg, D. P. Scogin, D. R. Storm, and R. B. Gennis, *Biochemistry*, 1980, **19**, 3353.
[26] C. M. Deber, *Can. J. Biochem.*, 1980, **58**, 865.



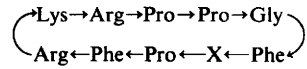
(17)



(18) X = Glu or Asp, R^1 = H or Bzl
R^2 = n-decyl or cyclohexyl

of the cyclic product shows that, of five sequences examined, Pro-Val-Orn(Z)-Leu-D-Phe gave the highest ratio of dimer : monomer (80 : 19). With Orn(Z) and Pro as the *C*-terminal amino-acids, cyclodimerization predominated also. Preferential formation of cyclic monomer occurred with *C*-terminal Leu and Val, comparable studies being effected using the azide cyclization method.[27] The azide method has also been used to prepare [2,2′-Ser]-gramicidin S; the product shows some activity against Gram-positive micro-organisms but of an order less than gramicidin S itself.[28] The resonances of the four non-equivalent backbone amide protons of gramicidin S in ^2H_2O have been assigned by decoupling of the amide protons from their respective α-*C*-protons while exchange of the amide protons for deuterons was occurring.[29] An examination of the replacement of peptide NH group protons by chlorine in both gramicidin S and tuberactinomycin shows that hydrogen bonded NH protons are much more susceptible to substitution than solvent exposed ones.[30]

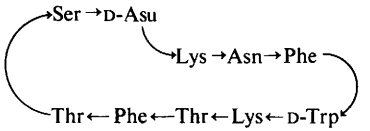
Syntheses of [8,9-Phe]- and [8,9-Leu]-tyrocidine E have been reported, azide cyclization being used; on bioassay the former showed weak antibacterial properties, but the latter had no activity.[31] A cyclic decapeptide analogue of kallidin (19) has also been prepared, in this case using an *NN*′-dicyclohexyl-carbodi-imide 1-hydroxysuccinimide mediated cyclization. At doses of 50 μg kg^{-1}



(19) X = Gly or Ser

[27] Y. Minematsu, M. Waki, K. Suwa, T. Kato, and N. Izumiya, *Tetrahedron Lett.*, 1980, 2179.
[28] M. Tamaki, M. Takimoto, S. Sofuku, and I. Muramatsu, *J. Antibiotics*, 1980, **33**, 105.
[29] A. J. Fischman, D. H. Live, W. M. Wittbold, jun., and H. R. Wyssbrod, *J. Magn. Reson.*, 1980, **40**, 527.
[30] M. Kando, K. Okamoto, I. Nishi, M. Yamamoto, T. Kato, and N. Izumiya, *Chem. Lett.*, 1980, 703.
[31] S. Ando, H. Takiguchi, H. Nishikawa, and N. Izumiya, *Fukuoka Daigaku Rigaku Shuho*, 1980, **10**, 7 (*Chem.* Abstr., 1980, **93**, 150643*w*).

in rats the hypotensive effects resembled bradykinin, but the activity was more prolonged.[32] A cyclic analogue of somatostatin (20) containing 11 amino-acid residues has been made using the *p*-nitrophenyl ester of the side-chain carboxy-group of the 2-aminosuberic acid residue to effect cyclization,[33] but no results of biological testing are reported.



(20) Asu = 2-aminosuberic acid

Conformational Studies.—Once again, in this section cyclic peptides are considered in an order based on increasing numbers of ring amino-acid residues. Two cyclotripeptides containing only tertiary amide bonds have been the subjects of an *X*-ray crystallographic study. *Cyclo*(-*N*-Bzl-Gly-)$_2$-Pro adopts a crown conformation. In solution, n.m.r. reveals that this exists in equilibrium with the boat conformation, whereas triproline was earlier shown to adopt only the boat form. *Cyclo*(-Pro$_2$-D-Pro-) adopts the boat conformation in both the crystalline form and in solution, but there are two independent forms quite similar in geometry.[34] Spectroscopic studies on two peptides related to desdimethylchlamydocin, *cyclo*(-Gly-Phe-D-Pro-Ala-) and *cyclo*(-D-Phe-Pro-D-Phe-Pro-), indicate that the conformation adopted is solvent dependent. In $CDCl_3$ the former has four *trans* amide bonds, and at least one 3 → 1 intramolecular hydrogen bond; the latter also adopts four *trans* amide bonds, but shows two inverse γ-turns. As [$^2H_6$]DMSO is added to the solutions, a conformational shift to *cis* X-Pro amide bonds begins to occur.[35]

The solution conformation of two diastereoisomeric cyclic tetrapeptides, *cyclo*(-D- and L-Tyr(Me)-Ile-Pro-Leu), analogues of the phytotoxic peptide Cyl-2 in which Pro and Leu replace L-pipecolic acid and 2-amino-8-oxo-9,10-epoxy-decanoic acid respectively, has also been examined. A unique *trans–cis–trans–cis* conformation is proposed for the D-Tyr analogue. In cyclizing the linear precursors, once again marked differences in yield were observed, the presence of the D-residue again increasing the efficiency of the reaction.[36] Theoretical calculations on the conformations likely to be adopted by cyclic pentapeptides that contain glycine and L- or D-alanine residues and have two intra-ring 3 → 1 hydrogen bonds suggest four types are possible.[37] Monte Carlo simulation of the structure of the 16 ordered and disordered water molecules in the unit cell of crystals of *cyclo*(-Ala-Pro-D-Phe-)$_2$ gives results showing positions very close to those earlier observed in *X*-ray crystallographic pictures.[38]

A Raman spectral study of *cyclo*(-Pro-Gly-)$_3$ in both the solid state and solution gives results in agreement with the conformational forms previously proposed.

[32] G. I. Cipens, F. Mutulis, O. Landa, and N. V. Myshlyakova, *Ger. P.*, 2 939 522 (*Chem. Abstr.*, 1980, **93**, 239 938).
[33] S. K. K. Shiraimatsu, *Jap. P.*, 80 59 152 (*Chem. Abstr.* 1980, **93**, 168 612*v*).
[34] J. W. Bats and H. Fuess, *J. Am. Chem. Soc.*, 1980, **102**, 2065.
[35] D. H. Rich and R. D. Jasensky, *J. Am. Chem. Soc.*, 1980, **102**, 1112.
[36] A. Yasutake, H. Aoyagi, T. Kato, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1980, **15**, 113.
[37] C. Ramakrishnan and B. N. Narasinga Roo, *Int. J. Pept. Protein Res.*, 1980, **15**, 81.
[38] A. T. Hagler, J. Moult, and D. J. Osguthorpe, *Biopolymers*, 1980, **19**, 395.

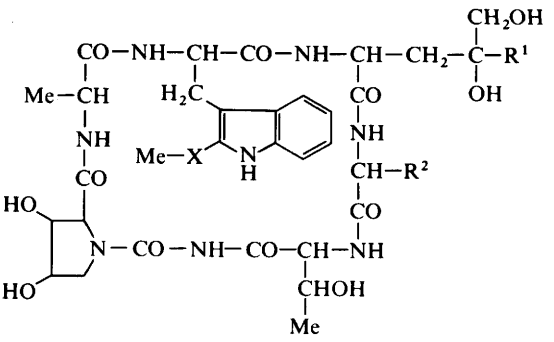
When complexed with Ca^{II}, the glycyl carbonyl groups appear to be involved in the metal ion binding; however, with monovalent cations binding seems to occur at the prolyl carbonyl groups, especially in the solid state. The spectrum of the Na complex of the lower homologue *cyclo*(-Pro-Gly-)$_2$ suggests an asymmetric structure.[39] A ^{27}Al-n.m.r. study of alumichrome, an isomorphous analogue of ferrichrome, at 65.1 MHz indicates a non-cubic ligand field configuration.[40] A more conventional n.m.r. examination of the cyclododecapeptide *cyclo*(-Ala-Pro-Gly-Val-Gly-Val-)$_2$, which contains the repeat sequence of tropoelastin, finds the conformation solvent dependent. In $CHCl_3$ alone, the ^{13}C spin–lattice relaxation times are indicative of a relatively rigid molecule. Two β-turns between Ala^{1}NH and Val^{4}CO (type II′) and Val^{4}NH and the Ala^{1}CO (type II) are proposed, the former also being present in the linear precursor.[41] Addition of DMSO to the solution changes the conformation to one in which the glycine flanked valine is *gauche*, but the other valine *trans*. The type II′ β-turn is retained, but the Gly^{3}NH and the Gly^{5}CO are now linked in an α-turn, which is also present in the linear counterpart.[42]

An *X*-ray analysis of a cyclic trimer of the repeating pentapeptide sequence of elastin, *cyclo*(-Val-Pro-Gly-Val-Gly-)$_3$, shows three β(II) turns joined by Val-Gly-Val bridges. Hydrophilic and hydrophobic channels that run parallel to the *x*-axis are formed by the stacking of cyclic peptide molecules on threefold axes. The presence of β(II) turns was earlier predicted from n.m.r. studies.[43]

Cyclic Peptides from *Amanita* Species.—The mushroom *Amanita virosa* has been the subject of a detailed chemical study as far as its toxic principles are concerned. Most of these fall into a group called the virotoxins, six of these having been structurally characterized (21). They are monocyclic peptides containing D-serine in place of the L-cysteine residue found in the phallotoxins. They also contain two new amino-acids not previously found in nature, *trans*-3,4-dihydroxy-L-proline and 2′-(methylsulphonyl)-L-tryptophan. Their biological activity is comparable to the phallotoxins, and they also bind to actin.[44] Also from *Amanita virosa* has been isolated amaninamide, a toxin closely related to the amatoxins. It differs from the well known α-amanitin only in that it lacks the 6′-hydroxy-group of the tryptophan unit. It is suggested that it is the biosynthetic precursor of α-amanitin.[45]

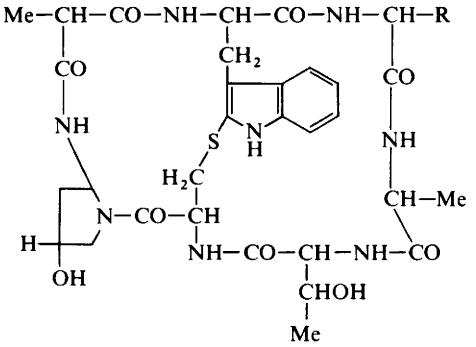
Ketophalloidin (22), obtained by periodate oxidation of phalloidin (23), has been converted into a dithiolane (24) with 3-aminopropane-1,2-dithiol. This basic 'handle' has been used to affix the phalloidin nucleus to sepharose for affinity chromatography, to attach fluorescein isothiocyanate to give a fluorescent phallotoxin, and for acylation with radiolabelled carboxylic acids. All these derivatives, like phalloidin itself, bind specifically to the receptor protein actin.[46]

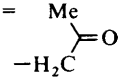
[39] I. M. Asher, G. D. J. Phillies, R. B. Geller, and H. E. Stanley, *Biochemistry*, 1980, **19**, 1805.
[40] M. Llinas and A. DeMarco, *J. Am. Chem. Soc.*, 1980, **102**, 2226.
[41] M. A. Khaled, A. Sugano, and D. W. Urry, *J. Chem. Soc., Perkin Trans. 2*, 1980, 206.
[42] M. A. Khaled, C. M. Venkatachalam, T. L. Tropane, H. Sugano, and D. W. Urry, *J. Chem. Soc., Perkin Trans. 2*, 1980, 1119.
[43] W. J. Cook, H. Einspahr, T. L. Tropane, D. W. Urry, and C. E. Bugg, *J. Am. Chem. Soc.*, 1980, **102**, 5502.
[44] H. Faulstich, A. Buku, H. Bodenmuller, and Th. Wieland, *Biochemistry*, 1980, **19**, 3334.
[45] A. Buku, Th. Wieland, H. Bodenmuller, and H. Faulstich, *Experientia*, 1980, **36**, 33.
[46] Th. Wieland, A. Deboben, and H. Faulstich, *Liebigs Ann. Chem.*, 1980, 416.

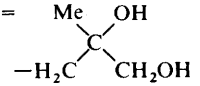


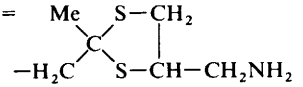
	X	R^1	R^2	% *of total*
Viroidin	SO_2	Me	$CHMe_2$	18
Desoxoviroidin	SO	Me	$CHMe_2$	4
[Ala^1]-viroidin	SO_2	Me	Me	10
[Ala^1]-desoxoviroidin	SO	Me	Me	
Viroisin	SO_2	CH_2OH	$CHMe_2$	49
Desoxoviroisin	SO	CH_2OH	$CHMe_2$	19

(21)



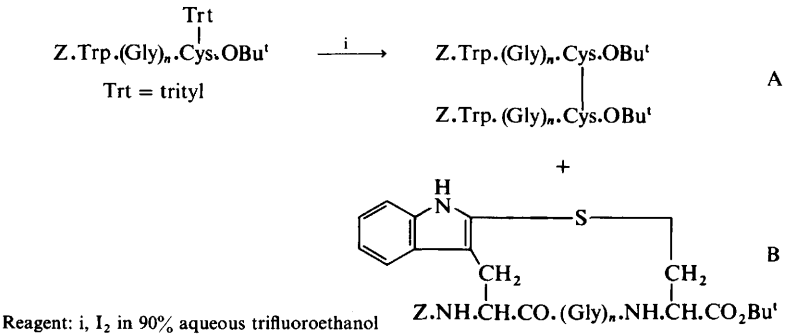
(22) R = 

(23) R = 

(24) R = 

Further experiments at the formation of tryptophan-2-thioether links, which relate to the synthesis of *Amanita* toxins, have been reported. Removal of *S*-trityl protecting groups from cysteine residues with iodine proceeds through sulphenyl iodides. Under normal conditions of iodine oxidation, tryptophan-2-thioethers are produced in insignificant amounts. However, in 90% aqueous trifluoroethanol, which retards disulphide bond formation, they may be major products. This was

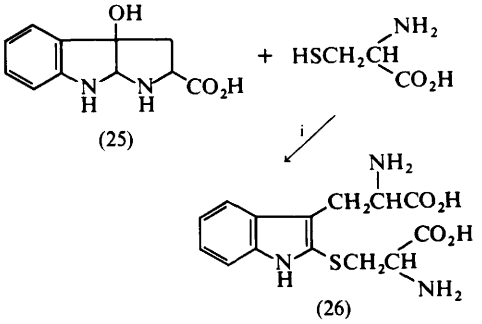
found with a somatostatin derivative.[47] Work with a series of model peptides has shown that the separation of the two residues concerned markedly influences the yield of cyclic product (Figure 1).[47] A derivative (25) formed from tryptophan by



Reagent: i, I_2 in 90% aqueous trifluoroethanol

n	A	B
1	80%	not detectable
2	80%	not detectable
3	45%	40%
4	10%	70%

Figure 1 *Products formed on removal of* S-*trityl protecting groups with iodine* (Reproduced by permission from *Acta Crystallogr.*, 1980, **B36**, 2651)



Reagent: i, H^+

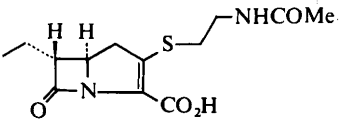
Scheme 10

mild peracetic acid oxidation has been found to react with thiols under acidic conditions to generate 2-thioethers. Cysteine, for example, reacts to give the double amino-acid tryptathionine (26; Scheme 10) directly.[48]

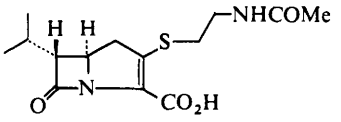
Highly Modified Cyclic Peptides.—In this category are included naturally occurring β-lactams, but synthetic aspects of β-lactam antibiotics are not covered at all in this chapter. The structure of PS-5, a new antibiotic from *Streptomyces cremeus* or *S. fulvoviridis*, has now been determined. This compound (27) is active against β-lactamase producing organisms resistant to the known β-lactam antibiotics.[49]

[47] P. Sieber, B. Kamber, B. Riniker, and W. Rittel, *Helv. Chim. Acta*, 1980, **63**, 2358.
[48] W. E. Savige and A. Fontana, *Int. J. Pept. Protein Res.*, 1980, **15**, 102.
[49] K. Yamamoto, T. Yoshioka, Y. Kato, N. Shibamoto, K. Okamura, Y. Shimauchi, and T. I. Ishikura, *J. Antibiotics*, 1980, **33**, 796.

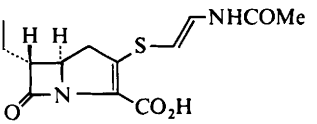
Alongside PS-5 occur two minor components, (28) and (29), also containing the carbapenem nucleus. The former, PS-6, is the first compound in the thienomycin family of β-lactams to have a C_3 side-chain at C-6.[50] From another *Streptomyces*



(27)

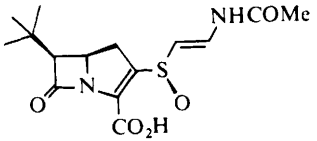


(28)



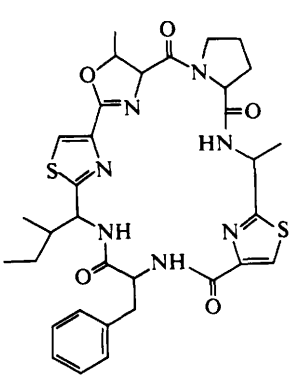
(29)

species, KC-6643, have been obtained carpetimycins A and B (30a and 30b). The latter was convertible into the former, whose structure was determined, as its *p*-bromobenzyl ester, by *X*-ray crystallography. They are active against both Gram-positive and Gram-negative bacteria, including β-lactamase-producing strains.[51]

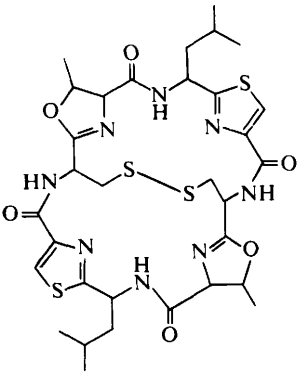


(30) a; R = H
b; R = SO_3H

Two unusual cyclic tetrapeptides, ulicyclamide (31) and ulithiacyclamide (32), have been isolated from the ascidian *Lissoclinum patella*. The lack of any free



(31)

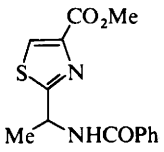


(32)

[50] N. Shibamoto, A. Koki, M. Nishino, K. Nakamura, K. Kiyoshima, K. Okamura, M. Ohabe, R. Okamoto, Y. Fukagawa, Y. Shimonouchi, and T. Ishikura, *J. Antibiotics*, 1980, **33**, 1128.

[51] M. Nakayama, A. Iwasaki, S. Kimura, T. Mizoguchi, S. Tanabe, A. Murakami, I. Watanabe, M. Okuchi, H. Itoh, Y. Saino, F. Kobayashi, and T. Mori, *J. Antibiotics*, 1980, **33**, 1388.

hydroxy-group in the peptides although threonine was isolated after acid hydrolysis led to the identification of the oxazoline rings. The presence of thiazole rings was established by comparison with a synthetic model thiazole (33) and other ^{13}C-n.m.r. and mass spectral examination.[52]

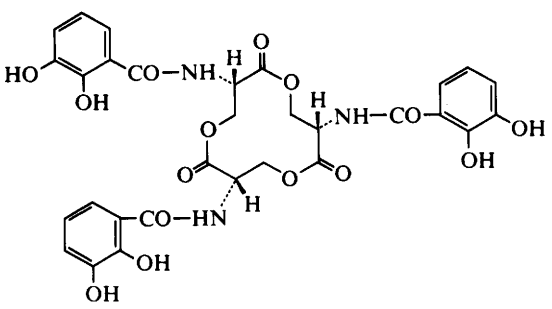


(33)

3 Cyclic Depsipeptides

All four stereoisomers of a cyclic didepsipeptide containing lysine and 2-hydroxy-2-phenylpropanoic acid have been synthesized and their susceptibility to trypsin has been examined. The diastereoisomers containing L-lysine hydrolysed rapidly, but their enantiomers were attacked only very slowly. Trypsin is already known not to affect *cyclo*(-L-Lys-L-Phe-).[53] The *X*-ray crystal structure determination of another cyclic didepsipeptide, *cyclo*(-D-MeVal-D-Hyi-), has been reported.[54]

A new synthesis of enterobactin, the iron-binding ionophore of enteric bacteria, has been described. Its antipode enantioenterobactin (34) was also prepared for



(34)

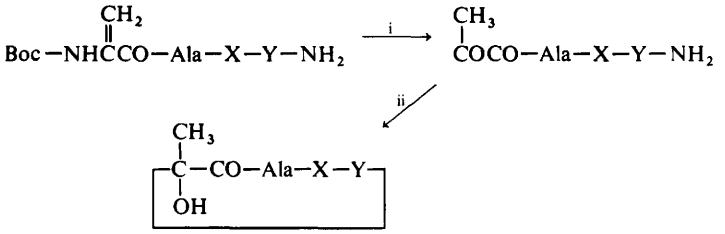
the first time from D-serine and its Fe^{III} complex found to exhibit the unnatural Λ-cis configuration at the metal centre.[55] Although composed solely of amino-acids, enterobactin contains no amide bonds in the ring. Conversely, three analogues of the cyclodepsipeptide toxin from *Alternaria mali* have been prepared, which, although they contain an α-hydroxy α-amino-acid residue, have a ring containing only amide bonds. These analogues were preparad from pyruvoyl tripeptide amides, although these were not purified, and cyclization (Scheme 11) was stereospecific, forming an α-hydroxyalanine residue of only one configuration.

[52] C. Ireland and P. J. Sheuer, *J. Am. Chem. Soc.*, 1980, **102**, 5688.

[53] A. Yasutake, K. Miyazaki, H. Aoyagi, T. Kato, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1980, **16**, 61.

[54] N. E. Zhukhlistova and G. N. Tischenko, *Kristallografiya*, 1980, **25**, 274 (*Chem. Abstr.*, 1980, **93**, 132784*p*).

[55] W. H. Rostetter, T. J. Erickson, and M. C. Venuti, *J. Org. Chem.*, 1980, **45**, 5011.



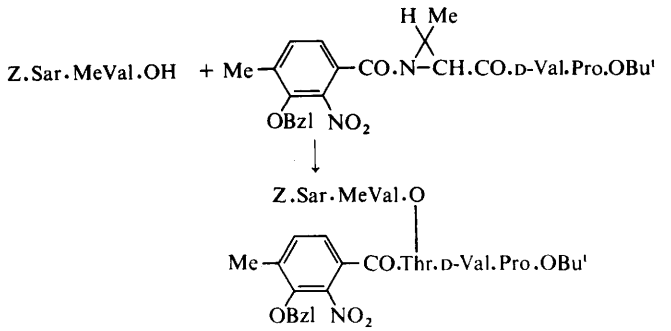
X-Y = Val-Phe, Hmb-Phe, or Hmb-Tyr, where
Hmb = 2-hydroxy-3-methylbutanoic acid

Reagents: *i*, HCl–HOAc; *ii*, HF

Scheme 11

These analogues were not active in causing necrosis in apple leaves.[56] The beauverolides are cyclotetradepsipeptides isolated from a strain of the entomopathogenic fungus *Beauveria bassiana*. Beauverolides H and I were earlier characterized, and the structures of four more of the series have now been reported. In this later work, however, a different strain of the organism was used, and isoleucine in the original metabolites is replaced by alloisoleucine in the new compounds. Beauverolides E and F are *cyclo*(-3-hydroxy-4-methyloctanoyl-Val-Phe-D-aIle-) and *cyclo*(-3-hydroxy-4-methyloctanoyl-Phe-Phe-D-aIle-), respectively. Beauverolide B is identical with isarolide A and beauverolide C is identical with isarolide C; both contain 3-hydroxy-4-methyldecanoic acid.[57]

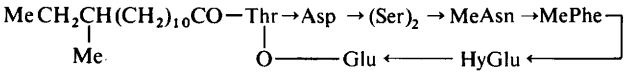
A total synthesis of actinomycin D has been described which uses a novel method to form the ester bond between *N*-methylvaline and threonine. Heating at 110 °C for 5 h a component containing the former with a free carboxy-group and a peptide containing a 3-methyl-2-aziridinecarboxylic acid residue gave 50% of the ring-opened product containing the required ester link (Scheme 12).[58] A cyclo-octadepsipeptide structure (35) has been proposed for lipopeptin A, an antifungal antibiotic from a streptomycete which resembles *Streptomyces violaceochromo-*



Scheme 12

[56] K. Noda, Y. Shibata, Y. Shimahigoshi, and N. Izumiya, *Tetrahedron Lett.*, 1980, 763.
[57] J. F. Elsworth and J. F. Grove, *J. Chem. Soc., Perkin Trans. 1*, 1980, 1795.
[58] T. Tanaka, K. Nakajima, and K. Okawa, *Bull. Chem. Soc. Jpn.*, 1980, **54**, 1352.

genes. This compound inhibits *in vitro* peptidoglycan synthesis of *E. coli*.[59] The rather similarly named peptidolipin NA (36) has been the subject of a 400 MHz ^{1}H-n.m.r. study. It is proposed that the Pro-D-aIle sequence forms a relatively rigid γ-turn stabilized by a ProCO,D-aIleNH interaction and by a supplementary



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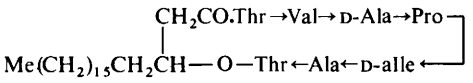
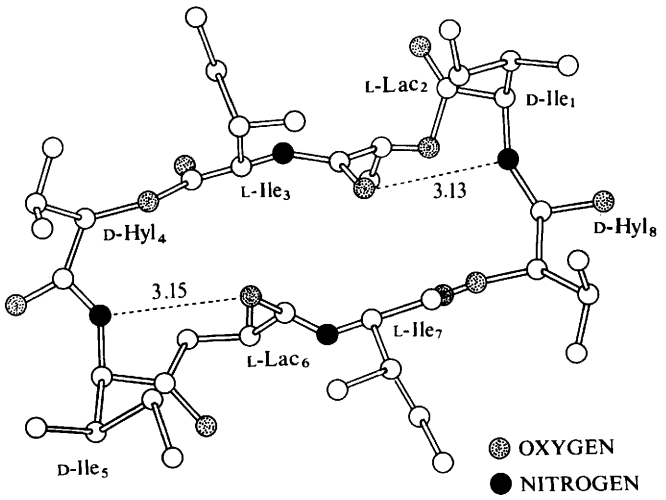


(36)

D-aIleCO,D-AlaNH hydrogen bond. The conformation of the Ala-Thr sequence seems somewhat flexible, and the molecule self associates; inter-molecular hydrogen bonding between Val and Ala groups may be the cause.[60]

An *X*-ray study of *cyclo*(-D-Ile-Lac-Ile-D-Hyi-)$_2$, a cyclic octadepsipeptide unable to form complexes with metal ions, shows two type IV β-turns generating a rectangular-shaped molecule with D-residues at the corners and containing pseudorotational symmetry (37). There are hydrogen bonds between the third and fourth residues in the β-turns; although these bonds are weak they appear to be the cause of severe distortion of the CO-bonds within them. This is the first



(37)

[59] M. Nishii, T. Kihara, K. Isono, T. Higashijima, T. Miyazawa, S. K. Sei, and J. A. McCloskey, *Tetrahedron Lett.*, 1980, 4627.

[60] M. Ptak, A. Hetz, M. Guinand, and G. Michel, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 1311.

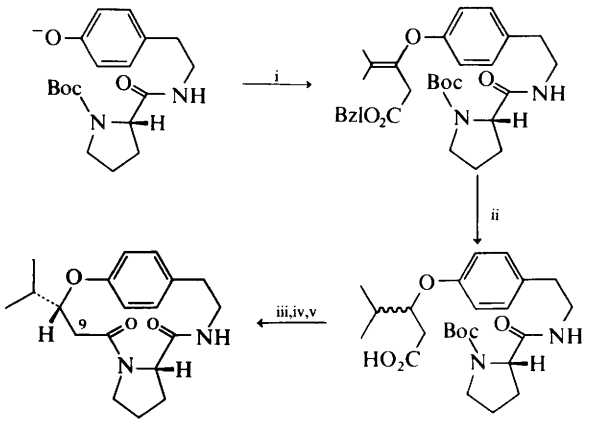
observation of a hydrogen bond of any sort in such a β-bend.[61] A novel conformation of the barium complex of valinomycin has been reported, which has been described as an extended depsipeptide chain without internal hydrogen bonds wound in the form of an ellipse with the two barium atoms located at the foci.[62] Several valinomycin analogues have also been the subject of conformational analysis. The crystal structures of prolinomycin rubidium picrate, a synthetic analogue in which proline substitutes for the α-hydroxy-acid and is therefore a peptide not a depsipeptide, shows two conformations, both very similar to those earlier found for valinomycin potassium tetrachloroaurate(III) complex. The cage is very rigid as before, but prolinomycin is much closer to a threefold molecular symmetry.[63] Isoleucinomycin, *cyclo*(-D-Ile-Lac-Ile-D-Hyi-)$_3$, on *X*-ray examination shows a completely asymmetric structure. There are six intramolecular hydrogen bonds, five being of the 4 → 1 type and one of the 5 → 1 type. Side chains are located on the molecular periphery. The conformation is intermediate between the corresponding crystalline states of valinomycin and meso-valinomycin. It is suggested that complexation could proceed *via* entry of the ion at the face possessing the Lac residues, the less crowded face.[64] Valinomycin analogues in which the ester groups are substituted by amide or *N*-methylamide groups show the bracelet conformation characteristic of valinomycin in non-polar media. However, the presence of two amide substitutions destabilizes this conformation, and the introduction of *N*-methyl groups significantly restricts conformational mobility.[65]

4 Peptide Alkaloids

The ^{13}C-n.m.r. spectra of four cyclopeptide alkaloids have been examined. The styrene carbons of the 13-membered ring of zizyphine A and the 15-membered ring of zizyphine D show evidence of enamide as well as styrene conjugation. There are significant differences in chemical shift from the corresponding carbons in the 14-membered alkaloids amphibine D and E, in accord with earlier observations of differences in u.v. absorption of 13- and 15-membered rings with respect to 14-membered rings.[66] Further work on the synthesis of the *p*-phencyclopeptidine ring characteristic of the peptide alkaloids has been reported. The model compound (38), which lacks the styrene double bond and the nitrogen atom at C-9 of natural peptide alkaloids, was prepared (Scheme 13) by a conventional amide bond formation from a linear *p*-nitrophenyl ester; a single diastereoisomer was obtained in 36% yield, asymmetric induction occurring during ring formation.[67]

Four novel linear peptide alkaloids, the celenamides, have been isolated from the sponge *Cliona celata*. These were characterized as their poly-acetyl derivatives;

[61] W. L. Duax, G. D. Smith, C. M. Weeks, V. Z. Pletnov, and N. M. Galitsky, *Acta Crystallogr.*, 1980, **B36**, 2651.
[62] S. Devorcjan, C. M. K. Nair, K. R. K. Easwaran, and M. Vijayan, *Nature*, 1980, **286**, 640.
[63] J. A. Hamilton, M. N. Sabeson, and L. K. Steinrauf, *Acta Crystallogr.*, 1980, **B36**, 1052.
[64] V. Z. Pletnev, N. M. Galitsky, G. D. Smith, V. M. Weeks, and W. L. Duax, *Biopolymers*, 1980, **19**, 1517.
[65] V. T. Ivanov, L. A. Fonina, L. B. Senyavina, Yu. A. Ovchinnikov, I. I.Chervin, and G. I. Yakovlev, *Biorg. Khim.*, 1980, **6**, 1008.
[66] D. M. Hindenlang, M. Shamma, C. A. Miana, A. H. Shah, and B. K. Cassels, *Liebigs Ann. Chem.*, 1980, 447.
[67] D. Goff, J. C. Lagarios, W. C. Shih, M. P. Klein, and H. Rapaport, *J. Org. Chem.*, 1980, **45**, 4813.

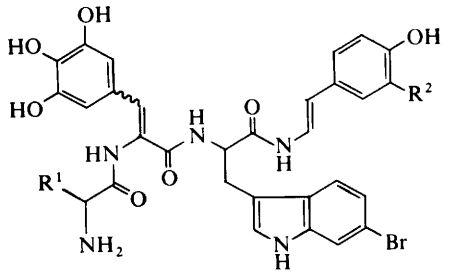


(38)

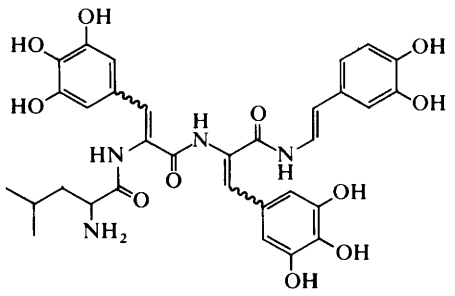
Reagents: i, benzyl propiolate; ii, Pd–H_2; iii, *p*-nitrophenyl trifluoroacetate; iv, TFA; v, pyridine, high dilution

Scheme 13

the two major components, celenamides A (39a) and B (39b), differ only in the substitution of valine for leucine at the *N*-terminus in the latter, and contain the novel amino-acids 6-bromotryptophan and $\alpha\beta$-didehydro-3,4,5-trihydroxyphenylalanine. Celenamide D (40) possesses two residues of the latter, while



(39) a; $R^1 = Me_2CHCH_2$—, $R^2 = OH$
b; $R^1 = Me_2CH$—, $R^2 = OH$
c; $R^1 = Me_2CHCH_2$—, $R^2 = H$



(40)

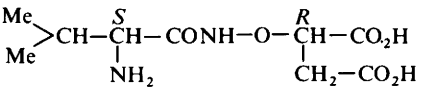
celenamide C (39c) lacks one of the phenolic hydroxy-groups of celenamide A.[68,69] These are the first peptide alkaloids to be found in species other than terrestrial plants.

5 Linear Peptides

Dipeptides.—Topo-opthalmic acid (41) has been synthesized and its inhibition of γ-glutamyltransferase examined.[70] A new antibiotic from *Streptomyces lydicus*, malioxamycin (42), inhibits cell wall peptidoglycan synthesis, but has only weak activity against a limited number of Gram-negative bacteria. It has been

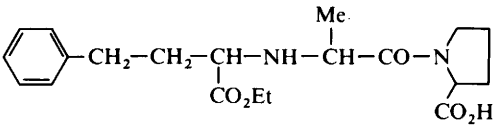
$$HO_2C.CH_2.CO.\text{D-Abu}\text{—}\underset{H.Dab.OH}{}$$

(41) Dab = α,γ-diaminobutyric acid
Abu = α-aminobutyric acid



(42)

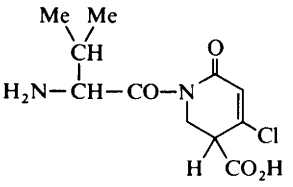
synthesized by treating the 1-succinimidyl ester of *N*-benzyloxycarbonyl-L-valine with (*R*)-amino-oxysuccinic acid and hydrogenolysing the product.[71] A programme aimed at finding angiotensin-converting enzyme inhibitors that do not contain a mercapto-function, to try and reduce the side-effects on clinical use of earlier compounds, these effects resembling those of penicillamine, has been successful. Of the new class of compounds *N*-(1-carboxy-3-phenylpropyl)-L-alanyl-L-proline (43) is now being used in clinical studies. It is suggested that these compounds should be classed as transition state inhibitors.[72]



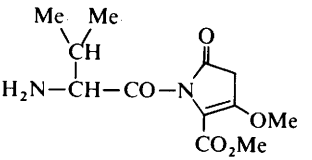
(43)

A new antibiotic from *Streptomyces xanthocidus* (44) contains a 2,5-dihydro-5-oxopyrrole ring. The double bond in this ring has a tendency to migrate; treatment with diazomethane gives (45), and mild acid also causes this sort of shift.[73] A number of papers concern γ-glutamyl dipeptides. A cationic rich fraction of the

[68] R. J. Stanard and R. J. Anderson, *J. Org. Chem.*, 1980, **45**, 3687.
[69] R. J. Stanard and R. J. Anderson, *Can. J. Chem.*, 1980, **58**, 2121.
[70] A. Kubik, I. Z. Siemion, W. Stachowiak, A. Szewczuk, and W. Klio, *Pol. J. Chem.*, 1980, **54**, 435.
[71] S. Takahashi, M. Takeuchi, M. Inukai, and M. Arai, *J. Antibiotics*, 1980, **33**, 1220.
[72] A. A. Patchett, E. Harris, E. W. Tristram, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, J. ten Broeke, L. G. Payne, D. L. Oudeyka, E. D. Thorsett, W. J. Greenlee, N. S. Lohr, R. D. Hoffsommer, H. Joshua, W. V. Ruyle, J. W. Rothrak, S. D. Aster, A. L. Maycock, F. M. Robinson, R. Hirschmann, C. S. Sweet, E. H. Ulm, D. M. Cross, T. S. Vossil, and C. A. Stone, *Nature*, 1980, **288**, 280.
[73] Y. Kuroda, M. Okuhara, T. Goto, M. Okamoto, M. Yamashita, M. Kohsaka, H. Aoki, and H. Imanaka, *J. Antibiotics*, 1980, **33**, 267.



(44)

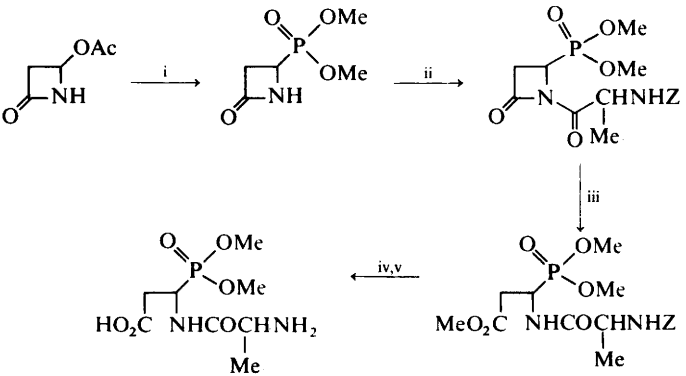


(45)

dried mushroom *Lentinus edodes* has yielded γ-glutamylcystine and *NN*-bis-γ-glutamylcystinylglycine,[74] while γ-glutamyl-leucine, previously found in plant tissues, has now been detected in the roots of *Rumex obtusifolius*.[75] The dipeptide L-γ-(*threo*-β-methyl)glutamyl-L-γ-aminobutyrate has been prepared and found to be a selective substrate for γ-glutamyl cyclotransferase, being converted into α-aminobutyrate and 3-methyl-5-oxoproline. It is not acted upon by γ-glutamyl transpeptidase. The desmethyl derivative β-aminoglutaryl-L-α-aminobutyrate is also a useful biochemical tool, being a specific inhibitor of γ-glutamyl cyclotransferase.[76]

Daunorubicin (DNR) is composed of an anthracycline linked to an amino-sugar and widely used in the treatment of acute leukaemia. A number of amino-acid and dipeptide *N*-derivatives of daunorubicin have been synthesized as potential pro-drugs. Leu-DNR, Ala-Leu-DNR, and Leu-Leu-DNR are much more active than DNR itself against subcutaneous leukaemia and less cardiotoxic. The hydrophobic side-chains are thought to aid transport to the site of tumour, but are susceptible to aminopeptidase cleavage of the site of action.[77]

A new synthesis of α-aminophosphonic acid dipeptides containing aspartic acid from 4-acetoxyazetidin-2-ones has been described (Scheme 14). The starting

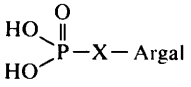


Reagents: i, $P(OMe)_3$, heat; ii, couple with Z-DL-Ala; iii, HCl, MeOH; iv, aq. NaOH; v, HBr–AcOH

Scheme 14

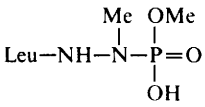
[74] S. Aoyagi, H. Sasaki, T. Sugahara, T. Hasegawa, and T. Suzuki, *Agr. Biol. Chem.*, 1980, **44**, 2667.
[75] T. Kasai, M. Okuda, and S. Sakamura, *Agr. Biol. Chem.*, 1980, **44**, 2723.
[76] R. J. Bridges, O. W. Griffith, and A. Meister, *J. Biol. Chem.*, 1980, **255**, 10787.
[77] M. Masquelier, R. Baurain, and A. Trouet, *J. Med. Chem.*, 1980, **23**, 1166; M. Masquelier, D. D. Campeneere, and A. Trouet, *ibid.*, 1171.

material has been the basis of many recent syntheses of β-lactams, the acetoxy-group being readily displaced by heteronucleophiles.[78] Four *N*-phosphorylated dipeptide aldehydes (46) have been synthesized that combine features of both



(46) X = Leu, Phe, Tyr, or Ala

leupeptin (arginal), which inhibits thermolysin-like activity, and phosphor-amidon (an *N*-phosphoryl group), which inhibits trypsin-like specificity. All four compounds inhibit both enzymes. Those with aromatic rings show enhanced inhibition of thermolysin, but aliphatic residues increase the inhibition of trypsin.[79] An antibiotic particularly active against *E. coli* has been isolated from a new strain of *Streptomyces unzenensis*. Cleavage of the phosphorohydrazidate group of this compound (47) occurs with dilute acid, leucine-*N'*-methylhydrazide being formed.[80]



(47)

X-Ray crystallographic studies have shown that pivaloy-L-prolyl-*N'*-isopropyl-glycinamide adopts a βII-bend and isobutyryl-L-alanyl-*N'*-isopropyl-L-prolin-amide an opened form stabilized by intermolecular hydrogen bonds,[81] while in both L-prolylsarcosine monohydrate and Boc-L-Pro-Sar-OBzl the peptide bond is *cis*.[82]

Peptides Containing α-Aminoisobutyric Acid.—The effect of the synthetic alamethicin fragment (48) and smaller peptides on the divalent cation permeability of phospholipid vesicles has been examined. The heptadecapeptide has a pronounced effect, although it is not as active as alamethicin itself. The methyl ester is more potent than the free carboxylic acid. The 1—13 sequence of (48) is weakly active and smaller peptides are inactive.[83] A peptide (49) containing the *N*-terminal undecapeptide sequence of alamethicin has been synthesized on polyoxyethylene as a solubilizing support and liberated as the crystalline hydrazide. As in alamethicin, trifluoroacetolysis enables selective cleavage of the Aib-Pro bond. C.d. and ^{13}C-n.m.r. studies demonstrated the helical conformation of the peptide.[84] The larger synthetic peptide (50) with a helical content of 44% exhibited membrane-modifying activity comparable to alamethicin, whose helical content is

[78] M. N. Campbell and N. Carruthers, *J. Chem. Soc., Chem. Commun.*, 1980, 730.
[79] H. N. Khatri, C. H. Stammer, M. M. Bradford, and R. A. McRorie, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 163.
[80] Y. Kuroda, H. Tanaka, M. Okamoto, T. Gato, M. Kohsaka, H. Aoki, and H. Imanaha, *Tetrahedron Lett.*, 1980, **33**, 280.
[81] P. A. Aubry, J. Protas, G. Boussard, and M. Marraud, *Acta Crystallogr.*, 1980, **B36**, 2822.
[82] H. Kojima, T. Kido, H. Itoh, T. Yamane, and T. Ashida, *Acta Crystallogr.*, 1980, **B36**, 326.
[83] R. Nagaraj, M. K. Mathew, and P. Balaram, *FEBS Lett.*, 1980, **121**, 365.
[84] W. Mayr and G. Jung, *Liebigs Ann. Chem.*, 1980, 1489.

5 10 15
Ac.Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib.OH
(48)

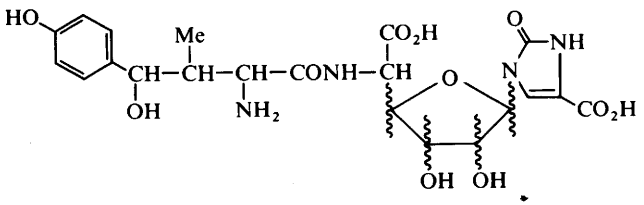
Ac.Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly.NH.NH_2
(49)

Boc.$(Aib\text{-}Ala)_5$-Gly-Ala-Aib-Pro-Ala-Aib-Aib-Glu(OBzl)-Gln.OMe
(50)

35%. When the *C*-terminal glutamine residue is replaced by glycine the helical content drops to 24% and the peptide is inactive.[85]

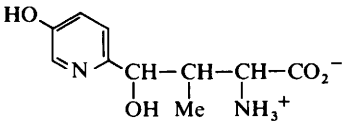
The protected alamethicin fragment Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe adopts a conformation with 4 → 1 and 5 → 1 hydrogen bonds, while the *N*-terminal hexapeptide sequence of alamethicin forms a 3_{10} helical structure.[86] An *X*-ray crystallographic study of Z-Aib-Aib-Ala-OMe shows a type III'-β-turn conformation with the CO of the Z-group hydrogen bonded to the NH of the Ala residue,[87] and the crystal structure of the oxazolinone of Z-Aib-Aib confirms its structural assignment.[88]

Peptides Containing Other Unusual Amino-acids.—The structure of a third member of the nikkomycin group of antibiotics, nikkomycin B (51), has been



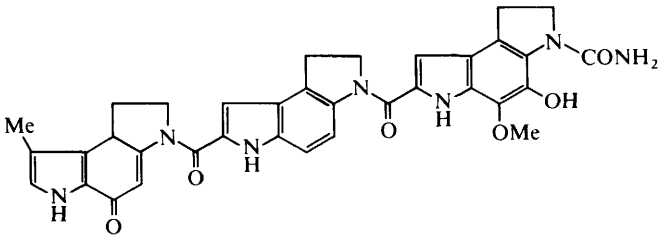
(51)

determined. A *p*-hydroxyphenyl residue replaces the 3-hydroxypyridine residue found in the *N*-terminal amino-acid of the two nikkomycins previously studied, but the *N*-glycoside unit (4-formyl-4-imidazolin-2-one) is that found in nikkomycin X. Nikkomycin was isolated from the culture filtrate of *Streptomyces tendac*.[89] The *N*-terminal amino-acid of nikkomycins X and Z (52) has been shown by an *X*-ray study to be of the 2*S*,3*S*,4*S*-configuration.[90] *X*-Ray crystallography has also shown the new antitumour antibiotic CC-1065 (53) from



(52)

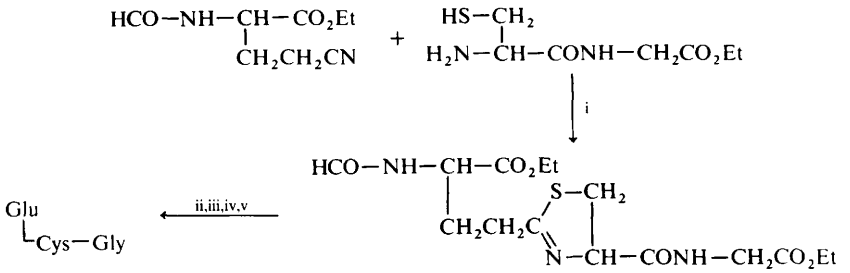
[85] R. Oekamonopoulos and G. Jung, *Biopolymers*, 1980, **19**, 203.
[86] C. P. Rao, R. Nagaraj, C. N. R. Rao, and P. Balaram, *Biochemistry*, 1980, **19**, 425.
[87] B. V. V. Prasad, N. Shamala, R. Nagaraj, and P. Balaram, *Acta Crystallogr.*, 1980, **B36**, 107.
[88] C. M. K. Nair and M. Vijayan, *Acta Crystallogr.*, 1980, **B36**, 1498.
[89] W. A. Konig, W. Hass, W. Dehler, H.-P. Fiedler, and H. Zahner, *Liebigs Ann. Chem.*, 1980, 622.
[90] W. A. Konig, K.-P. Pfaff, H.-H. Bartsch, H. Schmall, and H. Hagenmaier, *Liebigs Ann. Chem.*, 1980, 1728.



(53)

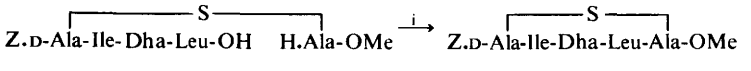
Streptomyces zelensis, which is reported to be remarkably potent against L-1210 cells in culture, to contain three tricyclic amide-linked residues.[91]

A synthesis of glutathione has been developed involving minimal protection in which the glutamic acid and cysteine residues are initially linked as a thiazoline (Scheme 15) that can subsequently be opened by acid (pH 4) after alkaline hydrolysis of the ester groups.[92] A protected fragment of the antibiotic nisin (54),



Reagents: i, EtOH, reflux; ii, OH^-; iii, H_3O^+; iv, Cu_2O: v, H_2O

Scheme 15



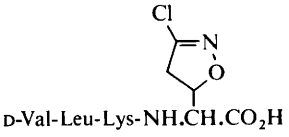
(54)

Reagents: i, *NN'*-dicyclohexylcarbodi-imide, l-hydroxybenzotriazole

Scheme 16

which contains a cyclic non-symmetrical lanthionyl peptide, has also been synthesized (Scheme 16), the dehydroalanyl residue being prepared from serine after incorporation of the latter into the peptide.[93] A tripeptide has been linked to the glutamine analogue (α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid and the product (55) examined for its potential in cancer chemotherapy. The same tripeptide was also attached to the alkylating agent phenyldiamine mustard (56). These peptidyl drugs *in vitro* were five-fold as selective as the free drugs for cancer cells. It is thought that such pro-drugs are locally activated by plasmin,

[91] D. G. Martin, C. G. Chidester, D. J. Duchamp, S. A. Mizsah, *J. Antibiotics*, 1980, **33**, 902.
[92] Y. Ozawa, T. Tsuji, and Y. Ariyoshi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2592.
[93] I. Photaki, S. Caronikas, I. Samoulidis, and L. Zervas, *J. Chem. Soc., Perkin Trans. 1*, 1980, 1965.



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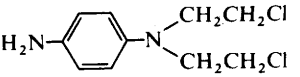
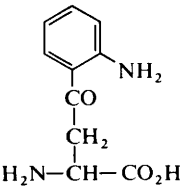


(56)

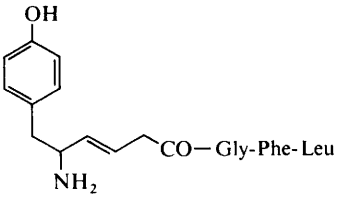
which is associated with tumour cells because of their increased plasminogen activator activity.[94]

The Strecker reaction has been used to prepare (2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoic acid, the required aldehyde being prepared by reduction of Z-D-Leu-OMe, and the diastereoisomeric mixture resolved. Extension at its carboxy-group with valylvalylaspartic acid has enabled the aminopeptidase inhibitor amastatin to be made without the necessity of separation from the 2*R*,3*R*-isomer at the last stage.[95] The incorporation of L-kynurenine (Kyn; 57), an important



(57)

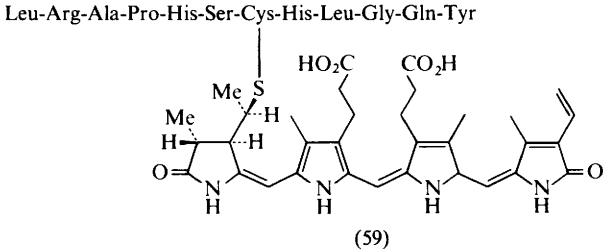
degradation product of tryptophan, into peptides has been explored with the side chain unprotected. It was found possible to couple Boc-Kyn successfully using Woodwards reagent K, enabling [Kyn^4]-enkephalin to be prepared.[96] Peptides in which a peptide bond has been replaced by a *trans*-olefinic linkage, 'double bond isosteres', have been synthesized as hormone analogues. The first compound of this type to be prepared was a [Leu^5]-enkephalin analogue (58), whose high biological activity showed that a Tyr-Gly peptide bond is not essential for opiate activity.[97] Double bond isosteres have also been reported for the Gly-Gly peptide bond in enkephalin, and for Phe-Phe and Phe-Gly in substance P.[98]



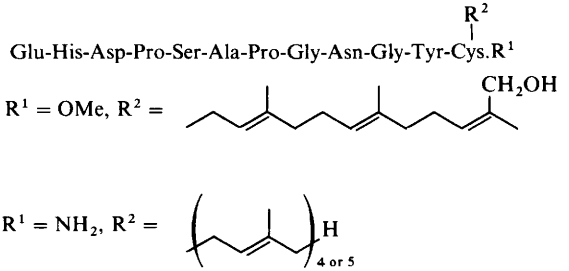
(58)

[94] P. L. Carl, P. K. Chakravarty, J. A. Katzenellenbogen, and M. J. Weber, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2224.
[95] D. H. Rich, B. J. Moon, and A. S. Baparai, *J. Org. Chem.*, 1980, **45**, 2288.
[96] F. H. C. Stewart, *Aust. J. Chem.*, 1980, **38**, 633.
[97] M. H. Hann, P. G. Sammes, P. D. Kennewell, and J. B. Taylor, *J. Chem. Soc., Chem. Commun.*, 1980, 234.
[98] M. T. Cox, J. J. Gormley, C. F. Hayward, and N. N. Pelter, *J. Chem. Soc., Chem. Commun.*, 1980, 800.

***S*-Alkyl Cysteinyl Peptides.**—After treatment of the lipoprotein phytochrome P_R with pepsin and thermolysin, an undecapeptide (59) has been isolated in which the chromophore is still linked to cysteine.[99] The peptide tremerogen A-10 (60a) from the culture filtrate of *Tr. mesenterica* was earlier shown to control the mating

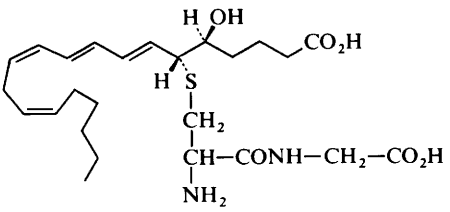


(59)



reaction of this yeast. It has now been reported that loss of the hydroxy-group of the *S*-alkyl substituent only slightly reduces the biological activity, and replacement of the ester group by an amide group has no effect on activity, although if the cysteine carboxyl is unprotected the peptide is inactive. Removal of the *N*-terminal glutamic acid residue also destroys the activity, but analogues having four or five prenyl units (60b) are respectively eight and sixteen times as potent as the natural material.[100]

However, the most important area of activity in *S*-alkyl cysteinyl compounds concerns the slow reacting substances or leukotrienes, important agonists in various forms of immediate hypersensitivity, including asthma. Total syntheses of leukotriene D (61), which accounts for >90% of the biological activity in

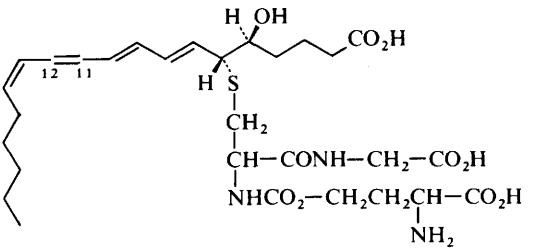


(61)

[99] J. C. Lagarias and H. Rapaport, *J. Am. Chem. Soc.*, 1980, **102**, 4821.

[100] M. Fujino, C. Kitada, Y. Sakagami, A. Isogai, S. Tamura, and A. Suzuki, *Naturwissenschaften*, 1980, **67**, 406.

hypersensitized human lung, and 11-*trans*-leukotriene C (62a; previously known as leukotriene C_1) have been reported.[101] At the last stage of the latter synthesis glutathione was added to the appropriate 5,6-epoxy-eicosa-tetraenoic acid methyl ester, the ester group being subsequently removed by hydrolysis.[102] Natural leukotriene C has also been found to be identical with synthetic 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (62b), *i.e.* 11-*cis*-leukotriene C.[103] Two positional isomers of leukotriene C in which the cysteine



(62) a; 11-*trans*
b; 11-*cis*

sulphur is attached to other positions of the hydrocarbon chain, which were previously considered as being possible members of the family of slowly reacting substances, have been synthesized and found biologically inactive.[104] Besides leukotrienes C and D, the third major component of the slow reacting substance from rat peritoneal cavity, leukotriene E, has been identified as leukotriene D minus the glycine residue.[105] Leukotriene E has also been found in cat paw slow reacting substance.[106]

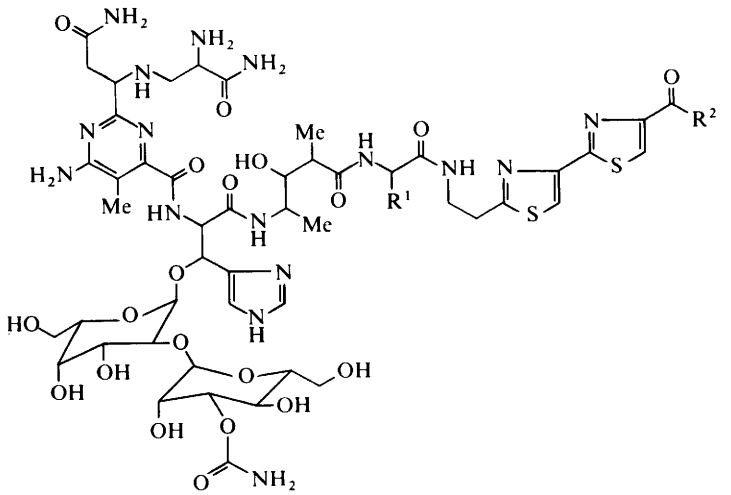
Conformational Studies.—*X*-Ray fibre diagrams of Boc(L-Nva)$_6$OMe and the corresponding hydrochloride after removal of the *N*-protecting group show the characteristic reflections of the cross-β-structure, but it was not possible to establish if the arrangement of the chains within the sheets is parallel or antiparallel.[107] The ^{1}H-n.m.r. spectra of the sarcosine oligomers Boc(Sar)$_n$OMe, where $n = 1$ to 5, in $^2H_6$-DMSO proved to be very complex and not fully interpretable above the dipeptide.[108] Oligomers of the form Boc(L-Val-D-Val)$_n$OMe have proved surprisingly volatile compared to their corresponding all-L counterparts; this is attributed to the lack of interchain hydrogen bonding. The octapeptide could be sublimed at 240 °C under high vacuum, and the underivatized dodecapeptide showed a molecular ion on e.i.–m.s.[109]

[101] E. J. Corey, D. A. Clark, A. Marfat, and G. Goto, *Tetrahedron Lett.*, 1980, **21**, 3143.
[102] E. J. Corey, D. A. Clark, G. Goto, A. Marfat, C. Mioskowski, B. Samuelson, and S. Hammarstrom, *J. Am. Chem. Soc.*, 1980, **102**, 1436.
[103] S. Hammarstrom, B. Samuelsson, D. A. Clark, G. Goto, A. Marfat, C. Mioskowski, and E. J. Corey, *Biochem. Biophys. Res. Commun.*, 1980, **92**, 946.
[104] E. J. Corey and D. A. Clark, *Tetrahedron Lett.*, 1980, **21**, 3547.
[105] R. A. Lewis, J. M. Drazen, K. F. Austen, D. A. Clark, and E. J. Corey, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 271.
[106] J. Houglum, J.-K. Pai, D.-E. Sok, and C. J. Sik, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5688.
[107] P. Spadou and A. DelPra, *Int. J. Pept. Protein Res.*, 1980, **15**, 54.
[108] C. Toniolo, G. M. Bonara, F. Schilling, and F. A. Bovey, *Macromolecules*, 1980, **13**, 1381.
[109] L. Tomasic, A. Stefani, and G. P. Lorenzi, *Helv. Chim. Acta*, 1980, **63**, 2000.

An examination of the solution conformation of gramicidin A using i.r., c.d., and fluorescence spectra shows that the four dimeric species present are predominantly antiparallel $\pi\pi_{LD}$ helices in equilibrium with smaller amounts of head-to-tail associated π_{LD} helices. The $\pi_{\overrightarrow{LD}}\pi_{\overleftarrow{LD}}$ dimer, thought to be the one in which gramicidin A carries out its channel function, turned out to be a minor form in non-polar solvents such as dioxan.[110] A Raman and i.r. study of gramicidin A also finds evidence for the existence of four distinct conformations of the peptide chain, depending on whether the compound is in the solid state, cast from film, or in methanol or DMSO solution.[111] Use of ^{23}Na-n.m.r. spectroscopy indicates that sodium binding does occur when malonyl gramicidin A is incorporated into lysolethicin micelles, where transmembrane channels are known to occur. Relaxation times are consistent with multiple-ion occupancy in the channels.[112]

6 Glycopeptides

Glycopeptide Antibiotics.—The bleomycins, some of which are widely used in the chemotherapy of certain human tumours, continue to be a very active field of research. The recently revised structure for bleomycin B_2 (63a) has been confirmed



(63)a; $R^1 = -CHOHMe$, $R^2 = -NH(CH_2)_4NHC(=NH)NH_2$

b; $R^1 = -CHOHMe$, $R^2 = -NH(CH_2)_3\overset{+}{S}Me_2$

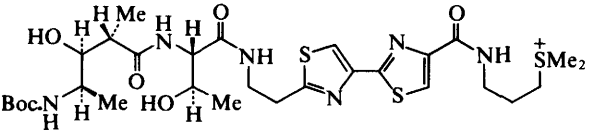
c; $R^1 =$  , $R^2 = -NH(CH_2)_4NHC(=NH)NH_2$

[110] S. V. Sychev, N. A. Nevskaya, St. Jordanov, E. N. Shepel, A. I. Miroshnikov, and V. T. Ivanov, *Bioorg. Chem.*, 1980, **9**, 121.

[111] Z. Iqbal and E. Weidekamm, *Arch. Biochem. Biophys.*, 1980, **202**, 639.

[112] C. M. Venkatachalam and D. W. Urry, *J. Magn. Reson.*, 1980, **41**, 313.

by a field desorption m.s. investigation, this being the first example of an antibiotic containing a guanido-group to be successfully characterized in this way. A short acetylation time in the reaction with acetic anhydride gives a mono-acetyl derivative more suitable for m.s. studies than the product obtained in the presence of triethylamine, where further acetylation occurs.[113] The presence of a free secondary amino-group in bleomycin A_2 (63b) has been further demonstrated by reductive methylation; a trimethyl derivative being obtained as a primary amino-group is also present.[114] A mutant produced by u.v. irradiation has yielded cleomycin (63c), a new type of bleomycin structure containing a cyclopropane ring,[115] and the synthesis of a tetrapeptide fragment (64) of bleomycin A_2 has been reported.[116]



(64)

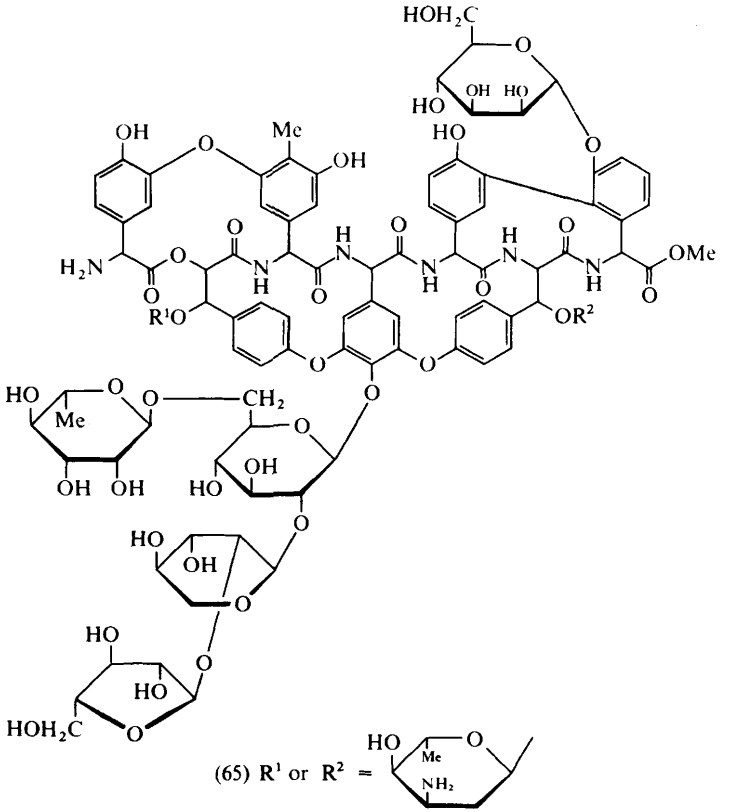
A ^{1}H-n.m.r. study of Fe^{II} bleomycin suggests that the metal ion co-ordinates with the α-amino-group, the imidazole N^{π}, the carbamoyl nitrogen, the valeric acid OH or CO, the pyrimidine N, and/or the secondary amino-group.[117] *N*-Acetylation of bleomycin destroys the ability of its Fe^{II} complex to bind and/or reduce oxygen and its ability to cause strand scission of DNA, but its chelating ability for metals is unaltered. The amino-terminal end of the molecule therefore seems more than a simple metal chelating site.[118] The formation of a ternary complex of bleomycin A_2–(poly dA-dT)–metal ion is indicated by the observation of spin diffusion in the ^{1}H-n.m.r. spectra when nuclear Overhauser experiments are carried out on samples containing these three components. Metal ion co-ordination seems to be minimally disturbed by polynucleotide binding.[119]

As measured by ^{13}C-n.m.r. spectroscopy, significant chemical shifts on protonation are limited to carbon atoms of the *N*-terminal tetrapeptide, suggesting that the *C*-terminal tripeptide extends into the solvent and interacts to a minimal extent with the rest of the molecule. The observed protonation shifts fail to correlate with earlier reported shifts of Zn^{II} complexation, indicating that ligation sites cannot be unambiguously determined from these complexation shifts.[120] A biosynthetic study of 3-morpholinopropyl-bleomycin (3-morpholinopropylamine was added to the culture medium) adding ^{13}C-enriched Met and Ala indicates that the methyl

[113] A. Dell, H. R. Morris, S. M. Hecht, and M. D. Levin, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 987.
[114] T. Fukuoka, Y. Muraoka, A. Fujii, H. Naganawa, T. Takita, and H. Umezawa, *J. Antibiotics*, 1980, **33**, 114.
[115] H. Umezawa, Y. Muraoka, A. Fujii, H. Naganawa, and T. Takita, *J. Antibiotics*, 1980, **33**, 1079.
[116] M. D. Levin, K. Subrabamanian, H. Katz, M. B. Smith, D. J. Burlett, and S. M. Hecht, *J. Am. Chem. Soc.*, 1980, **102**, 1452.
[117] R. P. Pillai, R. E. Levkinski, T. T. Sakai, J. M. Geckle, N. R. Khrishna, and J. D. Glickson, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 341.
[118] N. J. Oppenheimer, L. O. Rodriguez, and S. M. Hecht, *Biochemistry*, 1980, **19**, 4096.
[119] R. P. Pillai, N. R. Khrishna, T. T. Saka, and J. D. Glickson, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 270.
[120] E. S. Mooberry, J. L. Dallas, T. T. Sakai, and J. D. Glickson, *Int. J. Pept. Protein Res.*, 1980, **15**, 365.

group of the pyrimidine moiety originates from the Met Me and the carbon skeleton of the pentanoic acid is formed from Ala, acetate, and Met Me.[121] Bleomycin has been found an inhibitor of both dopamine-β-hydroxylase, a copper-containing mono-oxygenase,[122] and tyrosine hydroxylase.[123]

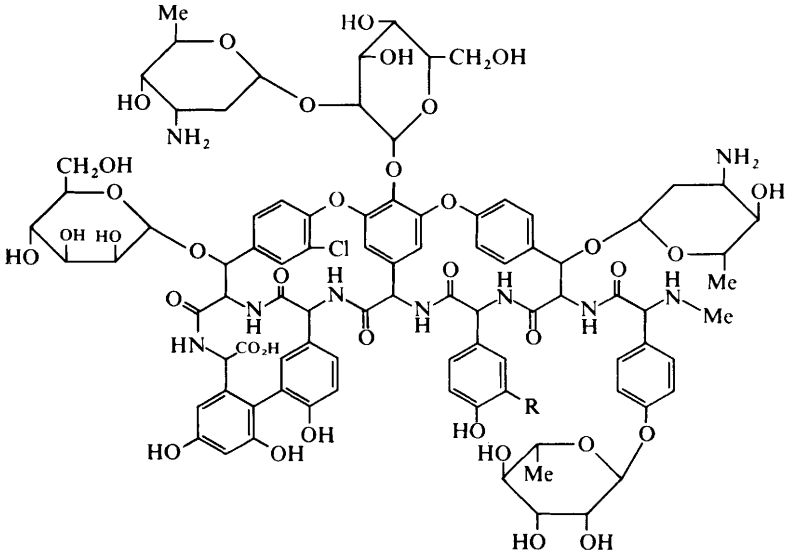
The structure of the carbohydrate component of the antibiotic ristocetin A (65) has been elucidated, and this compound now appears to be identical to ristomycin



A.[124] The absolute stereochemistry of eight of the nine asymmetric centres of ristocetin A has been proposed on the basis of a negative nuclear Overhauser effects analysis of the ^{1}H-n.m.r. spectrum in DMSO.[125] A ^{1}H-n.m.r. study of a ristocetin A–Ac-D-Ala-D-Ala complex in DMSO indicates a binding site similar to that earlier proposed for vancomycin, but strengthened by bonding of the carboxyl

[121] T. Nakatani, A. Fumii, H. Naganawa, T. Takita, and H. Umezawa, *J. Antibiotics*, 1980, **33**, 717.
[122] M. Matsui, T. Kato, C. Yamamoto, T. Takita, T. Takeuchi, H. Umezawa, and T. Nagatsu, *J. Antibiotics*, 1980, **33**, 435.
[123] K. Oka, T. Kato, T. Takita, T. Takeuchi, H. Umezawa, and T. Nagatsu, *J. Antibiotics*, 1980, **33**, 1043.
[124] F. Sztaricskai, C. M. Harris, A. Neszmelyi, and T. M. Harris, *J. Am. Chem. Soc.*, 1980, **102**, 7093; F. Sztaricskai, A. Neszmelyi, and R. Bognor, *Tetrahedron Lett.*, 1980, 2983.
[125] J. R. Kalman and D. H. Williams, *Tetrahedron Lett.*, 1980, 897.

of the dipeptide to several of the NH protons of ristocetin A.[126] The structures of avoparcin α (66a) and β (66b), two members of the vancomycin group of antibiotics, have been determined. The only uncertainties are the position of the chlorine atom on the triphenyl ether grouping and the positions of attachment of the sugars ristosamine and mannose; these may be interchanged.[127]



(66)a; R = H
b; R = Cl

Cell Wall Glycopeptides.—A solid phase synthesis of the tridecapeptide fundamental structural unit of the peptidoglycan from *Staphylococcus aureus* (67) has been reported. Pyrogenic effects were observed with all heteropeptides larger than the tetrapeptide. Immunoadjuvant effects were observed with the glycopeptide

N-Ac-Mur-Ala-D-iGln-Lys-D-Ala.OMe
|
(X)$_5$
|
N-Ac-Mur-Ala-D-iGln-Lys(Ac)-D-Ala ┘

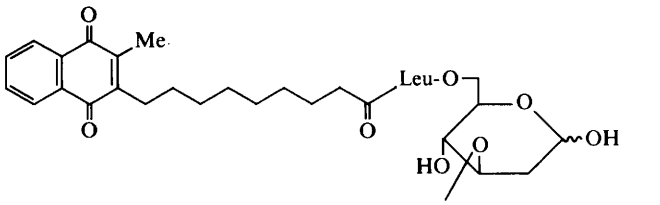
(67) X = Ala or Gly, iGln = isoglutaminyl

products, but also surprisingly with the sugar-free nonapeptide Ala-D-iGln-N^{ε}-AcLys-D-Ala-(Gly)$_5$-OMe.[128] The glycopeptide of the glomerular basement membrane, the *O*-(2-*O*-α-D-glucopyranosyl)-β-D-galactopyranoside of δ-hydroxy-L-lysylglycine, has been synthesized from δ-hydroxy-L-lysine-lactone,[129] and a

[126] J. R. Kalman and D. H. Williams, *Tetrahedron Lett.*, 1980, 906.
[127] W. J. McGahren, J. H. Martin, G. O. Martin, R. J. Hargreaves, R. A. Leese, F. M. Lovell, G. A. Ellestad, E. O'Brien, and J. S. E. Holker, *J. Am. Chem. Soc.*, 1980, **102**, 1671.
[128] M. Zaoral, J. Jezck, V. Krehnak, and P. Straka, *Coll. Czech. Chem. Commun.*, 1980, **45**, 1324.
[129] H. J. Koeners, C. Shattenkerk, J. Verhoeven, J. H. VanBoom, *Tetrahedron Lett.*, 1980, **21**, 2373.

short (4 step) synthesis of the adjuvant muramyl dipeptide from commercially available starting materials has been developed.[130] The metabolic fate of the peptidoglycan Glc-*N*-Ac-Mur-L-Ala-D-iGln-Dap-D-Ala-D-Ala (where Dap = *meso*-diaminopimelic acid) has been followed after ^{14}C-labelling in the *N*-acetyl and iGln residues. Most of the material (60—80%) is excreted in 3 h, part unchanged and part as the free pentapeptide.[131]

Analogues of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, often known as muramyl dipeptide (MDP), continue to be actively explored. The presence of an α-amino-acid adjacent to the lactic acid moiety is thought to be important for high biological activity, the valine analogue having the most favourable effect.[132] MDP-L-LysNH$_2$ has full immunoadjuvant activity,[133] while the *N*-acetyl-6-amino-6-deoxymuramyl derivative of serylisoglutamine[134] and the quinone derivative (68) are both more potent than MDP.[135]



(68)

Other Glycopeptides.—On the basis of c.d. and ^1H-n.m.r. experiments chiefly on 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-asparagine a proposal has been made for the conformation of the glycopeptide linkage in asparagine-linked glycoproteins. The glycosylated asparagine residue is in the second corner position of a type 1 β-turn, with the *N*-acetylglucosamine residue in a $^4C_1$ chair; the amide exocyclic angles are both 120°, and both amide side-chain dihedral angles are 60°.[136] In the presence of imidazole, 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose can be esterified with *N*-benzyloxycarbonyl tripeptide pentafluorophenyl esters. The resulting α-anomers, after separation from the β-anomers also produced, could be deprotected by catalytic hydrogenolysis in the presence of strong acid. In the absence of the acid a 1 → 2 acyl migration occurred during deprotection.[137]

[130] S. N. Schwartzman and E. Ribi, *Biochem. Prep.*, 1980, **10**, 255.
[131] J. Tomasic, B. Ladesic, Z. Valinger, and J. Hrsak, *Biochem. Biophys. Acta*, 1980, **629**, 77.
[132] S. Kobayashi, T. Fukuda, H. Yukimasa, M. Fujino, I. Azuma, and Y. Yamamura, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2570.
[133] F. Audibert, M. Parant, C. Damais, P. Lefrancier, M. Derrien, J. Choay, and L. Chedid, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 915.
[134] A. Hasegawa, H. Okumura, M. Kiso, I. Azuma, and Y. Yamamura, *Carbohydr. Res.*, 1980, **79**, C20.
[135] S. Kobayshi, T. Fukuda, H. Yukimasa, I. Imada, M. Fujino, I. Azuma, and Y. Yamamura, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2917.
[136] C. A. Bush, A. Duben, and S. Ralapati, *Biochemistry*, 1980, **19**, 501.
[137] S. Valentekovic and D. Keylevic, *Carbohydr. Res.*, 1980, **82**, 31.

5

Chemical Structure and Biological Activity of Hormones and Related Compounds

With contributions by H. C. BEYERMAN, D. BRANDENBURG, D. H. COY, G. W. HARDY, W. A. KLIS, M. MANNING, J. RAMACHANDRAN, P. D. ROY, D. SAUNDERS W. H. SAWYER, AND D. VOSKAMP

1 Hypothalamic Releasing Hormones

Contributed by D. H. Coy

Thyrotrophin Releasing Hormone (TRH).—As in the 1979 reporting period, most of the basic biological and also analogue studies on TRH have been devoted to the central nervous system and to gastrointestinal effects of the peptides. A previously reported 3-methylproline homologue of the TRH tripeptide (Glp-His-Pro-NH_2) which possessed enhanced anti-depressant activity has now been reported [1,2] also to increase analogue stability in both *in vivo* and *in vitro* systems.

Another new analogue with a larger γ-butyrolactone-γ-carboxylic acid ring in place of the Glp residue was also found to be more active than TRH in several anti-depression paradigms.[3]

TRH has now joined the list of peptides affecting appetite. Morley and Levine[4] found dose dependent reduction in stress- and starvation-induced appetite in the rat after both cerebroventricular and parenteral administration. The very large doses employed, particularly for peripheral injections (4—8 $mg\,kg^{-1}$), would suggest that a general toxicity effect cannot be discounted. In another study,[5] TRH infusion (20 $\mu g\,kg^{-1}\,h^{-1}$) into cats significantly reduced gastric acid and pepsin responses to infusion of insulin.

The generation of small, active peptide fragments from large precursor forms by enzymatic cleavage at paired, basic amino-acid dipeptide sequences is proving to be a widespread biological event. Formation of TRH from a pro-hormone form has not been demonstrated; however, the concept was tested[6] by the synthesis of [Val-Lys-Lys-Gln^1]-TRH, which could be converted into TRH in the body. This peptide exhibited low (*ca.* 0.2%) but significant TRH activity *in vitro*. It is not known whether this activity was intrinsic or due to TRH formation; *in vivo* activity was not reported.

[1] D. Brewster and M. J. Rance, *Biochem. Pharmacol.*, 1980, **29**, 2619.
[2] D. Brewster, P. W. Dettmar, A. G. Lynn, G. Metcalf, B. A. Morgan, and M. J. Rance, *Eur. J. Pharmacol.*, 1980, **66**, 65.
[3] M. Miyamoto, N. Fukuda, S. Narumi, Y. Nagai, Y. Saji, and Y. Nagawa, *Life Sci.*, 1981, **28**, 861.
[4] J. E. Morley and A. S. Levine, *Life Sci.*, 1980, **27**, 269.
[5] A. D. Gascoigne, B. H. Hirst, J. D. Reed, and B. Shaw, *Br. J. Pharmacol.*, 1980, **69**, 527.
[6] K. Folkers, J. Leban, N. Sakura, G. Rampold, E. Lundanes, J. Dahmen, M. Lebek, M. Ohta, and C. Y. Bowers, in 'Polypeptide Hormones', ed. R. F. Beers and E. G. Bassett, Raven Press, New York, 1980, p. 149.

Luteinizing Hormone-Releasing Hormone (LH-RH).— In a similar study on the generation of LH-RH (Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) from a pro-hormone form, Folkers *et al.*[6] synthesized [Lys-Lys-Gln[1]]-LH-RH. When tested *in vivo* in the rat, this analogue was 500-times less active than LH-RH itself in releasing gonadotrophins.

Most new analogue studies on LH-RH have been concentrated on competitive antagonist development for contraceptive use. Recent advances in this area continue to be based on the substitution of D-*p*-chlorophenylalanine [D-Phe(*p*-Cl] in position 2 and have been quite recently reviewed.[7, 8] The most potent analogues in the standard blockade of rat ovulation assay continue to have *N*-acetyl-D-aromatic amino-acids in position 1,[7] although Spatola and Agarwal[9] have just demonstrated that [*N*-Ac-Gly [1],D-Phe(*p*-Cl) [2],D-Trp [3,6]]-LH-RH is also very active despite the absence of a position 1 side-chain. Another analogue of the same general type, (*N*-Ac-dehydro-Pro [1],D-Phe(*p*-Cl) [2],D-Trp [3,6],*N*-Me-Leu [7]]-LH-RH,[10] completely blocked ovulation at a minimum dose of 20 μg per rat and was able to disrupt reproductive functions in the male rat after prolonged administration.

Even some of the older, less effective LH-RH antagonists have been found effective in preventing endogenous LH-RH action in other animal species, most importantly the primates. [Glp-Pro[1], D-Phe[2], D-Trp[3,6]]-LH-RH [11] eliminated pre-ovulatory peaks of LH and FSH and also ovulation in rhesus monkeys.[12] Several antagonists of varying activities in the rat have been found [13] to be effective in preventing gonadotrophin release in women.

Replacement of the peptide bond by groups that retain as much of the stereochemistry as possible and yet inhibit enzymatic cleavage is becoming increasingly popular in peptide analogue design. Spatola *et al.*[14] have developed techniques for synthesizing pseudo-dipeptide units containing —CH_2S— in place of the regular —CONH— and incorporating the dipeptides into biologically interesting peptides by the solid-phase method. In LH-RH, peptide bond replacements at either positions 5—6, 6—7, or 9—10 gave virtually inactive analogues. This would indicate that conformational distortions or other related problems, such as loss of hydrogen-bonding capabilities, are introduced. A similar series of inhibitory analogues of LH-RH based on [D-Glp[1], D-Phe[2], D-Trp[3,6]]-LH-RH was also prepared with the addition of a position 1—2 peptide bond replacement. The two analogues containing *N*- and *C*-terminal —CH_2S— linkages were the only

[7] D. H. Coy, I. Mezo, E. Pedroza, M. V. Nekola, A. V. Schally, W. Murphy, and C. A. Meyers, in ref. 6, p. 185.
[8] A. V. Schally, D. H. Coy, and A. Arimura, *Int. J. Gyn. Obstet.*, 1980, **18**, 318.
[9] A. Spatola and N. S. Agarwal, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 1571.
[10] C. Rivier, J. Rivier, and W. Vale, *Science*, 1980, **210**, 93.
[11] C. Y. Bowers, J. Humphries, T. Wasiak, K. Folkers, G. A. Reynolds, and L. E. Reichert, *Endocrinology*, 1980, **106**, 674.
[12] J. W. Wilks, K. Folkers, C. Y. Bowers, J. Humphries, B. Schirks, and K. Friebel, *Contraception*, 1980, **22**, 190.
[13] E. S. Canales, H. Montvelinsky, A. Zarate, A. J. Kastin, D. H. Coy, and A. V. Schally, *Int. J. Fertil.*, 1980, **25**, 190; D. Gonzalez-Barcena, H. Trevino-Ortiz, F. Gordon, A. J. Kastin, D. H. Coy, and A. V. Schally, *Int. J. Fertil.*, 1980, **25**, 185.
[14] A. F. Spatola, N. S. Agarwal, A. L. Bettag, J. A. Yankeelov, C. Y. Bowers, and W. Vale, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 1014.

ones to exhibit inhibitory activities. These were, however, diminished in relation to the parent analogue.

Somatostatin.—Much effort has been concentrated in the last year on the isolation of large forms of somatostatin from various animal species. Their syntheses and the examination of biological properties in comparison to the somatostatin tetradecapeptide have also been undertaken.

The 28-residue peptide (Figure 1), originally isolated and characterized by Pradayrol *et al.*[15] from porcine intestine, has now been extracted and identified from porcine [16] and bovine [17] hypothalamic tissue. Bovine extracts also yielded a shorter, 25-residue peptide in which the *N*-terminal three amino-acids were missing.[17] Reports [18, 19] have also appeared on the synthesis of somatostatin-28 by solid-phase methods and the use of high performance liquid chromatography for homogeneity determinations. In one report, the synthetic product was also sequenced.[19]

Despite the presence of the regular somatostatin sequence at its *C*-terminus (Figure 1), somatostatin-28 does have interesting biological properties of its own. Presumably because of its size and slower rate of breakdown, the large peptide is considerably longer acting than somatostatin in the rat.[18] Activities calculated on the basis of peak inhibition in the rat at two dose levels indicated that somatostatin-28 was roughly equipotent for inhibition of GH release, twice as active for glucagon, and 12-times as active for inhibition of insulin release.[18] This was in comparison to somatostatin on a molar concentration basis. Although the same report [18] showed that somatostatin-28 was virtually equipotent with somatostatin for inhibition of GH and prolactin release from monolayer cultures of pituitary cells, other papers [20] have claimed that the larger peptide is up to 14-times more active for inhibition of *in vitro* GH release. One possible reason for the difference could be the relative stability of somatostatin-28 compared to somatostatin itself in an enzyme-rich *in vitro* incubation system. Culture systems that were relatively enzyme free would clearly give different results. Somatostatin-28 was also reported [21] to be more active than somatostatin in inhibiting pancreatic secretion in response to secretin or caerulein in the dog.

Clinical trials with somatostatin-28 have already begun.[22] Studies on its abilities to inhibit arginine-induced GH and prolactin release quite clearly revealed greater and prolonged activity as compared to somatostatin. Certainly, the prolonged activity of somatostatin-28 makes it of therapeutic interest, particularly if

[15] L. Pradayrol, H. Jornvall, V. Mutt, and A. Ribet, *FEBS Lett.*, 1980, **109**, 55.
[16] A. V. Schally, W.-Y. Huang, R. C. C. Chang, A. Arimura, T. W. Redding, R. P. Miller, M. W. Hunkapiller, and L. Hood, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4489.
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[19] N. Ling, F. Esch, D. Davis, M. Mercado, M. Regno, P. Bohlen, P. Brazeau, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 945.
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[21] C. Susini, J. P. Esteve, N. Vaysse, L. Pradayrol, and A. Ribet, *Gastroenterology*, 1980, **79**, 720.
[22] M. D. Rodriguez-Arnao, A. Gomez-Pan, S. J. Rainbow, S. Woodhead, A. M. Comaru-Schally, A. V. Schally, C. A. Meyers, D. H. Coy, and R. Hall, *Lancet*, 1981, 353.

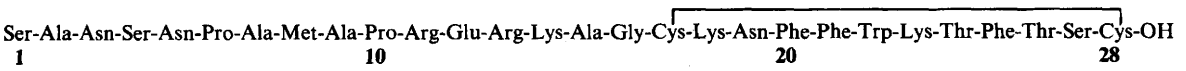

Figure 1 *Amino-acid sequence of somatostatin*-28, *of which somatostatin comprises residues* 15—28

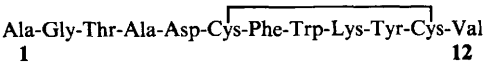

Figure 2 *Amino-acid sequence of urotensin II*

synthetic material could be made economically by, for instance, genetic engineering techniques.

The sequences of pro-somatostatin molecules present in other animal species are also becoming available. Goodman *et al.*[23] determined the nucleotide sequence of DNA cloned from mRNA coding for anglerfish pancreatic pre-prosomatostatin, from which the amino-acid sequence of a 119-residue protein could be derived. This contained the standard somatostatin-14 sequence at its *C*-terminus. A 12.5×10^3 dalton protein was identified analytically in rat pancreatic islets and also appeared to have somatostatin-14 at its *C*-terminus, which was released during cellular processing.[24] No sequencing data were determined. A 12×10^3 dalton protein was also synthesized by messenger RNA taken from catfish pancreatic islets and translated in a wheat germ, cell-free system.[25]

A curious peptide with smooth muscle contracting and osmoregulatory effects was recently isolated[26] from fish urophyses and called urotensin II. This 12-residue peptide (Figure 2) has a sequence which bears some close resemblances to somatostatin with sequence homology of the critical Phe-Trp-Lys portion. The possible somatostatin-like biological properties of urotensin II remain to be evaluated.

Relatively few reports have appeared concerning new analogue structure–activity work. We were able to extend observations with substituted-Trp^8-analogues to effects on gastric acid and pepsin release in the cat.[27] Several halogenated-Trp^8-somatostatin analogues were only slightly more active than somatostatin in this assay despite being up to 30-times more active in inhibiting *in vitro* GH release. Caution must, however, be excercised in comparing *in vitro* and *in vivo* data.

Long-awaited receptor assays for somatostatin are now being reported. In one,[28] utilizing synaptosomal membrane fractions, a number of somatostatin analogues showed binding activities that paralleled but were usually lower than *in vitro* GH release inhibiting activities. [D-Trp(5-F)^8]-somatostatin was outstanding in being 20—30-times more active than somatostatin in both assay systems.

Somatostatin will inhibit the electrically induced contractions of mouse *vas deferens* preparations in a dose dependent fashion, which is not blocked by opiate antagonists. This assay was evaluated for testing of analogues, and potencies derived from it were very similar to those obtained from gastric acid release experiments.[28] It was suggested that this rapid assay method might be particularly suitable in screening for competitive antagonists of somatostatin.

As has been reviewed in previous editions of this series, numerous truncated, cyclic analogues of somatostatin have been made that have good biological activity. One of these, *cyclo*(-Aha-Cys-Phe-D-Trp-Lys-Thr-Cys-) (Aha = aminohexanoic acid), was chosen as a sufficiently simple candidate for

[23] R. H. Goodman, J. W. Jacobs, W. W. Chin, P. K. Lund, P. C. Dee, and J. F. Habener, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5869.
[24] C. Patzelt, H. S. Tager, R. J. Carroll, and D. F. Steiner, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2410.
[25] H. Oyama, K. O'Connell, and A. Permutt, *Endocrinology*, 1980, **107**, 845.
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[27] B. H. Hirst, B. Shaw, C. A. Meyers, and D. H. Coy, *Reg. Peptides*, 1980, **1**, 97.
[28] C. A. Meyers, A. J. Kastin, A. V. Schally, and D. H. Coy, *Digestion*, 1981, **21**, 21.

conformational energy calculations.[29] Several conclusions emerged, including a type II bend at the D-Trp-Lys peptide bond and close proximity of Trp and Lys side-chains. It was suggested that Phe-D-Trp aromatic ring stacking occurs upon binding to the receptors.

Another somatostatin analogue, [(des-Ala^1,Gly^2),His^{4,5},D-Trp^8]-somatostatin, has been reported previously as having selective GH and glucagon release inhibiting properties and prolonged activity. A paper[30] has now appeared that reports significant LH-RH-like activity for the analogue, which causes various fertility effects. Before too much attention is given to this report it should be noted that other laboratories have thus far failed to duplicate this unusual result, although their findings have not been published.

2 Anterior Pituitary Hormones

Contributed by J. Ramachandran

Introduction.—This review will be concerned with the structure–function relationships of the adenohypophyseal hormones. Since this subject was covered previously[1] in Volume 12 of these reports, the literature for 1980 will be covered in this review. The nomenclature is according to the suggestions of the IUPAC-IUB commission on Biochemical Nomenclature.[2] The format will be similar to that used in Volume 12 of these reports and the anterior pituitary hormones will be considered under three structurally and functionally related groups.[1]

Hormones of Group I.—All the hormones of this group are derived from a single high molecular weight precursor which has been called pro-opiocortin[3] or pro-opiomelanocortin (POMC).[4] POMC has a molecular weight of 31 000 and contains β-lipotropin (β-LPH) at the carboxy-terminal. Corticotropin (ACTH) is in the middle and a glycopeptide of apparent molecular weight 16 000 is present at the amino-terminal of POMC. Nakanishi *et al.*,[5] who deduced the amino-acid sequence of POMC from the nucleotide sequence of the DNA complimentary to the mRNA of POMC, pointed out that the amino-terminal region of POMC contains a new melanotropin (MSH) sequence flanked by paired basic residues. This segment of 12 amino-acid residues was named γ-MSH by Nakanishi *et al.*[5] Thus, γ-MSH is contained in the amino-terminal peptide of POMC. α-MSH and corticotropin-like intermediate lobe peptide (CLIP) are derived from ACTH by proteolytic processing in the intermediate lobe of the pituitary gland. β-MSH and β-endorphin (β-EP) are formed from β-LPH (see ref. 1 or 5 for structure of POMC).

[29] F. A. Momany, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 61.
[30] F. J. Bex, A. Corbin, D. Sarantakis, and E. Liens, *Nature*, 1980, **284**, 342.
[1] J. Ramachandran, in 'Amino-acids, Peptides, and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), The Royal Society of Chemistry, London, 1981, Vol. 12, p. 469.
[2] *J. Biol. Chem.* 1975, **250**, 3215.
[3] M. Rubinstein, S. Stein, and S. Udenfriend, *Proc. Natl. Acad. Aci. USA*, 1978, **75**, 669.
[4] M. Chretien, S. Benjannet, F. Grossard, C. Gianoulakis, P. Crine, M. Lis, and N. G. Seidah, *Can. J. Biochem.*, 1979, **57**, 1111.
[5] S. Nakanishi, A. Inouyl, T. Kita, M. Nakamura, A. C. Y. Chang, S. N. Cohen, and S. Numa, *Nature*, 1979, **278**, 423.

γ-MSH and the Amino-terminal Fragment of POMC. γ-MSH itself has not been isolated from pituitary extracts until now. However, larger peptides containing the γ-MSH sequence have been isolated from porcine [6] and human [7–9] pituitaries and detected in bovine pituitary extracts.[10] Hakanson *et al.*[6] reported the isolation of a glycopeptide with an apparent molecular weight of 11 000 from pig pituitaries and showed that the amino-terminal amino-acid sequence of this peptide is identical to the amino-terminal end of bovine POMC. This peptide was found to be 0.34% as potent as α-MSH in melanotropic activity and 1% as active as β-LPH in stimulating lipolysis. Benjannet *et al.*[7] isolated the γ-MSH-containing peptide from human pituitaries and found that the amino-acid composition of a tryptic peptide corresponding to γ-MSH was identical to that of the bovine γ-MSH peptide. The isolation and characterization of a glycosylated human γ-MSH precursor peptide from human pituitaries was reported by two groups. Seidah *et al.*[8] found a high degree of homology between the amino-terminal half of the human and bovine γ-MSH precursor peptides. The peptide isolated from human pituitaries contained 103 amino-acid residues and was glycosylated at asparagine in position 65. The human γ-MSH precursor peptide did not exhibit any steroidogenic activity up to a concentration of 1 μg ml^{-1} on adult or foetal adrenal cells.

Estivariz *et al.*[9] isolated a peptide from human pituitaries with an apparent molecular weight of 16 000, an amino-terminal tryptophan residue and a melanotropic potency of 1×10^{-5} relative to α-MSH. This peptide did not stimulate lipolysis nor did it potentiate the adrenal weight maintaining activity of ACTH (1—24) when administered to hypophysectomized rats. Since synthetic peptides corresponding to the γ-MSH sequence also have less than 0.1% of the melanotropic activity of α-MSH,[11] the name γ-MSH may be inappropriate.

The physiological role of this peptide remains to be elucidated. Pedersen and Brownie [12] have proposed that the amino-terminal peptide of POMC may play a role in the regulation of adrenocortical steroidogenesis. They observed that the amino-terminal peptide (known as 16K fragment), prepared from the mouse pituitary tumour cell line A_tT-20/D_{16v}, has a slight but significant potentiating action on the stimulation of steroidogenesis by ACTH (1—24). Prior treatment of the 16K fragment with trypsin for 30 s increased this dose-dependent synergism. In hypophysectomized female rats, the 16K fragment stimulated cholesterol esterase activity in the adrenal cortex but failed to activate cholesterol side-chain cleavage. A synthetic peptide composed of 27 amino-acid residues containing the γ-MSH sequence with a carboxy-terminal extension also potentiated [13] the steroidogenic action of ACTH(1—24). This peptide caused a synergistic augmentation of corticosterone and aldosterone production when administered to hypophysecto-

[6] R. Hakanson, R. Ekman, F. Sundler, and R. Nilsson, *Nature*, 1980, **283**, 789.
[7] S. Benjannet, N. G. Seidah, P. Routhier, and M. Chretien, *Nature*, 1980, **285**, 415.
[8] N. G. Seidah, S. Benjannet, R. Routhier, G. DeSerres, J. Rochemont, M. Lis, and M. Chretien, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1417.
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[11] N. Ling, S. Ying, S. Minick, and R. Buillemin, *Life Sci.*, 1979, **25**, 11 773.
[12] R. C. Pedersen and A. C. Brownie, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 2239.
[13] R. C. Pedersen, A. C. Brownie, and N. Ling, *Science*, 1980, **208**, 1044.

mized female rats in the presence of ACTH(1—24). This peptide also activated cholesterol esterase but not cholesterol side-chain cleavage.

ACTH and CLIP. The isolation and characterization of ACTH from turkey pituitary glands have been described.[14] The biologically important amino-terminal half of the avian hormone is highly homologous to mammalian hormones but there are a number of differences in the amino-acid sequences of the carboxy-terminal regions of turkey and mammalian ACTH. It is not surprising that turkey ACTH was found to be nearly as potent as ovine ACTH in stimulating steroidogenesis in rat adrenocortical cells.

A new form of ACTH containing 38 amino-acid residues instead of the usual 39 residues was reported.[15] In addition to ACTH(1—39), Brubaker *et al.*[15] found ACTH(1—38) and CLIP [ACTH(18—39)] in extracts of calf anterior pituitaries. Tritium labelled ACTH(1—39) did not give rise to radioactive ACTH(1—38) or CLIP during isolation, indicating that ACTH(1—38) was not formed artifactually from ACTH(1—39). CLIP was also isolated from an alkaline extract of the pituitary gland of the salmon.[16] The amino-acid sequence of salmon CLIP exhibited greater homology to the sequence of avian (ostrich) CLIP than to that of dogfish. A large molecular weight glycosylated form of CLIP was identified in extracts of intermediate and anterior lobes of the pituitary glands of female wistar rats.[17]

It has been suggested that CLIP or a closely related peptide from the neurointermediate lobe of obese mice may stimulate insulin release in isolated pancreatic islets.[18] Two closely related peptide fractions smaller than CLIP have been isolated from neurointermediate lobe of the ob/ob mouse by gel chromatography.[19] These two fractions cross-reacted with antiserum directed against the carboxy-terminal region of ACTH and also rapidly stimulated insulin release in the perfused rat pancreas. The release of insulin by these peptide fractions was blocked by the ACTH antiserum. CLIP itself did not stimulate insulin release.

Photoreactive derivatives of ACTH have been prepared, characterized, and used for photoaffinity labelling of ACTH receptors on rat adipocytes and rat adrenocortical cells.[20-23] Three new photoreactive arylsulphenyl chlorides, namely 2,4-dinitro-5-azidophenylsuphenyl chloride,[20] 2-nitro-4-azido-phenylsulphenyl chloride,[21] and 2-nitro-5-azidophenylsulphenyl chloride,[21] were synthesized and used for the selective modification of the single tryptophan residue in ACTH. Tritiated [2,4-dinitro-5-azidophenylsulphenyl-Trp-9]-ACTH was found to attach covalently to a macromolecular component of the rat adipocyte plasma membrane upon photolysis.[22] Tritiated [2-nitro-5-

[14] W. C. Chang, D. Chung, and C. H. Li, *Int. J. Pept. Protein Res.*, 1980, **15**, 261.
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[16] H. Kawauchi, K. Abe, and A. Takahashi, *Bull. Jpn. Soc. Sci. Fisheries*, 1980, **46**, 743.
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[18] A. Beloff-Chain, J. A. Edwardson, and J. Hawthorn, *J. Endocrinol.*, 1976, **73**, 28p.
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[20] E. Canova-Davis and J. Ramachandran, *Biochemistry*, 1980, **19**, 3275.
[21] K. Muramoto and J. Ramachandran, *Biochemistry*, 1980, **19**, 3280.
[22] J. Ramachandran, E. Canova-Davis, and C. Behrens, in 'Synthesis and Release of Adenohypophyseal Hormones', ed. M. Jutisz and K. W. McKerns, Plenum Publications Corporation, New York, 1980, p. 363.

azidophenylsulphenyl-Trp-9]-ACTH was employed for labelling receptors on intact rat adrenocortical cells.[23] A protein with an approximate molecular weight of 100 000 (measured in the absence of β-mercaptoethanol) was specifically labelled upon photolysis.

A radioimmunoassay using specifically tritiated ACTH of high specific radioactivity and full biological potency has been reported.[24] The effects of pituitary corticotropin-inhibiting peptide [ACTH(7–38)] on the actions of ACTH have been investigated in detail.[25] Analysis of the inhibition of ACTH-induced steroidogenesis and cAMP production indicated that two functionally separate receptors may be involved in mediating these responses. Stimulation of cyclic nucleotide-independent protein kinase activity in intact adrenocortical cells by ACTH has been reported.[26] ACTH receptors on rat adipocytes have been identified by direct binding studies with tritiated ACTH.[27] Binding of ACTH to adipocytes was found to be of high affinity (K_d = 5.23 ± 1.92 nM) and paralleled closely the stimulation of lipolysis (K_m = 2.09 ± 0.35 nM). The number of receptors per adipocyte was calculated to be 16 300.

The Melanotropins. Kawauchi *et al.*[28] have isolated and characterized yet another melanotropin (MSH) from the pituitary gland of the teleost *Oncorhynchus keta* (Chum salmon). The new MSH is a pentadecapeptide that differs in size from salmon α-MSH (13 residues) and β-MSH I and II (17 residues each). The amino-acid sequence of the new peptide was found to be: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Ile-Gly-His-OH. The sequence is very similar to, yet distinct from, that of salmon α-MSH. This peptide, named α-MSH-II, is the third line of evidence in the salmon that the teleost pituitary contains two different forms of processed hormones, for which the precursors are coded on separate genes. A new peptide similar to the known equine β-MSH was isolated from horse pituitaries.[29] Amino-acid sequence analysis revealed it to be equine β-MSH without the amino-terminal aspartic acid residue. [Des-Asp[1]]-β-MSH was twice as active as equine β-MSH in stimulating lipolysis in rabbit adipocytes.

A synthetic analogue of α-MSH with high potency and prolonged biological activity has been prepared.[30] The analogue containing norleucine in position 4 in place of methionine and D-phenylalanine in position 7 in place of the L-isomer was resistant to degradation by serum enzymes. [Nle-4-D-Phe-7]-α-MSH exhibited increased biological activity in frog-skin bioassay, activation of mouse melanoma adenylate cyclase, and tyrosinase activity. The preparation of several other analogues related to α-MSH and β-MSH has been reported.[31]

[23] J. Ramachandran, K. Muramoto, M. Kenez-Keri, G. Keri, and D. I. Buckley, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3967.
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[26] M. McPherson and J. Ramachandran, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 1057.
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The isolation of *NO*-diacetylserine-1-α-MSH from porcine pituitary extracts has been reported.[32] The presence of the extra acetyl group on the amino-terminal serine was confirmed by high resolution proton n.m.r. spectra. The *o*-acetyl analogue of α-MSH was found to be as active as α-MSH in stimulating lipolysis in isolated rabbit adipocytes. The preparation of specifically tritiated α-MSH with high specific radioactivity has been described.[33] α-MSH was iodinated and [3,5-diiodoTyr-2]-α-MSH was isolated by reverse phase h.p.l.c. Catalytic dehalogenation in the presence of tritium resulted in the formation of [3,5-ditritioTyr-2]-α-MSH.[33]

LPH. β-LPH was isolated from fin whale[34] and turkey[14] pituitary glands and characterized. The primary structure of the whale hormone was found to be highly homologous to other mammalian hormones. Whale β-LPH was as active as human β-LPH in stimulating lipolysis. The turkey hormone differed considerably from mammalian hormones in the amino-acid sequence of the segment of β-LPH corresponding to β-MSH. This structural difference may account for the low lipolytic activity of the turkey β-LPH (5% of ovine β-LPH).

The isolation and characterization of human γ-LPH has been reported.[35] It consists of 56 amino-acid residues. Comparison with the structure of ovine and porcine γ-LPH reveals that the 23 amino-acid residue sequence at the carboxy-terminal is highly conserved. The amino-acid sequence of human β-LPH has been revised.[36] The human peptide contains 89 amino-acid residues compared to 91 for the ovine hormone. The lipolytic potencies of human and ovine β-LPH were identical.

Regulation of Aldosterone Secretion. It is known that the secretion of aldosterone from the *zona glomerulosa* of the mammalian adrenal cortex is stimulated by angiotensin, potassium, and ACTH. There are suggestions in the literature that pituitary factors other than ACTH may play a role in regulating aldosterone secretion.[37] During the past year two reports have appeared suggesting that α-MSH[38] and β-LPH[39] may also be involved in aldosterone regulation. Vinson *et al.*[38] purified a factor from bovine posterior pituitary extracts which stimulated glomerulosa cells. This factor was identified as α-MSH. It was reported that ACTH was equipotent in both glomerulosa cells and fasciculata cells giving half maximal stimulation of steroidogenesis at an ACTH concentration of 66–69 pM; α-MSH, on the other hand, stimulated glomerulosa cells but not fasciculata cells. Half maximal stimulation was achieved at an α-MSH concentration of 10 000 pM. Matsuoka *et al.*[39] reported that β-LPH stimulated aldosterone production in isolated rat adrenal capsular cells. Half maximal stimulation was induced by 30 000 pM β-LPH. Again, the capsular (glomerulosa) cells respond better than the fasciculata cells to β-LPH. Although these results are interesting, it is clear that neither α-MSH nor β-LPH is able to stimulate aldosterone production at

[32] D. I. Buckley, R. Joughten, and J. Ramachandran, *Int. J. Pept. Protein Res.*, 1981, **17**, 508.
[33] D. I. Buckley and J. Ramachandran, *Int. J. Pept. Protein Res.*, 1981, **17**, 514.
[34] H. Kawauchi, D. Chung, and C. H. Li, *Int, J. Pept. Protein Res.*, 1980, **15**, 171.
[35] C. H. Li, D. Chung, and D. Yamashiro, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 7214.
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[37] W. P. Palmore, R. Anderson, and P. J. Mulrow, *Endocrinology*, 1970, **86**, 728.
[38] G. P. Vinson, B. J. Whitehouse, A. Dell, T. Etienne, and H. R. Morris, *Nature*, 1980, **284**, 464.
[39] H. Matsuoka, P. J. Mulrow, and C. H. Li, *Science*, 1980, **209**, 307.

physiological concentrations. ACTH on the other hand is 150—500 times more potent than α-MSH or β-LPH. Previous studies of the structure–function relationships of these peptides have shown that steroidogenesis can be induced by any peptide containing the sequence His-Phe-Arg-Trp although pharmacological concentrations were required for activity.[40] Since both α-MSH and β-LPH contain this sequence in common with ACTH it is understandable that pharmacological concentrations of α-MSH and β-LPH can elicit steroidogenesis. The preferential stimulation of glomerulosa cells is perplexing since previous studies have shown that α-MSH can induce corticosterone production in fasciculata cells both *in vitro*[41] and *in vivo*.[42] Since both Vinson *et al.*[38] and Matsuoka *et al.*[39] performed incubation with isolated cells in the absence of proteolysis inhibitors, the observed differences may reflect differences in degradation of the peptides by glomerulosa and fasciculata cells.

Hormones of Group 2.—*Growth Hormone.* Growth hormone (GH) was isolated from fin whale[43] and sturgeon[44] pituitary glands and characterized. The molecular weight of the fin whale hormone was found to be 20 000 by sodium dodecyl sulphate gel electrophoresis and 40 000 by exclusion chromatography on Sephadex G-100 at pH 8.2. The whale hormone is similar to bovine and ovine growth hormones in this respect, existing as dimers under physiological conditions. The amino-acid composition and secondary structure of fin whale GH were found to be similar to those of other mammalian hormones. Sturgeon GH was found to be highly active in a mammalian GH assay, the rat tibia test. Chemical characterization of sturgeon GH indicated that this GH is very similar to mammalian GHs in terms of molecular weight, disc gel electrophoretic behaviour, and amino-acid composition. These data suggest that the molecular structure of GH has been highly conserved during evolution.

Selective cleavage of the peptide bond between residues 134 and 135 in human GH was accomplished by digestion with bovine thrombin conjugated to Sepharose.[45] Thrombin-digested human GH (TD-hGH) was equipotent with hGH in promoting growth in hypophysectomized rats, in stimulating the *in vitro* oxidation of glucose by isolated adipose tissue of hypophysectomized rats, in inducing *N*-acetylgalactosamine synthase activity in mammary tissue explants from pregnant mice, and in competing with [^{125}I]hGH for binding hGH antibodies. Reduction and alkylation of TD-hGH in 6 M guanidine HCl resulted in the formation of peptides (1—134) and (135—191) of hGH. Peptide (1—134) had a low order of growth-promoting and insulin-like activities but retained 25—50% of the diabetogenic activity of intact hGH in the ob/ob mouse. Peptide (135—191) was inactive.

The methionine residues of bovine GH were chemically modified with

[40] J. Ramachandran, *Hormonal Prot. Polypept.*, 1973, **2**, 1.
[41] J. Ramachandran, S. W. Farmer, S. Liles, and C. H. Li, *Biochim. Biophys. Acta*, 1976, **428**, 347.
[42] D. G. Grahame-Smith, R. W. Butcher, R. L. Ney, and E. W. Sutherland, *J. Biol. Chem.*, 1967, **242**, 5535.
[43] M. Tubokawa, H. Kawauchi, and C. H. Li, *J. Biochem. (Tokyo)*, 1980, **88**, 1407.
[44] S. W. Farmer, T. Hayashida, H. Papkoff, and A. L. Polenov, *Endocrinology*, 1981, **108**, 377.
[45] J. B. Mills, J. L. Kostyo, C. R. Reagan, S. A. Wagner, M. H. Moseley, and A. E. Wilhelmi, *Endocrinology*, 1980, **107**, 391.

chloramine-T at pH 7.4.[46] Methionine in position 4 was found to be the most reactive followed in decreasing order by methionines 148, 123, and 178. With a 50-fold molar excess of chloramine-T over methionine residues almost full oxidation of all the methionine residues was achieved. The fully oxidized bovine GH retained its growth properties.

A purification scheme for the isolation of the 20 000 dalton variant of hGH^{1-47} from human pituitary glands has been described.[48] Sequence analysis of this 20 000 dalton hGH has confirmed that residues 32–46 of hGH are absent in this variant hGH. Physicochemical comparison of hGH and the 20 000 dalton hGH indicate that the folding of the polypeptide chain of the two proteins is similar but not identical.

Purification of GH from a rat pituitary tumour cell line (GH_3) and generation of sulphation factor activity by proteolytic modification of rat GH have been reported.[49] Rat GH from GH_3 cells was found to be a single component of 22 000 daltons. Treatment of rGH with trypsin resulted in the generation of sulphation factor activity in porcine costal cartilage. Incubation of the purified rGH preparation at 37 °C for 18 h, by itself, also generated sulphation factor activity. The rGH preparation with sulphation factor activity revealed two low molecular weight bands of 14 000 daltons and 8000 daltons on sodium dodecyl sulphate gel electrophoresis in the presence of mercaptoethanol. This alteration of rGH was apparently mediated by an endogenous protease also present in purified preparations of rat and bovine GH. The growth hormone protease was characterized as a heat-sensitive serine protease capable of specifically cleaving the rGH molecule at an Arg-Ile bond. These results suggest that growth hormone may exert biological effects on peripheral tissues by a mechanism involving specific proteolytic modification.

Prolactin. Isolation and characterization of secreted hamster pituitary prolactin (PRL) has been reported.[50] Hamster PRL resembled other mammalian PRLs in its molecular weight and biological activity. PRL was also isolated from turkey pituitary glands.[51] Turkey PRL has a molecular weight of 26 000 and displays multiple bands in disc electrophoresis at pH 8.3. The turkey hormone was active in stimulating pigeon crop-sac development (standard assay for PRL) but did not give a parallel dose-response to ovine PRL. The development of a homologous RIA for turkey PRL was also described.[51]

Three forms of human pituitary PRL, separable at alkaline pH in a highly purified preparation, were isolated by means of electrophoresis in agarose suspension.[52] The most acidic component showed a significantly lower immunochemical reactivity but a higher biological activity than the other two components, which were approximately equipotent in both assays. Amino-acid analysis indicated close similarity between the three components and no size

[46] O. Cascone, M. J. Biscoglio de Jimenez Bonino, and J. A. Santone, *Int. J. Pept. Protein Res.*, 1980, **16**, 299.
[47] U. J. Lewis, L. F. Bonewald, and L. J. Lewis, *Biochem. Biophys. Res. Commun.*, 1979, **92**, 511.
[48] G. E. Chapman, K. M. Rogers, and T. Brittain, *J. Biol. Chem.*, 1980, **256**, 2395.
[49] T. Maciag, R. Forand, and S. Ilsley, *J. Biol. Chem.*, 1980, **255**, 6064.
[50] P. Colosi, E. Markoff, A. Levy, L. Ogren, N. Shine, and F. Talamantes, *Endocrinology*, 1981, **108**, 850.
[51] W. H. Burke and H. Papkoff, *Gen. Comp. Endocrinol.*, 1980, **40**, 297.
[52] F. Nyberg, P. Roos, and L. Wide, *Biochim. Biophys. Acta*, 1980, **625**, 255.

heterogeneity was observed by SDS electrophoresis in polyacrylamide gel. The electrophoretic behaviour of the two adjacent isohormones was consistent with a difference in a single net charge.

A new form of prolactin containing a cleavage in peptide chain enclosed by the large disulphide loop in the hormone has been identified.[53] The new form, referred to as cleaved PRL, was synthesized and secreted by rat pituitary glands radioactively labelled *in vitro*. Cleaved PRL was detected by SDS polyacrylamide gel analysis and identified by two dimensional mapping of tryptic digests. The two-chain molecule can be separated into an amino-terminal 16000 dalton and a carboxy-terminal 8000 dalton fragment by the reduction of the intervening disulphide bridge. The cleaved prolactin appears to be a post-translationally modified form of the hormone whose production is regulated by physiological and pharmacological stimuli. Pregnancy, pseudopregnancy, and treatment with perphenazine or estradiol, which are all associated with increased mammary growth, showed a higher ratio of cleaved PRL to intact PRL. Mittra[54] further demonstrated that the 16000 dalton chain obtained from cleaved PRL by gel electrophoresis significantly increased the rate of DNA synthesis and cell division in the mammary epithelial cells when injected subcutaneously. Intact PRL was ineffective. Mittra[53, 54] suggests that cleaved PRL may function as the mammary mitogenic factor and intact PRL may regulate function characteristic of the differentiated state (synthesis of milk-specific proteins).

Human placental lactogen or human chorionic somatomammotropin (hCS) is structurally and functionally closely related to hGH and PRL. Plasmin treatment of hCS results in the removal of the hexapeptide (135—140). By reduction and alkylation hCS (1—134) as well as a dimer of hCS (1—134) linked by a disulphide bridge were prepared.[55, 56] Whereas hCS (1—134) had less than 5% of the activity of hCS to bind to mammary gland receptors, hCS (1—134) dimer was 30% as active as intact hCS. Physicochemical studies of hCS and the plasmin cleaved hCS products indicated that important elements of the native conformation are retained in biologically active derivatives of hCS.[57] Chemical modification of the single tryptophan residue in hCS also suggests that the tryptophan *per se* is not essential for biological activity but loss of activity following modification is due to conformational changes.[58] Peptide fragments (1—134) and (141—191) obtained from hGH and hCS by plasmin treatment and reduction were used to form hybrid recombinant molecules.[59] The recombinant with hGH (1—134) and hCS (141—191) possessed both lactogenic and growth-promoting activity. The recombinant with hCS (1—134) and hGH (141—191) possessed lactogenic activity alone. These results suggest that the biological activity is contained in the (1—134) fragment and the role of the (141—191) fragment appears to be one of maintaining the overall conformation.

[53] I. Mittra, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1750.
[54] I. Mittra, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1760.
[55] J. Russell, A. B. Schneider, J. Katzhendler, and L. M. Sherwood, *J. Biol. Chem.*, 1979, **254**, 2296.
[56] J. Russell, L. M. Sherwood, K. Kowalski, and A. B. Schneider, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 1204.
[57] A. B. Schneider, F. Barr, J. Russell, K. Kowalski, and L. M. Sherwood, *J. Biol. Chem.*, 1981, **256**, 301.
[58] J. Russell, J. Katzhendler, K. Kowalski, A. B. Schneider, and L. M. Sherwood, *J. Biol. Chem.*, 1981, **256**, 304.
[59] J. Russell, L. M. Sherwood, K. Kowalski, and A. B. Schneider, *J. Biol. Chem.*, 1981, **256**, 296.

Hormones of Group 3.—*Lutropin (LH).* Three isohormones of lutropin have been isolated from ostrich pituitary glands and characterized.[60] The three hormones appear to be very similar in terms of molecular weight, amino-acid and carbohydrate composition, and electrophoretic mobility. The differences between the isohormones are attributed to differences in sialic acid content.

The carbohydrate units of the glycoprotein hormones of the pituitary gland are linked to asparagine residues of the polypeptide chains through the side-chain amide nitrogen atom. These carbohydrates are unusual in that they contain one residue of *N*-acetylgalactosamine in addition to the mannose, fucose, and *N*-acetylglucosamine typical of *N*-linked oligosaccharides. The oligosaccharides of bovine LH exhibit complete resistance to several exoglycosidases.[61] Stability of the hexosamines of LH to periodate oxidation has been traced to the presence of a sulphate group covalently linked to the hexosamine residues. Sulphation of the hexosamines is also considered the most probable reason for the resistance to enzymatic deglycosylation. The α-subunits of bovine TSH and human pituitary LH also contain sulphate, in contrast to human chorionic gonadotropin (hCG). The results indicate that sulphating enzymes are present in the pituitary and that sulphation of hexosamines may have a protective function similar to that of peripheral sialic acids in the placental gonadotropin.[61] Partial structures for the oligosaccharide units of bovine [61, 62] and ovine [62] LH have been proposed.

The effects of deglycosylation of ovine LH subunits on their interaction and biological activity have been described.[63] Brief exposure to the isolated α- and β-subunits of ovine LH to anhydrous HF resulted in effective but incomplete removal of the oligosaccharide moiety. Fucose and hexoses were completely eliminated while hexosamine content was considerably reduced. The partially deglycosylated subunits retained their capability to recognize each other and their unmodified counterparts. The recombinant of native α + partially deglycosylated β was fully active but the recombinant of deglycosylated α and deglycosylated β antagonized the action of unmodified LH in stimulating steroidogenesis in isolated Leydig cells.

Chorionic Gonadotropin (CG). The chorionic gonadotropin of the donkey (dCG) has been isolated from the serum of pregnant donkeys.[64] Whereas pregnant mare CG (eCG) has both LH and FSH activities, dCG was found to be predominantly an LH in biological tests. Specific rat testis radioreceptor assays for LH and FSH also showed dCG to be at least nine times more potent in LH than in FSH activity. Donkey CG had significantly less carbohydrate (31%) than had eCG (45%) and several differences were noted in a comparison of amino-acid compositions.

The effects of oxidation of histidine residues in eCG on the biological activities of the hormone have been examined.[65] The kinetics of loss of histidine due to Rose Bengal-sensitized photo-oxidation was biphasic, a rapid decrease followed by a slower decline. An average loss of 15—20% of the total histidines in the molecule

[60] M. M. J. Oosthuizen, W. Oelogsen, J. C. Schabort, A. W. H. Neitz, and C. C. Viljoen, *Int. J. Pept. Protein Res.*, 1980, **15**, 181.
[61] T. F. Parsons and J. G. Pierce, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 7089.
[62] O. P. Bahl, M. S. Reddy, and G. S. Bedi, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 1192.
[63] M. R. Sairam, *Arch. Biochem. Biophys.*, 1980, **204**, 199.
[64] B. B. Aggarwal, S. W. Farner, and H. Papkoff, *J. Endocrinol.*, 1980, **85**, 449.
[65] B. B. Aggarwal and H. Papkoff, *Arch. Biochem. Biophys.*, 1980, **202**, 121.

resulted in 70—80% loss of LH activity with no change in immunochemical reactivity. The loss of LH activity paralleled the decline of FSH activity. Further destruction of histidine of up to 70—90% of the total resulted in over 99% loss of biological activity without any significant alteration of immunochemical reactivity. The oxidized eCG was unable to compete with native eCG in the rat Leydig cell assay.

The immunological properties of hCG and its subunits have been investigated in detail in order to obtain antisera that can selectively neutralize hCG but not human pituitary LH, for possible use as contraceptive agents.[66-68] Ghai *et al.*[66] investigated the effects of chemical and enzymatic modifications of the immunochemical properties of the β-subunit of hCG. These detailed studies have shown that the carbohydrate does not play a significant role in the immunological activity of hCGβ and that the antigenic determinants of the polypeptide chain of hCG are conformational rather than sequential in nature. It was further inferred that hCGβ has two types of antigenic determinants, those unique to hCG and those common to both hCG and hLH. The controlled reduction and alkylation of hCGβ yielded derivatives that retained significant immunoreactivity in the hCGβ immunoassay but not in the hLH immunoassay.

Antibodies were raised against these analogues of hCGβ prepared by controlled reduction and alkylation.[67] These antibodies neutralized the specific binding of [^{125}I]hCG but not of [^{125}I]hLH to ovarian hCG/LH receptors. Also, the antibody was able to neutralize *in vivo* the effect of hCG on ovarian ascorbic acid depletion but had no effect on the depletion caused by hLH.

Birken *et al.*[68] also investigated the immunochemical determinants unique to hCG. The carboxy-terminal of hCGβ contains a peptide segment not contained in hLHβ. Studies with antisera raised against this peptide segment suggested that antisera of high affinity to the native hormone may be obtained by the use of peptides with their natural complement of sialic acid intact rather than by the use of asialo or synthetic peptides. These authors also concluded that there are at least two immunochemical determinants in hCG that are absent in hLH.

The availability of antibodies directed against the unique carboxy-terminal segment of hCGβ enabled Matsuma *et al.*[69] to detect and isolate an hCG-like substance from human pituitary glands. The hCG-like material (hCG′) was found to be highly similar in isoelectric point, molecular size, and immunological and biological activities to hCG, but distinct from hLH.

Follitropin (FSH). FSH was isolated from chicken adenohypophyses and found to be similar in amino-acid composition to turkey FSH.[70] Purified chicken FSH consisted of two components with different isoelectric points. The less acidic fraction had high FSH potency in both radioreceptor assay and radioimmunoassay. The more acidic component of chicken FSH had high potency in radioimmunoassay but not in radioreceptor assay.

[66] R. D. Ghai, T. Mise, M. R. Pandian, and O. P. Bahl, *Endocrinology*, 1980, **107**, 1556.
[67] M. R. Pandian, R. Mittra, and O. P. Bahl, *Endocrinology*, 1980, **107**, 1564.
[68] S. Birken, R. Canfield, R. Lauer, G. Agosto, and M. Gabel, *Endocrinology*, 1980, **106**, 1659.
[69] S. Matsuma, M. Ohashi, H. C. Chen, R. C. Shownkeen, A. S. Hartree, L. E. Reichert, V. C. Stevens, and J. C. Powell, *Nature*, 1980, **286**, 740.
[70] J. Sakai and S. Ishii, *Gen. Comp. Endocrinol.*, 1980, **42**, 1.

The location of the disulphide bridges in human FSH subunits has been elucidated.[71] In the α-subunit cysteine, residues in positions 7 and 10, 28 and 87, and 82 and 84 are involved in disulphide bonds. In the β-subunit disulphide bridges are formed between cysteine residues in positions 3 and 28, 17 and 51, and 32 and 104.

A glycopeptide isolated from fetuin was conjugated to human FSH and its subunits through photoactivation of an azidobenzoyl derivative of the glycopeptide.[72] This modification increased the carbohydrate content of human FSH by 2.7-fold with a concomitant 2-fold increase in biological activity. Human FSH α conjugate, when recombined with untreated human FSH β, showed a 50% increase in the biological activity over the control.

Thyrotropin (TSH). The α- and β-subunits of human TSH were purified by preparative agarose-suspension electrophoresis.[73] Each subunit preparation was resolved into four isoforms, which all had the same amino-acid composition. Recombination of two major components led to high biological activity whereas recombination of two minor components resulted in a product with low biological activity. Storage of native TSH resulted in a decrease in TSH activity (about 40% in 10 weeks).

TSH and its free subunits have been isolated from a mouse thyrotrope tumour by affinity chromatography on concanavalin A sepharose, gel filtration, and preparative electrophoresis.[74] A proportion of the free TSH α, but not TSH α derived from intact TSH, had undergone limited proteolysis at specific sites. The amino-acid composition of the intact hormone from the mouse tumour differed from those of the bovine and human hormones, but the α-subunits were similar in all three species. Purification of TSH and other glycoprotein hormones by immunoaffinity chromatography has been described.[75] Asialo choriogonadotropin, prepared by neuraminidase treatment of highly purified hCG, was found to act as a competitive antagonist of TSH on human thyroid membranes in terms of TSH binding adenylate cyclase stimulation.[76]

3 Posterior Pituitary Peptides

Contributed by M. Manning, W. A. Klis, and W. H. Sawyer

This report covers the literature from early 1980 to early 1981. Forty new analogues were reported during this period. For clarity of presentation, discussion, and readability, they have been subdivided into the following categories:

1. Antagonists of *in vivo* antidiuretic responses to arginine vasopressin (AVP) (Table 1).
2. Antagonists of *in vitro* and *in vivo* oxytocic responses to oxytocin (Table 2).
3. Antagonists of *in vivo* vasopressor responses to AVP (Table 3).
4. Agonistic analogues of oxytocin (Table 4).

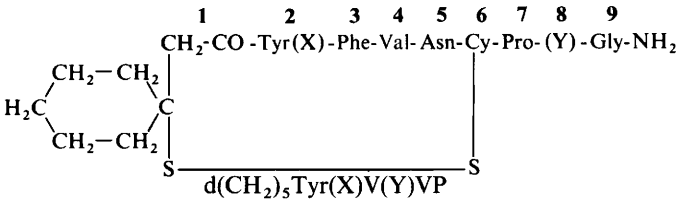
[71] Y. Fujiki, P. Rathnam, and B. B. Saxena, *Biochem. Biophys. Acta*, 1980, **624**, 428.
[72] P. Rathnam and B. B. Saxena, *Biochem. Biophys. Acta*, 1980, **624**, 436.
[73] G. Jacobson, P. Roos, and L. Wide, *Biochim. Biophys. Acta*, 1980, **625**, 146.
[74] W. W. Chin, J. F. Habener, M. A. Martorana, H. T. Kentmann, J. D. Kieffer, and F. Maloof, *Endocrinology*, 1980, **107**, 1384.
[75] F. Pekonen, D. A. Williams, and B. D. Weintraub, *Endocrinology*, 1980, **106**, 1327.
[76] P. Carayon, S. Amr, and B. Nisula, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 69.

5. Agonistic analogues of AVP and analogues of AVP prepared for special studies (Table 5).

Severe space constraints have necessitated keeping comments to a minimum and readers are advised to read the original reports for a more detailed discussion of the many highly interesting analogues presented in Tables 1—5.

Antagonists of *in vivo* Antidiuretic Responses to AVP (Table 1).—The four analogues in Table 1 are the first known effective antagonists of both exogenous and endogenous antidiuretic responses to AVP (see Vol. 11, p. 395). They are thus potentially useful pharmacological tools for studies of the roles of AVP in regulating water balance in normal and pathophysiological states in animals and in humans. They may also be of therapeutic use for the treatment of hyponatremia secondary to the inappropriate secretion of the antidiuretic hormone (SIADH or the Schwartz–Bartter syndrome).

Table 1 *Anti-antidiuretic potencies of four new antagonists of arginine vasopressin (AVP)*



Antagonists[c]	X	Y	*Anti-antidiuretic Effective dose*[a] /nmol kg^{-1}	*Ref.*
1. d(CH$_2$)$_5$Tyr(Me)VDAVP	Me	D-Arg	15 ± 3 (4)[b]	1*a*
2. d(CH$_2$)$_5$Tyr(Et)VDAVP	Et	D-Arg	5.70 ± 0.5 (4)	1*a*
3. d(CH$_2$)$_5$Tyr(Me)VAVP	Me	L-Arg	3.10 ± 0.4 (4)	1*a*
4. d(CH$_2$)$_5$Tyr(Et)VAVP	Et	L-Arg	1.90 ± 0.2 (4)	1*a*

[a] The effective dose is defined as the dose (in nmol kg^{-1}) that reduced the response to 2*x* units of agonist to equal the response to *x* units of agonist administered 20 min after antagonist. [b] Mean ± S.E., number of assay groups in parentheses. [c] The abbreviations and their full names are as follows: d(CH$_2$)$_5$Tyr(Me)VDAVP = [1-(*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH$_2$)$_5$Tyr(Et)VDAVP = [1-(*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH$_2$)$_5$Tyr(Me)VAVP = [1-(*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine,4-valine]arginine-vasopressin; d(CH$_2$)$_5$Tyr(Et)VAVP = [1-(*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,4-valine]arginine-vasopressin.

1*a* W. H. Sawyer, P. K. T. Pang, J. Seto, M. McEnroe, B. Lammek, and M. Manning, *Science*, 1981, **212**, 49.

Antagonists of *in vitro* and *in vivo* Oxytocic Responses to Oxytocin (Table 2).—Nine new antagonists of oxytocin together with two previously reported antagonists (see Vol. 12, p. 485) are presented in Table 2. The six Orn^8 analogues are all highly potent antagonists of *in vivo* oxytocic responses to oxytocin. dEt$_2$Tyr(Me)OVT and d(CH$_2$)$_5$Tyr(Me)OVT with *in vivo* antioxytocic pA_2 values of 7.35 and 7.37 are the most potent *in vivo* antagonists of oxytocin reported to date. Increasing the size of the alkyl substituents on the tyrosine residues at position two in these

Table 2 *Antagonists of oxytocic responses to oxytocin*

Peptide[d]	*Antioxytocic* (in vitro) pA_2 *No* Mg^{2+}	0.5 mm Mg^{2+}	*Rat uterus* in situ *Effective dose* /nmol kg^{-1} [a]	*Estimated* pA_2[b]	*Rat milk ejection* *Effective dose* /nmol kg^{-1} [a]	*Estimated* pA_2[b]	*Ref.*
dEt_2OVT	7.81 ± 0.07	6.91 ± 0.08	9.2 ± 1.5[c]	7.07 ± 0.12	10.3 ± 2.8	6.84 ± 0.10	2*a*
$d(CH_2)_5OVT$	7.69 ± 0.05	7.21 ± 0.12	6.0 ± 1.1	7.12 ± 0.08	9.7 ± 0.8	6.85 ± 0.04	2*a*
$dEt_2Tyr(Me)^2OVT$	8.91 ± 0.07	7.86 ± 0.13	3.3 ± 0.7	7.35 ± 0.08	5.3	7.11	2*a*
$dEt_2Tyr(Et)^2OVT$	8.50 ± 0.05	7.83 ± 0.11	6.9 ± 1.5	7.08 ± 0.12	5.3 ± 0.3	7.10 ± 0.02	2*a*
$d(CH_2)_5Tyr(Me)^2OVT$	8.52 ± 0.10	7.88 ± 0.40	4.2 ± 1.6	7.37 ± 0.17	4.7 ± 0.6	7.16 ± 0.05	2*a*
$d(CH_2)_5Tyr(Et)^2OVT$	8.25 ± 0.05	7.55 ± 0.11	8.4 ± 1.0	6.91 ± 0.05	3.4 ± 0.2	7.30 ± 0.03	2*a*
$PThr^4OT$	7.55						2*b*
$PPhe^2Thr^4OT$	7.67						2*b*
$dPThr^4OT$	7.52 ± 0.04	6.23 ± 0.11	34 ± 4	6.31 ± 0.05	agonist		2*c*
$dPPhe^2Thr^4OT$	7.49 ± 0.05	7.58 ± 0.06	61 ± 14	6.10 ± 0.12	10 ± 1	6.28 ± 0.04	2*c*
$AcTyr(Me)^2AVP$	7.29 ± 0.08	6.73 ± 0.14					2*d*

[a] The effective dose is defined as the dose (in mol kg^{-1}) that reduces the response to $2x$ units of agonist to equal the response to x units of agonist. [b] Estimated *in vivo* 'pA_2' values represent the negative logarithms of the 'effective doses' divided by the estimated volume of distribution of the antagonists. [c] Means ± S.E., number of assay groups in parentheses. [d] The abbreviations and their full names are as follows: dEt_2OVT = [1-(β-mercapto-β,β-diethylpropionic acid),8-ornithine]vasotocin; $d(CH_2)_5OVT$ = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),8-ornithine]vasotocin; $dET_2Tyr(Me)OVT$ = [1-(β-mercapto-β,β-diethylpropionic acid),2-*O*-methyltyrosine,8-ornithine]-vasotocin; $dEt_2Tyr(Et)OVT$ = [1-(β-mercapto-β,β-diethylpropionic acid),2-*O*-ethytyrosine,8-ornithine]vasotocin; $d(CH_2)_5Tyr(Me)OVT$ = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine,8-ornithine]vasotocin; $d(CH_2)_5Tyr(Et)OVT$ = [1-(β-mercapto-β,β-(cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,8-ornithine]vasotocin; $PThr^4OT$ = [1-penicillamine,4-threonine]oxytocin; $PPhe^2Thr^4OT$ = [1-penicillamine,2-phenylalanine,4-threonine]oxytocin; $dPThr^4OT$ = [1-deamino-penicillamine,4-threonine]oxytocin; $dPPhe^2Thr^4OT$ = [1-deamino-penicillamine,2-phenylalanine,4-threonine]oxytocin; $AcTyr(Me)^2AVP$ = N^a-acetyl[2-*O*-methyltyrosine]arginine-vasopressin.

2*a* K. Bankowski, M. Manning, J. Seto, J. Haldar, and W. H. Sawyer, *Int. J. Peptide Protein Res.*, 1980, **16**, 382.
2*b* V. J. Hruby, H. I. Mosberg, M. E. Hadley, W. Y. Chan, and A. M. Powell, *Int. J. Peptide Protein Res.*, 1980, **16**, 372.
2*c* W. H. Sawyer, J. Halder, D. Gazis, J. Seto, K. Bankowski, J. Lowbridge, A. Turan, and M. Manning, *Endocrinology*, 1980, **106**, 81.
2*d* D. A. Jones, jun. and W. H. Sawyer, *J. Med. Chem.* 1980, **23**, 696.

Table 3 *Antagonists of the vasopressor response to arginine vasopressin*

Peptide *	*Antivasopressor potency* *Effective dose*	pA_2	*Antidiuretic activity* $U\,mg^{-1}$	*Ref.*
dEt_2OVT	1.8 ± 0.3	7.62 ± 0.06	0.006	1*a*
$d(CH_2)_5OVT$	4.0 ± 0.09	7.26 ± 0.09	0.006	1*a*
$dEt_2Tyr(Me)^2OVT$	0.7 ± 0.1	8.02 ± 0.08	0.04	1*a*
$dEt_2Tyr(Et)^2OVT$	0.6 ± 0.2	8.11 ± 0.07	0.18	1*a*
$d(CH_2)_5Tyr(Me)^2OVT$	0.7 ± 0.2	7.96 ± 0.10	0.009	1*a*
$d(CH_2)_5Tyr(Et)^2OVT$	1.3 ± 0.3	7.84 ± 0.09	0.005	1*a*
$AcTyr(Me)^2AVP$		7.18 ± 0.08	0.026 ± 0.002	2*d*

* Abbreviations as in Table 1.

peptides did not lead to increases in antagonistic potencies. Rather the *O*-ethyl–*O*-methyl interchange led to a halving of antagonist potency in each case. This is an interesting contrast to the effects of identical interchanges in the antidiuretic antagonists listed in Table 1. In both instances, anti-antidiuretic potencies were increased two-fold by the replacement of *O*-ethyl for *O*-methyl on the tyrosine residue at position two.

Antagonists of Vasopressor Responses to AVP (Table 3).—The seven analogues in Table 3 are taken from Table 2 because, besides being potent anti-oxytocics, they have antivasopressor pA_2 values ranging from 7.18 to 8.11. They are thus nearly all equal in antivasopressor potency to all but two previously reported AVP vasopressor antagonists (see Vol. 12, p. 487). In addition, the low antidiuretic potencies of a number of these antagonists endows them with a degree of antivasopressor–antidiuretic selectivity hitherto unencountered or unreported in all but the antidiuretic antagonists given in Table 1.

Agonistic Analogues of Oxytocin (Table 4).—Seventeen new analogues of oxytocin have been reported. [8-Tryptophan]-oxytocin has very interesting and unusual properties. It behaved as a mixed agonist–antagonist in the *in vitro* rat uterus assay in the presence and in the absence of Mg^{2+}. On the basis of these findings, the substitution of tryptophan at position 8 in known antagonists of oxytocin is highly warranted and might lead to the development of more potent and selective antagonists of *in vivo* oxytocic responses to oxytocin. [8-α-Hydroxyisocaproic acid]-oxytocin was found to possess $\sim 160\,U\,mg^{-1}$ of oxytocic activity in the absence of Mg^{2+}, $\sim 1/3$ that of oxytocin. Its antidiuretic activity is only $0.06\,U\,mg^{-1}$, which is $\sim 1/50$th that of oxytocin. Its oxytocic/antidiuretic ratio (O/A) is thus >2600, a significant enhancement over that of oxytocin ($O/A < 130$).

On the basis of the properties of deamino-[9-glycolicamide]oxytocin it is concluded that the proposed hydrogen bond between the peptide N-H of Gly^9 and the C=O of Cys^6 in oxytocin is not critical for the biologically active conformation required for the interaction of oxytocin with its receptors in the uterus, mammary gland, and other target organs (see Vol. 5, p. 409; Vol. 6, p. 444).

Agonistic Analogues of AVP (Table 5).—Twelve new analogues have been reported in this category. [2-D-Tyrosine]AVP was inadvertently omitted from previous

Table 4 *Agonistic analogues of oxytocin* *

	Rat uterus		*Rat milk*	*Rat*	*Rat*	
	No Mg^{2+}	0.5 mM Mg^{2+}	*ejection*	*antidiuretic*	*pressor*	*Ref.*
Deamino-6-carba[2-*O*-methyltyrosine]oxytocin	3.14	†	18.0			4*a*, *b*
Deamino-6-carba[2-*O*-methyltyrosine]oxytocin sulphoxide	5.25		10.1			4*a*
Deamino-6-carba[2-isoleucine]oxytocin	3.1		90.0			4*a*
Deamino-6-carba[2-isoleucine]oxytocin sulphoxide	8.44		88.1			4*a*
Deamino-6-carba[2-methionine]oxytocin	4.74		7.3			4*a*
Deamino-6-carba[2-methionine]oxytocin bis-sulphoxide	0.13		<0.05			4*a*
Deamino[1,6-homolantionine,2-*O*-methyltyrosine]oxytocin	0.24		0.74			4*a*
Deamino[1,6-homolantionine,2-isoleucine]oxytocin	2.36		6.54			4*a*
[8-α-Hydroxyisocaprionic acid]oxytocin	158 ± 11	98 ± 13	63 ± 5	0.06 ± 0.02	0.36 ± 0.03	4*c*
Oxytocinoic acid dimethylamide				0.096 ± 0.004	<0.01	4*d*
[1-α-Mercaptoacetic acid, 5-isoasparagine]oxytocin	0.098 ± 0.002					4*e*
Deamino[9-glycolicamide]oxytocin	134 ± 13	355 ± 48	108 ± 8	2.5 ± 0.1	0.35 ± 0.3	4*f*
[8-Tryptophan]oxytocin	~55	~15				4*g*
Deamino-1-carba[4-valine]oxytocin	2.8		10.9	0.003	<0.2	4*h*
Deamino-1-carba[4-isoleucine]oxytocin	58.7		295.5	3.00	2.8	4*h*
Deamino-1-carba[4-leucine]oxytocin	0.4		0.5	<0.15	<0.2	4*h*
Deamino-1-carba[4-glutamic acid]oxytocin	1.0		39.6	0.04	<0.2	4*h*, *i*

* Biological activities expressed in units mg^{-1} ± S.E. † *In vivo* 75 U mg^{-1}.

4*a* M. Lebl, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1980, **45**, 2855.
4*b* T. Barth, I. Skopkova, M. Lebl, and K. Jost, *Collect. Czech. Chem. Commun.*, 1980, **45**, 3045.
4*c* J. Roy, D. Bazis, and I. L. Schwartz, *Int. J. Protein Res.*, 1980, **16**, 106.
4*d* Y. F. Ting, C. W. Smith, G. L. Stahl, and R. Walter, *J. Med. Chem.*, 1980, **23**, 693.
4*e* J. Roy, M Johnson, S. Dubin, D. C. Gazis, and I. L. Schwartz, *Int. J. Peptide Protein Res.*, 1980, **15**, 279.
4*f* J. Roy, M. Johnson, D. Gazis, and I. L. Schwartz, *Int. J. Peptide Protein Res.*, 1980, **16**, 55.
4*g* M. Bodansky, J. C. Tolle, J. Seto, and W. H. Sawyer, *J. Med. Chem.*, 1980, **23**, 1258.
4*h* M. Lebl, A. Machova, P. Hrbas, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*,1980, **45**, 2714.
4*i* P. Hrbas, T. Barth, J. Skopkova, M. Lebl, and K. Jost, *Endocrinol. Exper.*, 1980, **14**, 151.

Table 5 *Agonistic analogues of AVP*

	Rat uterus oxytocic	*Rat milk ejection*	*Rat antidiuretic*	*Rat pressor*	[H]0.5* /M	*Ref.*
[7-Glycine,8-ornithine]vasopressin	0.45	7	1.2	0.78	—	*5a*
Deamino[7-glycine,8-ornithine]vasopressin	1.6	7	0.61	0.63	—	*5a*
N^{α}-Glycyl-glycyl-glycyl[7-glycine,8-ornithine]vasopressin	0.006	—	0.032	—	—	*5a*
[2-(3′,5′-Deutero)tyrosine]lysine-vasopressin	—	—	—	337 ± 3	—	*5b*
[2-D-Tyrosine]arginine-vasopressin	1.53 ± 0.09	—	207 ± 10	194 ± 11	—	*5c*
[5-Aspartic acid]arginine-vasopressin	0.38 ± 0.03	—	86.5 ± 48	6.93 ± 0.5	—	*5d*
[2-(4′-amino)phenylalanine]arginine-vasopressin	—	—	—	—	—	*5e*
AVP	15 ± 1	—	332 ± 20	376 ± 6	8.8×10^{-9}	*5f, e*
[2-(4′-Azido)phenylalanine]arginine-vasopressin	—	—	—	—	3.8×10^{-8}	*5e*
[2-(4′-Bromoacetylamino)phenyalanine]arginine-vasopressin	—	—	—	—	7.9×10^{-8}	*5e*
N^{α}-(5-Dimethylaminonaphthalene sulphonyl)[2-(4′-azido)phenyl-alanine]arginine-vasopressin	—	—	—	—	6.1×10^{-7}	*5e*
[2-Phenylalanine,3-(4′-azido)phenylalanine]arginine-vasopressin	—	—	—	—	3.0×10^{-7}	*5e*
[2-Phenylalanine,3(4′-bromoacetylamino)phenylalanine]arginine-vasopressin	—	—	—	—	2.5×10^{-6}	*5e*

*Activation of adenylate cyclase by AVP and its analogues; [H]0.5 = hormone concentration for half maximal stimulation.

5a M. Lebl, T. Barth, J. Skopkova, and K. Jost, *Collect. Czech. Chem. Commun.*, 1980, **45**, 2865.
5b A. A. Bothner-by, B. Lemarie, R. Walter, R. Tiao-TeCo, L. D. Rabbani, and E. Breslow, *Int. J. Peptide Protein Res.*, 1980, **16**, 450.
5c W. J. Hruby, D. A. Upson, D. M. Yamamoto, C. W. Smith, and R. Walter, *J. Am. Chem. Soc.*, 1979, **101**, 2717.
5d C. W. Smith, G. Skala, and R. Walter, *Int. J. Peptide Protein Res.*, 1980, **16**, 365.
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reports. Its relatively high antidiuretic and vasopressor potencies are in striking contrast to the virtual abolishment of these activities and the drastic decrease in oxytocic potency obtained when D-tyrosine was substituted in oxytocin.[1] The obvious conclusion to be drawn from these findings is that the tyrosine residue in oxytocin plays a much more critical role in binding to and stimulating all receptors than does the tyrosine residue in AVP. These findings have been interpreted to support the proposed biologically active conformational models for oxytocin and AVP (see Vol. 5, p. 409; Vol. 6, p. 444).

Analogues modified at positions 2 and 3 with reactive chemical groups (*p*-azido, *N*-α-dansyl-*p*-azido, and *p*-bromoacetylamino) have been found to have interesting *in vitro* properties. The position-two modified analogues were found to have the same activities and binding affinities as AVP for the stimulation of bovine kidney adenylate cyclase. Such analogues have obvious potential for the labelling of vasopressin receptors in plasma membranes and for studies of covalent hormone–receptor complexes.

Other Studies.—A new active neurohypophysial peptide has been discovered in two kangaroo species.[2] It is [2-phenylalanine]-arginine vasopressin or 'phenypressin'. This peptide could have arisen from a point mutation in the arginine vasopressin genome. It is unusual in that it is the first natural peptide found containing an amino-acid other than tyrosine in the 2-position. It may also be worth noting that phenypressin, like four of the nine previously known vertebrate neurohypophysial principles, had been synthesized and pharmacologically characterized[3] long before it was isolated and identified from natural sources.

Earlier reports that large doses of LVP could improve memory in man (see Vol. 10, p. 432) have been confirmed and extended by Weingarten *et al.* (1981).[4] Careful attention to fluid restriction allowed these investigators to perform double-blind studies on patients receiving dDAVP or placebo intranasally for 2—3 weeks. The peptide enhanced performance of normal young adults in tests of learning and memory. It also improved memory in depressed patients and appeared to decrease the period of retrograde amnesia produced by electroconvulsive therapy. These results should encourage a search for vasopressin analogues that influence learning and memory but do not also cause water retention, as does dDAVP. These could be of potential value in the treatment of subjects with memory deficiencies.

4 Pancreatic Hormones

Contributed by D. Brandenburg and D. Saunders

The present state of insulin and diabetes research is well documented in the Proceedings of the 10th Congress of the International Diabetes Federation

[1] S. Drabarek and V. du Vigneaud, *J. Am. Chem. Soc.*, 1965, **37**, 3974.
[2] M. T. Chauvet, D. Hurpet, J. Chauvet, and R. Archer, *Nature*, 1980, **287**, 640.
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[4] H. Weingartner, P. Gold, J. C. Ballenger, S. A. Smallberg, R. Summers, D. R. Rubinow, R. M. Post, and F. K. Goodwin, *Science*, 1981, **211**, 603.

(Vienna) [1] and of the 2nd International Insulin Symposium (Aachen).[2] The former naturally emphasizes medical and biological aspects of the disease and contains sections on receptors, action, and release of insulin, as well as on somatomedins and IGFs (insulin-like growth factors). Of particular interest are contributions on the biosynthesis of insulin and glucagon and reviews on the structure of insulin and related peptides,[3] the binding surface,[4] and its chemistry and function.[5,6] The Aachen Proceedings contain papers on insulin (70), proinsulin, preproinsulin, and C-peptide (5), IGF (2), relaxin (2), glucagon (4), somatostatin, and pancreatic polypeptide. They deal with structural aspects, peptide synthesis, semisynthesis, chemical modification, and labelling. Other sections cover reversible and irreversible hormone–receptor interactions, structure, binding and activity, and degradation. Immunology, biosynthesis, storage, and evolution are also treated. Relevant contributions are included in this article unless full experimental papers have appeared in the meantime.

Reviews have appeared on recombinant DNA as a new source of insulin,[7] the role of receptors in membrane transduction,[8] hormone-induced clustering of membrane receptors,[9] and internalization of polypeptide hormones.[10] It is unclear whether internalization is linked to hormone action.

The four hormone families represented by insulin, glucagon, somatostatin, and pancreatic polypeptide,[11] and homologies between insulin and relaxin,[12] as well as nerve growth factor [13] have also been discussed.

The physico-chemical properties of insulin in solution become important with respect to artificial delivery systems, and aggregation in such devices is reviewed.[14]

Insulin.—Insulin has been detected in a broad range of extrapancreatic tissues in rats and humans, and may be synthesized by these tissues.[15] The recent demonstration of the presence of material with insulin activity and immunoreactivity in unicellular eukaryotes (*e.g. Neurospora crassa*) may indicate that insulin is evolutionarily much more ancient than has been thought.[16] During the 500 million years of vertebrate evolution only small changes in the hormone's

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[2] 'Insulin, Chemistry, Structure and Function of Insulin and Related Hormones', Proceedings of the 2nd International Insulin Symposium, Aachen, 4—7 September, 1979, ed. D. Brandenburg and A. Wollmer, Walter De Gruyter, Berlin/New York, 1980.

[3] G. G. Dodson, E. J. Dodson, C. D. Reynolds, and D. Vallely, in ref. 1, p. 129.

[4] J. E. Pitt, in ref. 1, p. 88.

[5] S.-C. Chu, D.-F. Cui, T.-F. Li, C.-P. Tsao, and Y.-S. Shang, in ref. 1, p. 124.

[6] D. Brandenburg, in ref. 1, p. 134.

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regulatory mechanism have occurred, as detailed studies with hagfish insulin have shown.[22]

Over 60 new analogues and derivatives have been prepared in 1980, and further data have been obtained with an equal number of known insulins (Tables 1 and 2).

Isolation, Synthesis, Semisynthesis, Chemical Modification. Insulins from porcupine (*Hystrix cristata*) and a Chinese non-venomous snake, *Zaocys dhumnades dhumnades* (Cantor),[17] have been isolated. The former has been sequenced and studied in detail[18,19] (see below).

Details of the production of human insulin in bacteria have not been published. Clinical trials have been carried out,[25] the first time that any hormone from recombinant DNA has been used for human therapy.[26] Peptide synthesis *via* fragment condensation in solution and subsequent combination with natural partner chains have yielded three analogues with modified A-chains [(2), (23), and (25)] and five with altered B-chains [(48)—(52)]. Benzyl,[50] trityl,[43,89] and

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thioethyl[63] groups were used for thiol protection. Enzyme-assisted semisynthesis is progressing apace.[32, 33, 65, 73, 74] The conversion of porcine into human insulin is possible *via* the tryptic coupling of Thr(OMe) and mild saponification,[32] as well as the use of *Achromobacter* protease,[33] which is specific for lysine. Synthetic octapeptides with Leu in position B24 or 25 have been coupled by trypsin to Boc_2-des-octapeptide insulin [(58) and (60)] in order to establish the structure of an abnormal insulin (59). Although chemical semisynthesis in this region is difficult, it remains the only possibility once Arg is removed, as in the preparation of the analogues (64) and (65) by coupling of tetrapeptides to des-nonapeptide insulin.[5] Limited digestion of (63) with carboxypeptidase A gave the des-heptapeptide analogue (62), and specific tryptic cleavage at B29 occurred after blocking Arg with cyclohexanedione.[64]

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Table 1 *Analogues and derivatives of insulin*

Compound[a]		*Structure*[b]	*Species*[c]	% *Biological activity*[d] in vivo	in vitro		% *Receptor binding*[d]		*Ref.*
Insulin									
1*a*	^{3}H-labelled								27, 28
b			beef[f]		105		99		29
c			casiragua		5		5		19
					6		3		20
d			chicken	100					17
e	^{125}I-labelled		cod						30
f			guinea-pig						20
g			hagfish		5		25		22, 23
	^{125}I-labelled								24
h			horse						31
i			human						32, 33, 25, 34
j	^{125}I-labelled		lamprey						30
*k**			porcupine[g]	30	4		25		18, 19
l			rabbit						35
m			rat						35
n			salmon						36
o			sheep				80		31, 37
*p**			snake[g]	84					17
A-Chain									
2	Arg^{A-2}-Arg^{A-1}-Arg^{AO}		b				125	L	37, 38
3	Arg^{A-1}-Arg^{AO}		b		35	C	90.5	L	37, 38
4	Lys^{A-1}-Arg^{AO}		b		36	C	80	L	37, 38
5	Arg^{AO}		b		39	C	107	L	37, 38
6	Ac	A1					23.2	L	37, 38
7	Aceto-Ac	A1							39, 40
8	Boc	A1					15.2	L	37, 38

9	Carbamoyl	A1	b			43	C	25.9	L	37, 38, 39, 40
10	Guanyl	A1	b					84.2	L	37, 38
11	P-SS-B	A1		36						90
12	Thiaz	A1	b					5.6	L	37, 38, 39, 40
13	[D-Ala^{A1}]		p	96	B	74	C	31	L	41
						107	K	19	H	41
14	[β-Ala^{A1}]		p	49	B	58	C	62	L	41
						45	K	71	H	41
15	[ε-Ahx^{A1}]		b	33	B	42	C	37	L	41
						41	K	38	H	41
16	[D-Glu^{A1}]		b	81	B	79	C	70	L	41
						112	K	23	H	41
17	[D-Leu^{A1}]		b	69	B	112	C	81	L	41
						105	K	79	H	41
18	[D-Lys^{A1}]		b	46	B	61	C	66	L	41
						47	K	61	H	41
19	[D-Phe^{A1}]		b	57	B	60	C	71	L	41
						35	K	62	H	41
20*	[^{14}C-Me$_2$-Gly^{A1}]					86	C			42
21*	[Phe^{A14}]		p			96	C			43
22*a*	[Tyr(3-^{125}I)^{A14}]		p							30, 44, 45
								+	Ad	46
						+	C	+	Ad	47
								+	L	48
22*b**	[Tyr(3-^{127}I)A^{14}]		p							49
23	[Phe^{A19}]		p			22.6	C			89
24*a*	[Tyr(3-^{125}I)^{A19}]		p							30, 44, 45
						+		+	Ad	47
								66	L	48
24*b*	[Tyr(3-^{127}I)^{A19}]		p							49
25*	[Asn-NH$_2{}^{A21}$]		o	72	A	14.8	C	51.4	Ad	50
						12	D	63.9	L	50

Table 1 *(cont.)*

Compound[a]	*Structure*[b]	*Species*[c]	% *Biological activity*[d] in vivo		% *Biological activity*[d] in vitro		% *Receptor binding*[d]		*Ref.*
B-Chain									
26*	Mesyl-Met^{BO}				59.9	C	51		51
27	Biotinyl	B1					100	L	52, 53
							100	L	37, 38
28	Carbamoyl	B1							39, 40
29*	Ethylcarbamoyl	B1	20	B	26	C	27	L	54
30*	Hexanoyl	B1	50	B	38	C	79	L	54
31*	1-Naphthylcarbamoyl	B1	0	B	22	C	75	L	54
32*	Octadecanoyl	B1	0	B	43	C	37	L	54
33*	PEG	p							54
34*	PEG_{100}	B1	100	B	26	C	43	L	54
35*	PEG_{200}	B1	100	B	30	C	48	L	54
36*	PEG_{1500}	B1	100	B	36	C	32	Ad	54
			prolonged	B			42	L	54
37*	Sulphopropionyl	B1	100	B	97	C	70	Ad	54
							77	L	34, 54, 55
38*	Undecanoyl	B1	10	B	10	C	37	L	54
39*	[Ala$(SO_3H)^{B1}$]		95—100	B					54
40*a*	[Desamino-Tyr(3-^{125}I)^{B1}]	b							56
40*b**	[Desamino-Tyr(3-^{127}I)^{B1}]	b			97.2	C			57
41	[Desamino-Tyr(3,5-$^{127}I_2$)^{B1}]	b			77.5	C			57
42*a**	[^{14}C-$(Me)_2$-Phe^{B1}]				95				42
42*b**	[^{3}H-$(Me)_2$-Phe^{B1}]				95				42
43	[^{3}H-Phe^{B1}]						+		58, 59, 60
44*a**	[Trp^{B1}]	b	70	A					61
44*b**		p	70	A					61
45	Des-Phe^{B1}	b							34, 62
		p							34
46	Des(B1—4)	b							35
47	[Lys^{B3}]	p							35

48*	[Ala^{B4}]-des(B1—3),des(B28—30)			50	A					63	
49*	[Ala^{B4,9,10,27}]-des(B1—3),des(B28—30)			20	A					63	
50*	[Ala^{B4,27}]-des(B1—3),des(B28—30)			50	A					63	
51*	[Ala^{B4},Val^{B6},Ala^{B9,10,27}]-des(B1—3),des(B28—30)			10	A					63	
52*	[Ala^{B9,10,27}]-des(B28—30)			50	A					63	
53*	[Arg(DHCH)^{B22}]		p							64	
54	[Arg(DHCH)^{B22}]-des-Ala^{B30}		p							64	
55*	[Met^{B23}]-des(B24—30)									65	
56*	Des(B22—30)		p	<0.6	A					5	
57	Des(B23—30)									35, 37	
										38, 66, 67, 69	
			b[h]		D, K					68	
			p[h]		D, K					68	
			p[h]		C					70	
58*a*	Leu^{B24}		h			12.2	C			73	
58*b*			p			+	D	9	Ad	74	
								8	H	75	
59	Leu$^{B24/25}$		h							71, 72	
60*a*	Leu^{B25}		h			3.4	C			73	
60*b*			p			+	D	1	Ad	74	
								1	H	74	
61*	[Ala^{B24,25,26}]			0.4	A					5	
62	Des(B24—30)			0	A					75	
63	Des(B26—30)		b							35	
			p					36	L	37, 38	
			p							76	
			p	30	A					5	
64*	Asp^{B22}-des(B26—30)		p	30—40	A					5	
65*	[Asp^{B22},D-Ala^{B23}]-des(B26—30)		p	30	A					5	
66	Ftc	B29						+	Ad	77	
67	Rtc	B29						+		78	
68	Suc	B29								40	
69*	Lys(^{14}C-Me$_2$)^{B29}					93	C			42	
70	[Thr^{B30}-OBu^t]		h							33	

Table 1 *(cont.)*

Compound[a]	*Structure*[b]		*Species*[c]	% *Biological activity*[d] in vivo		% *Biological activity*[d] in vitro		% *Receptor binding*[d]		*Ref.*
71	[Thr^{B30}-OMe]		h							32
72	Des-Ala^{B30}		p							33, 35, 64
A-+B-Chain										
73	$(Ac)_2$	A1, B29								39, 40
74	^{14}C-$(acetamidino)_2$	A1, B29	b			100	D			79
75*	^{14}C-$(acetamidino)_3$	A1, B1, B29	b			reduced	D			79
76*	$(Boc)_2$-des(B23—30)-N_2H_3	A1, B1								65
77	$(Carbamoyl)_3$	A1, B1, B29								39, 40, 31
			p							35
78	$(Guanyl)_2$	A1, B29	b					100	L	37, 38
79	$(Msc)_3$	A1, B1, B29	b							34
80*	[^{14}C-Me_2Gly^{A1},Me_2Phe^{B1},$Lys(Me_2)^{B29}$]					77	C			
81	$Tyr(3\text{-}NO_2)^{A14,19,B16,26}$		b	50—70	A	55	C			80
82	Des-Asn^{A21}, des-Ala^{B20}		b[h]				D, K			68
Cross-linked Insulins										
83	A_2Sub		b							81
84	$(Boc)^2$-Cys	A1, B29								39, 40
85	Cys	A1, B29								39, 40
86	Dodecane-dioyl	A1, B29								39, 40
87	Oxaloyl	A1, B29								39, 40
88	Sub	A1, B29								39, 40
89	Sub-des-Phe^{B1}	A1, B29								39, 40
90	$(Z\text{-}Lys)_2$-(Ad)	A1, B1								39, 40
91		A1—B29								39, 40

92*	Sub(insulin)$_2$		A1—A1′							82
				12	B	5	C	36	Ad	83
								21	L	83
		^{125}I						+	H	24
93*			A1—B1′							82
				18	B	25	C	96	Ad	83
								37	L	83
		^{125}I						+	H	24
94*			A1—B29′	10	B	28	C	66	Ad	83
								32	L	83
								+	H	24
95*	Sub(insulin)$_2$		B1—B1′							82
				20	B	6	C	29	Ad	83
								16	L	83
						15	C	70	Ad	84
		^{125}I						+	H	24
								+	Ad	84
96			B1—B29′							82
				14	B	29	C	82	Ad	83
								44	L	83
						38	C	92	Ad	84
		^{125}I						+	H	24
								+	Ad	84
97*	Dodecane-dioyl (insulin)$_2$		B29—29′							82
98*	Oxaloyl(insulin)$_2$		B29—29′							82
99	Sebacoyl(insulin)$_2$		B29—B29′							82
100	Sub(insulin)$_2$		B29—B29′							82
				9	B	7	C	41	Ad	83
								4	L	83
		^{125}I						+	H	24
101*	Alkaline phosphatase-mercaptobutyryl-insulin (A1)									90
102	Dextran-^{125}I-insulin									85

Compound[a]	*Structure*[b]	*Species*[c]	% *Biological activity*[d] in vivo	in vitro	% *Receptor bindi*	*Ref.*
103*	Diphtheria toxin fragment A-insulin					86
104	β-D-Galactosidase-mercaptosuccinyl-insulin	p				87
105	Poly(*N*-vinylpyrrolidone)-insulin (B29)					88
106*a*	Biotinyl-insulin–avidin complex (B1)					53
*b**	Biotinyl-insulin–SpHPP-avidin complex				+	52
*c**	Biotinyl-insulin–^{125}I-SpHPP-avidin complex				+	52
107*	Biotinyl-insulin–SpHPP-avidin–bioctinamide-Sepharose complex				+	52

[a] New compounds are marked by an asterisk. [b] Abbreviations: Ad = adiopoyl, Ahx = aminohexanoyl, DHCH = 1,2-dihydroxycyclohex-1,2-ylene, Ftc = fluoresceine thiocarbamoyl, PEG = polyethyleneglycol succinoyl, PSSB = 2-pyridyldithiobutyrimidyl, Rtc = rhodamine thiocarbamoyl, SpHPP = succinoyl-3-(4-hydroxyphenyl)-propionyl, Suc = succinoyl, TAN = 4-trimethylammonio-2-nitrophenyl, Thiaz = 2-dimethyl-3-formyl-L-thiazolidine-4-carbonyl, Z_2-LAL = α,α′-bis-benzyloxycarbonyl-(ε,ε′-adipoyl)-bis-lysyl. [c] Species: b = bovine, h = human, p = porcine, o = ovine. [d] % Potency or % binding based on insulin (porcine, bovine) = 100. Figures for dimers are also on weight basis. The following test systems are indicated: A = mouse convulsion assay, Ad = adipocytes or adipocyte membranes, B = *in vivo* blood glucose assay, C = lipogenesis in isolated adipocytes, D = glucose oxidation in isolated adipocytes, H = cultured human lymphocytes, K = ^{14}C-incorporation in diaphragma, L = liver cells or liver cell membranes. [f] Synthetic. [g] Crystalline. [h] Highly purified.

Careful purification of des-octapeptide (57) and des-Asn-des-Ala-insulin (82) by (repeated) isoelectric focusing or h.p.l.c. in combination with gel and ion-exchange chromatography has now been described in detail, as have purity tests.[68] The three amino-groups remain the major sites for specific chemical modifications[91] and are the starting points for sequential alterations. Msc-groups are particularly valuable for partial protection owing to their acid stability. They have been used in routes leading to replacements of A1-glycine [(13)—(19)], of B1-phenylalanine [(40), (41), and (44)], as well as specific reductive methylation [(20), (42), and (69)], the preparation of dimers [(92)—(100)], and a variety of photoreactive insulins (see below). Boc protection was used for acylations at B1 [(26), (29)—(38)], and citraconylation at A1 and B1 to prepare (66).

^{3}H- and ^{14}C-labelling of insulin to low specific activities has been accomplished by reductive methylation [(20), (42), (69), and (80)] and acetamidation of amino-groups [(74) and (75)], both of which maintain the positive charges, and by a modified Wilzbach procedure.[27] Iodination of insulin has been studied in detail, and homogeneous iodo-insulins were isolated by h.p.l.c.,[45] ion-exchange chromatography,[44, 92] disk electrophoresis,[30] or a combination of the two last mentioned procedures[49] [compounds (1*e*), (1*j*), (22), and (24)]. The fully active A14 derivative (22*a*) has been used in various receptor binding and also degradation studies. Semisynthetic (40*a*) is the first tracer iodinated in the B-chain to a high specific activity. ^{125}I-Avidin can be used to prepare labelled complexes like (106*c*); non-specific binding to membranes is reduced by succinoylation.[52]

Tetranitro-insulin (81) has now been fully characterized.[80] The reaction of insulin with tetranitromethane gave predominantly (*ca.* 70%) dimers and oligomers. Three homogeneous symmetrical dimers [(92), (95), and (97)—(100)] were obtained by crosslinking the three amino-groups of isomeric Msc_2-insulins and the three asymmetrical dimers [(93), (94), and (96)] were obtained in a two-step synthesis *via* activated intermediates.[82]

Insulin has been linked to other proteins by acylation with *S*-acetylmercapto-succinic anhydride,[87] butyrimidyl pyridine disulphide,[90] or DCC-coupling of cystamine,[86] and subsequent reaction *via* the introduced SH-group. The conjugates (102) and (104) were used in enzyme immunoassays, and (103) in internalization studies.

The synthesis of the two protected segments 1—45 and 46—86 of human proinsulin has been described.[93] The stepwise assembly of 16 segments gave a proinsulin preparation with 10% of the immunoreactivity of native proinsulin.[94] Semisynthesis with bovine proinsulin is being further explored, and analogues with the following extensions at the *N*-terminus have been prepared: Ala-Gln-Ala,[95] Boc-Met, Met, Boc, citraconyl,[96] as well as the side-chain protected derivative 29,59-Msc_2-proinsulin.[96] The semisynthesis of des-(1—13)- as well as a

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complete hybrid (rat/beef) preproinsulin sequence (oxidized form) have been accomplished.[95]

Structure, Receptor Binding, and Biological Activity. A comparison of insulin structures in 2-zinc and 4-zinc crystals,[97] and correlations between the structure in crystals and in solution, based on *X*-ray analysis and tyrosyl c.d. of insulin, des-Phe^{B1}, and A1-B29-crosslinked insulin (83), have been reported.[62, 81] C.d. studies have also been reported for porcupine (1*k*),[18] casiragua (1*c*),[20] guinea-pig,[20] and beef insulin[98] as well as compounds (23), (25), (26), and (81). Marked spectral changes (reduction of ellipticities, reduced response to concentration changes, and to zinc ions) are usually paralleled by low potency and binding. However, A_2Sub-insulin (83) appears to be structurally intact but has low binding, while tetranitro-insulin (81) is still quite potent but shows a much changed spectrum. Compounds (1*c*), (1*k*), and (81) do not bind zinc and are monomeric. Porcupine differs from bovine insulin at seven positions: Asp^{A4}, Thr^{A8}, Gly^{A9}, Gln^{A17}, Asn^{B21}, Asp^{B22}, and Arg^{B27}.

Zinc binding of bovine insulin and nerve growth factor has been studied.[99]

Higher receptor binding than *in vitro* potency, which was so far only confirmed for hagfish insulin,[23, 24] has now also been demonstrated for porcupine insulin[18] and a variety of analogues with modifications at quite different sites: the *N*-terminus of the A-chain [extensions by basic amino-acids, *e.g.* (2), (3), (5)], the *C*-terminus [replacement of Asn^{A21} by the amide, (25)], and the *N*-terminus of the B-chain [moderate with PEG-insulins, pronounced with the naphthylcarbamoyl derivative (31)]. Furthermore, all six isomeric dimers [(92)—(96), (100)] show a marked disparity.

A-Chain. Except for compounds (2)—(5) (see above) binding of several derivatives [*e.g.* (6)—(12)] to bovine liver membranes corresponds to binding to other tissues and to previously reported *in-vitro* potencies.[37, 38] Extensive studies with analogues (13)—(19) show generally good agreement between binding and lipogenic potency, but disparities with stimulation of glycogen synthesis in rat diaphragm, which may correlate with hypoglycaemic activity.[41] At the *C*-terminus, replacement of the negative charge by the amide group (25) affects the structure (c.d.) slightly, and *in vitro* potency more than binding. The Tyr^{A19} → Phe replacement results in loss of potency with minimal conformational changes, whereas the phenolic hydroxy-group in A14 (21) is dispensible, and the ring appears to possess high thermal mobility.[62] A similar situation is found for B1-phenylalanine, which is in close contact.

B-Chain and dimers. It is remarkable that only one *N*-terminal derivative exhibits full binding affinity (27) and only three [(37), (40), and (42)] full *in vitro* potency. High binding (75%) is found with substituents as different as the sulphopropionyl and the naphthylcarbamoyl group [(37) *vs.* (31)], whereas the ethylcarbamoyl group (29) reduces binding and lipogenic potency markedly. *In vivo* potencies range between 100% and 0%, and can even be zero in cases where considerable *in vitro* activity is observed [(31) and (32)]. Except for (26), which shows some conformational changes (these are more pronounced than with Met^{BO}-insulin, *cf.*

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Vol. 12), structural data are not available. The new observations are additional support for a new definition of the biological role of the *N*-terminus of the B-chain.

An abnormal insulin (59) has now been further characterized, but the assignment of its structure is still ambiguous. [B24Leu]-insulin has a low and [B25Leu]-insulin a very low potency. Although these findings would support the biological importance of this region, the low but significant biological activity of highly purified des-octapeptide insulin[68] and its ability to produce maximal insulin effects make it difficult to maintain the analogy between receptor binding and dimerization (*cf.* previous volumes). The key role of this region has also been questioned on the basis of *X*-ray analysis (3.1 Å spacing) and c.d. studies with the crosslinked insulin (83).[81] Conformational adjustment of parts of the insulin molecule may be important requirements for correct and efficient receptor binding. Initial *in vivo* data have been reported for a number of analogues [(48)—(52), (61), (62), (64), and (65)] and are indications, but not clear evidence, for the significance or insignificance of particular residues in structure–function considerations.

The insulin dimers (92)—(100) exhibit interesting and often unexpected biological properties, such as slower association to, and dissociation from, receptors,[24, 84] differences between *in vivo* and *in vitro* bioactivity, tissue-dependent binding (liver cells or membranes, adipocytes), and divergence between affinity and *in vitro* potency.[83, 84] Receptor-binding studies with ^{125}I-labelled dimers gave three different types of Scatchard plots indicating lost negative co-operativity and, in some cases, bivalent binding.[24]

Photo-reactive Insulins. Hormone derivatives containing photo-sensitive groups,[100] in particular insulin derivatives (Table 2), are becoming increasingly popular for affinity labelling studies of receptors. Specifically labelled receptors and receptor fragments have been characterized. Photo-sensitive groups incorporated so far are based on the 4-azidophenyl nucleus.[102, 107] Light sensitivity is increased substantially by incorporating a nitro-group.[102] Such photo-labels can be activated with light, which does not cause metabolic damage to living cells.[105, 108, 109] The syntheses of insulin derivatives follow established routes, and require only that the materials are not exposed to light. Uncharacterized radio-iodinated Nap-insulins have been used to label membrane receptors.[113] Although specific labelling could be observed, the level of non-specific labelling was high. Use of well characterized derivatives, made from partially protected insulin intermediates,[101, 107] gave low background labelling and high specific labelling of

[100] V. Chowdhry and F. H. Westheimer, *Ann. Rev. Biochem.*, 1979, **48**, 293.
[101] P. Thamm, D. J. Saunders, and D. Brandenburg, in ref. 2, p. 309.
[102] D. J. Saunders, P. Thamm, and D. Brandenburg, in ref. 2, p. 317.
[103] M. H. Wisher, M. D. Baron, R. H. Jones, P. H. Sönksen, D. J. Saunders, P. M. Thamm, and D. Brandenburg, *Biochem. Biophys. Res. Commun.*, 1980, **92**, 492.
[104] M. H. Wisher, P. Thamm, D. Saunders, P. H. Sönksen, and D. Brandenburg, in ref. 2, p. 345.
[105] A. R. Rees and M. R. Whittle, in ref. 2, p. 327.
[106] C. W. T. Yeung, M. L. Moule, and C. C. Yip, *Biochemistry*, 1980, **19**, 2196.
[107] C. C. Yip, C. W. T. Yeung, and M. L. Moule, in ref. 2, p. 337.
[108] C. Diaconescu, D. Saunders, P. Thamm, and D. Brandenburg, in ref. 2, p. 353.
[109] D. Brandenburg, C. Diaconescu, D. Saunders, and P. Thamm, *Nature (London)*, 1980, **286**, 821.
[110] L. Kuehn, H. Meyer, M. Rutschmann, and P. Thamm, *FEBS Lett.*, 1980, **113**, 189.
[111] C. C. Yip, C. W. T. Yeung, and M. L. Moule, *Biochemistry*, 1980, **19**, 70.
[112] C. C. Yip, M. L. Moule, and C. W. T. Yeung, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 1671.
[113] S. Jacobs, E. Hazum, and P. Cuatrecasas, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 1066.

Table 2 *Photo-insulins and results of irreversible binding to membranes and cells*

No.	*Photo-insulin*	*% Activity* (A) *or binding* (B)	*Photo-induced binding to*	*Analytical procedure*	*Results* (CB = *covalent binding*)	*Ref.*
1	A1-Apa					101
2	[Apa-Dab^{A1}]					102
			rat liver membrane	PAGE–SDS–thiol	CB 130 × 10^3	103
			rat liver membrane	7% gel	CB 130 × 10^3	104
				8% gel	CB 110 × 10^3	
				corrected	90 × 10^3	
3	[Nap-Dap^{A1}]	100 A	human placental microvillar membrane	PAGE–SDS–thiol	280—300 × 10^3	105
		100 B	rat adipocytes	glucose-transport enhanced	CB 130 × 10^3 80 × 10^3	105
4	B1-Abz	75 A, 53 B				106, 107
5	B1-Napa					101
6	B1-Nap[Gly^{B1}]	61 A	adipocytes	lipogenesis enhanced		108
7	[A-Phe^{B1}]	80—100 A	rat liver membrane		CB 130 × 10^3	104
				8% gel	CB 110 × 10^3	
				corrected	90 × 10^3	
8	B2-Napa-des-Phe^{B1}					101
		78 A	adipocytes	basal lipogenesis enhanced		108, 109
		100 B	pork liver solubilized rec.	PAGE–Triton X100	CB 300 × 10^3 CB 600 × 10^3	110

9	B29-Abz	67 A	rat liver, kidney, heart, lung, testis membranes, rat brain membrane	PAGE–SDS–thiol	CB 130 × 10^3 CB 90 × 10^3 CB 115 × 10^3	111 112
10	B29-Apa		rat liver membrane	PAGE–SDS–thiol PAGE–SDS–thiol	CB 130 × 10^3 corr. 90 × 10^3	104 103
11	B29-Nap					101
12	A1,B29-$(Abz)_2$	21 A	rat liver membrane	PAGE–SDS–thiol	CB 90 × 10^3 CB 130 × 10^3	111
13	B1,B29-$(Nap\text{-}Gly)_2$					101
14	A1,B1,B29-$(Abz)_3$	0 A				107
15	(B2-Apa-des-Phe^{B1})-B29—B29′-adipoyl-insulin dimer					101
16	Nap^x		rat liver membrane human placenta membrane	PAGE–SDS PAGE-SDS–thiol	CB 310 × 10^3 CB 135 × 10^3	113

x = mixture.

Abbreviations: Apa = 4-azidophenylacetyl; Abz = 4-azidobenzoyl; Dab = α,γ-D-diaminobutyryl; Napa = 2-nitro-4-azidophenylacetyl; Nap = 2-nitro-4-azidophenyl; A-Phe = 4-azido-L-phenylalanyl; Dap = α,β-D-diaminopropionyl.

unique bands. Control experiments have shown that compounds (2) and (4) can be iodinated using either lactoperoxidase or chloramine T with the usual specificity for the A-chain. No iodine was incorporated into the photo-label, and u.v.-induced transfer of iodine was ruled out.[102]

Specific labelling of the same components occurs regardless of the derivative used.[103, 104, 111] Photo-labels at the B1 position [103, 106] give good incorporation yields, although previous work had suggested that this region did not contribute to insulin binding. The solubilized labelled receptor has been shown to have a molecular weight of *ca.* 300 000 [103, 104, 105, 110] (and also 600 000 [110]).

After reduction, SDS-PAGE analysis shows that a band of material of mol. wt. 130 000 is predominantly labelled, with some groups claiming to find a less specific species of mol. wt. 90 000 also. Roles for these subunits have been proposed.[112] Anomalous electrophoretic behaviour indicates that the band of material of mol. wt. 130 000 is glycosylated; on correction, a real mol. wt. of 90 000 is found.[103] The apparent size can be altered by neuraminidase treatment, but not by β-galactosidase, although this alters binding to whole cells.[113] Specific fragments can be obtained by tryptic or chymotryptic digestions of solubilized labelled receptors, possibly indicating a domain structure for such molecules.[113]

Activation of photo-sensitive insulin compounds in the presence of isolated adipocytes leads to a long-term stimulation of lipogenesis [108, 109] and glucose transport.[105] This has been shown to be due to the crosslinking of the receptor–insulin complex.[108, 109]

Other Receptor Studies and Degradation. The fluorescent insulins (66) and (67) and the complexes of biotinylinsulin and avidin [(106) and (107)] have been used as tools to study hormone–receptor interactions. The involvement of the aromatic residues B24 and B25 in binding has been deduced from fluorescence polarization studies.[77] At 37 °C, Ftc-insulin and Rtc-insulin or Rtc-labelled antireceptor antibodies form a single cap on one pole of cultured human lymphocytes (co-capping).[78]

Degradation of insulin has further been studied (*e.g.* refs. 28 and 46), particularly using a ^{3}H-labelled analogue (43).[58, 59, 60] So far, there is no evidence that degradation is related to insulin action, as had been speculated earlier.

Properties of Insulin Fragments. The sequence B(15—27) and five related peptides exhibit a very low activity *in vivo*, which is highest (0.3%) with the asymmetrical disulphide A(19—21)/B(15—27).[63] Feng [114] reports that several fragments, which are inactive *in vivo*, bind to insulin receptors, and that B(22—26) enhances the binding of insulin to adipocytes. β-Ala-B(22—26)-NH_2, while inactive alone, enhances the binding and *in vitro* activity of insulin.[68, 115] No binding to IM-9 lymphocytes could be observed with the related hexapeptide B(22—27).[24]

Immunological Studies. A large number of analogues and derivatives has been used in various immunological studies. These include insulins from different species,[31, 34, 35] and insulins with modifications in the A-chain,[30, 39, 40, 44] at the *N*-terminal [34, 35, 39, 40, 51, 54] and the *C*-terminal region [35, 40, 67, 69] of the B-chain, with modifications in both chains,[31, 34, 35, 39, 40, 79] and with crosslinks.[39, 40, 87, 90]

[114] Y.-M. Feng, J.-L. Gu, X.-T. Zhang, Z.-L. Lu, W.-J. Xu, and J.-H. Zhu, in ref. 2, p. 455.
[115] K. Kikuchi, J. Larner, R, Freer, and A. R. Day, *FEBS Lett.*, 1980, **119**, 161.

Relations between the immune response and primary structure have been studied in mice[31,67,69] and larger animals.[34] The specificity of several antisera from guinea-pig[39,40] and rabbit[35] has been tested with a wide spectrum of insulins, and the latter used to characterize the antisera.

Glucagon.—A review covering the role of glucagon in hyperglycaemia has been published,[116] and also a review on the enteroinsular axis.[117] Work continues on the elucidation of the biosynthetic pathway of glucagon.[118–121] Immunoreactive glucagon-like material is heterogeneous not only in plasma,[122] but also in extracts of pancreatic[123] and gut[124] tissue.

The relation of gut glucagons (glicentin) to pancreatic glucagon is becoming clearer: the whole sequence of glucagon occurs as residues 64—92 of glicentin, and is followed by an octapeptide starting Lys-Arg.[125] It is possible that glicentin is a proglucagon in pancreatic tissue[126] as well as gut tissue.[127] As with several other hormones, glucagon reactivity is increasingly being found in unsuspected tissues,[120] and increasingly studied in lower orders of animals such as insects.[128,129] An electron microscopical study[128] on pancreatic A-cells of the teleost *Fuga rubripes* has suggested that rhombic dodecahedral crystal forms are present with the space group ($P2_3$) of cubic porcine glucagon crystals. The mean crystal size suggests 250 000 molecules of glucagon would be the average quantal release for this hormone.

A list of some recently published semisynthetic derivatives of glucagon and their biological properties is given in Table 3. In assessing the biological data, matters are considerably confused by the partial agonism, antagonism, and non-parallel responses shown by many of the derivatives with respect to native glucagon. These effects may indicate that such data have not been collected under steady-state conditions, although this is unlikely to be the whole answer. A recent report demonstrates that, in contrast to data showing it to be a competitive inhibitor of glucagon in the rat-liver plasma-membrane adenylate cyclase assay, N^{ε}-PTC, des-His[1]-glucagon is a full agonist with low potency (*ca.* 0.3%) but no glucagon-antagonist properties in the perfused rat liver.[131] This could be seen as support for

[116] Proceedings of the Serono Symposium, Rome, 1979, Vol. 30, ed. D. Andreani, P. J. Lefebre, and V. Marks, Academic Press, 1980.
[117] 'Enteroinsular Axis', ed. W. Creutzfeld, A. G. S. Karger, 1980.
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[119] A. J. Moody and F. Sundby, in ref. 1, p. 427.
[120] J. M. Conlon, in ref. 1, p. 432.
[121] C. Pazelt, S. J. Chan, P. S. Quinn, R. J. Caroll, H. S. Tager, and D. F. Steiner, in ref. 1, p. 119.
[122] R. W. J. Flanagan, R. F. Murphy, and K. D. Buchanan, *Biochem. Soc. Trans.*, 1980, **8**, 426.
[123] A. K. Tung, J. L. Ruse, and E. Cockburn, *Can. J. Biochem.*, 1980, **58**, 707.
[124] F. Sundby and A. J. Moody in *Gastrointestinal Horm.*, 1980, ed. G. B. Raven and M. Y. Raven, p. 307.
[125] J. J. Holst, *Biochem J.*, 1980, **187**, 337.
[126] M. Ravazzola and L. Orci, *Nature (London)*, 1980, **284**, 66.
[127] M. Ravazzola and L. Orci, *Diabetes*, 1980, **29**, 156.
[128] K. J. Kramer, H. S. Tager, and C. N. Childs, *Insect Biochem.*, 1980, **10**, 179.
[129] K. J. Kramer, *Neurohorm. Techn. Insects*, 1980, 116.
[130] R. H. Lange and K. Kobayashi, *J. Ultrastruct. Res.*, 1980, **72**, 20.
[131] B. A. Khan, M. D. Bregman, C. A. Nugent, V. J. Hruby, and K. Brendel, *Biochem. Biophys. Res. Commun.*, 1980, **93**, 729.

Table 3 *Semisynthetic derivatives of glucagon*

Derivative	*Binding*	*Adenylate cyclase activation (max. level)*	*Ref.*
Homoserine[27] des(Asn[28], Thr[29])-glucagon (1)	2.5%	2.5% (100%)	132
1-Hydrazide	2.5%	2% (100%)	134
1-Butylamide	2.5%	2% (100%)	134
1-Glycine	—	3% (100%)	134
1-(6-Aminohexylbiotinamide)	—	0.1% (100%)	134
[Homoarginine[12]]-glucagon	—	20% (100%)	135
	—	11% (100%)	
	—	(15% lipolysis)	
N^{α}-(Trinitrophenylsulphenyl)-[Homoarg[12]]-glucagon	—	strong inhibitor	135
[Homoarginine[12]]des-(His[1])-glucagon (2)	—	0.1% (22%)	135
N^{α}-(TNPS)-2	—	inhibitor	135
N^{α}-Carbamoyl-glucagon	—	5.5% (27%)	135
	33%	6%	144
N^{α},N^{ε}-Di-carbamoyl-glucagon	—	0.4% (17%)	135
N^{α},N^{ε}-Di-acetyl-glucagon	3.5%	0.15%	144
N^{α}-Carbamoyl-, N^{ε}-TNPS-glucagon	—	inhibitory	135
N^{ε}-PTC des-(His[1])-glucagon	—	0	
		0.3% (glycogenolysis)	131
N^{ε}-Acetamidino-glucagon	100%	100% (100%)	136
N^{α},N^{ε}-Di-acetamidino-glucagon	50%	50% (100%)	136
N^{ε}-(4-Hydroxyphenylamidino)-glucagon	10%	10% (100%)	136
N^{α},N^{ε}-Di-(4-mercaptobutyr-amidino)-glucagon	10%	10% (100%)	136
[2-(2-Nitro-4-azidophenylsul-phenyl)-Trp[25]]-glucagon	100%	100% (100%)	137
[2-(2,4-Dinitrophenylsulphenyl)-Trp[25]]-glucagon	100%	100% (100%)	137
[2-Thiotrp[25]]-glucagon	100%	100% (100%)	137
([2-Thiotrp[25]]-glucagon)$_2$ disulphide dimer	100% (+GTP) 25% (−GTP)	100% (100%)	137
Asp[8, 15, 21] tri-methyl ester glucagonyl methyl ester	0.02%	0.01%	144

the above suggestion, or as evidence for the activation of glucagon-sensitive systems by cAMP-independent mechanisms.

Cyanogen bromide treatment of glucagon leads to loss of Asn-28, and Thr-29, and the conversion of Met-27 into homoserine lactone in acid conditions.[132] This lactone intermediate has been used to acylate various amines.[133, 134] Only unhindered primary amines reacted well. A hydrazide was obtained from the lactone after reaction with hydrazine and could be converted to an azide which reacted with poor nucleophiles such as glycine.[134] No regain of biological activity

[132] M. C. Lin, D. E. Wright, V. J. Hruby, and M. Rodbell, *Biochemistry*, 1975, **14**, 1559.
[133] V. J. Hruby, D. E. Wright, M. C. Lin, and M. Rodbell, *Metabolism*, 1976, **25**, Suppl. 1, 1323.
[134] D. E. Wright, V. J. Hruby, and M. Rodbell, *Biochim. Biophys. Acta*, 1980, **631**, 49.

was observed with any of the derivatives. No damage to Trp-25 was reported despite the acidolytic and oxidative conditions used.

A different set of analogues has been made with a view to investigating the roles of the amino-groups of glucagon.[135] Reaction with *O*-methylisourea-H_2SO_4 [neutralized with $Ba(OH)_2$] gave [12-Homoarg]-glucagon. The free α-NH_2 group was subsequently allowed to react with trinitrobenzenesulphonic acid or PITC; the latter derivative was treated with TFA and then acylated with cyanate or TNBS. Partial reaction of glucagon with cyanate gave the N^{α}- and N^{α},N^{ε}-derivatives. The N^{α}-derivative was treated with TNBS. No yields were quoted. Several of the derivatives were shown to be partial agonists and inhibitory to glucagon-stimulated cyclase; all were significantly reduced in potency. Interestingly, N^{ε}-acetamidino-glucagon[134] was found to be fully potent in both binding and cyclase assays. The difference between this derviative and [12-Homoarg]-glucagon (potency *ca.* 20%) consists only of replacing a Me- for a NH_2-group, with no charge alteration, so the discrepancy in biological activities is remarkable.

[25-(2-Thiotryptophan)]-glucagon has been synthesized by thiolysis of {25-[2-(2,4-dinitrophenylsulphenyl)Trp]}-glucagon, and its use as a potential semi-synthetic intermediate described.[133] The disulphide-linked dimer of this intermediate is readily formed. In the presence of GTP (as required for the cyclase assay) the binding curves of glucagon, [25-(2-Thiotrp)]-glucagon, and the dimer were superimposable. In the absence of GTP the relative affinity of the dimer to glucagon dropped to *ca.* 25%. Although this phenomenon can be explained by a current model of the glucagon receptor,[138] it would be interesting to know whether the kinetics of association of this dimer are as severely affected as the insulin dimers.[24, 84] {25-[2-(2,4-Dinitrophenylsulphenyl)Trp]}-glucagon retains full potency.[137] {25-[2-(2-Nitro-4-azidophenylsulphenyl)Trp]}-glucagon has been synthesized in *ca.* 50% yield [137] and is also fully potent. It can be radio-iodinated, and shown to bind covalently to anti-glucagon antibodies and to albumin. This is obviously a more attractive photo-affinity analogue than N^{ε}-[2-nitro-4-azido-phenyl]-glucagon,[140] which binds well but has no cyclase stimulatory activity. It will be used to investigate the structure of the glucagon receptor and, analogous to photo-sensitive derivatives of insulin,[141] for investigations of the mechanism of the induction of biological activity. Related derivatives of corticotropin have also recently been reported.[142, 143]

The relation between receptor binding and biological activity has been discussed [144] in terms of the need to form an ampipathic helix at the *C*-terminus of glucagon to bind to phospholipids. Charged and polar groups both play important roles. The roles of hormone receptors and GTP-sensitive regulatory proteins in

[135] M. D. Bregman, D. Trevidi, and V. J. Hruby, *J. Biol. Chem.*, 1980, **255**, 11 725.
[136] D. E. Wright and M. Rodbell, *Eur. J. Biochem.*, 1980, **111**, 11.
[137] D. E. Wright and M. Rodbell, *J. Biol. Chem.*, 1980, 10 884.
[138] W. Schleger, E. S. Kempner, and M. Rodbell, *J. Biol. Chem.*, 1979, **254**, 5168.
[139] C. D. Demoliou and R. M. Epand, *Biochemistry*, 1980, **19**, 4539.
[140] M. D. Bregman and D. Levy, *Biochim. Biophys. Acta*, 1977, **78**, 584.
[141] D. Brandenburg, C. Diaconescu, D. J. Saunders, and P. Thamm, *Nature (London)*, 1980.
[142] E. Canova-Davis and J. Ramachandran, *Biochemistry*, 1980, **19**, 3275.
[143] K. Muramoto and J. Ramachandran, *Biochemistry*, 1980, **19**, 3280.
[144] R. M. Epand, in ref. 2, p. 363.

membrane transduction have also been examined.[145] Radio-iodinated glucagon has been purified by h.p.l.c. methods; lactoperoxidase is the catalyst of choice since chloramine T leads to extensive oxidation of methionine.[146] Solid phase syntheses of crystallizable glucagon have been reported.[147, 148] Boc-protected tri- and tetrapeptides were added stepwise to a chloromethylated styrene–divinylbenzene resin in high yield. The final material was recovered by treatment with anhydrous HF containing anisole. After purification by exclusion and ion-exchange chromatography a yield of 10.5% was obtained. Despite tryptophan being in the first coupled peptide, it seems to resist the seven treatments with TFA and HF handling.

Arguments relating crystal structures to the structure necessary for receptor binding are always suspect, particularly with relatively extended linear molecules such as glucagon. C.d. studies continue to show that significant amounts of ordered structures are present in glucagon only at high hormone concentrations ($1\,mg\,ml^{-1}$) or in the presence of detergent or phospholipid micelles.[149] In a 270 MHz ^{1}H-n.m.r. study a similar conclusion was obtained.[150] Some structure seems to be present in the *C*-terminal segment (residues 22—29), and changes on increasing the hormone concentration suggest that trimerization occurs with a K_a of $2.4 \times 10^5\,M^{-2}$. This value is close to that determined in other studies.[151, 152] The nature of the aggregation seems to be similar to Type 1 of the crystal[153] and not Type 2.

Pancreatic Polypeptide.—The physiological significance of this peptide remains unclear despite the demonstration that its level increases after meals[154] (as does C-peptide). It is known to be localized in the D-cells of pancreatic islets,[155] and it is becoming evident that it results from the processing of a higher molecular weight precursor.[156] Immunoreactive material with a molecular weight of *ca.* 9000 has been characterized from canine pancreas, after incubation with ^{3}H-leucine and ^{35}S-methionine, and shown to be rapidly processed to pancreatic polypeptide (mol. wt. 4300) and a smaller peptide (mol. wt. *ca.* 2500—3000). The pancreatic polypeptide sequence is *N*-terminal in the 9000 dalton peptide. Whether or not the smaller peptide has physiological significance is unclear.

The conformation of avian pancreatic polypeptide has now been determined[157] to be a collagen-like helix folded against an α-helical region, at least when a minimum of one zinc ion per protomer is present. The protomers are aligned

[145] M. Rodbell, *Nature (London)*, 1980, **284**, 17.
[146] J. Markussen and U. D. Larsen, in ref. 2, p. 161.
[147] Y.-C. Du, J.-H. Shen, and J.-P. Shi, *Sheng Wu Hua Hsneh Yu Sheng Wu Wu Li Hseuh Pao*, 1980, **12**, 93 (*Chem. Abstr.*, 1980, **93**, 150 647).
[148] Y.-T. Kung, D.-Y. Zhu, X.-D. Qiu, X.-W. Yuan, L.-T. Ke, and W. Wei, in ref. 2, p. 91.
[149] C. S. C. Wu and J.-T. Yang, *Biochemistry*, 1980, **19**, 2117.
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[156] T. W. Schwartz, R. L. Gingerich, and H. S. Tager, *J. Biol. Chem.*, 1980, **255**, 11 494.
[157] J. E. Pitts, T. L. Blundell, I. J. Tickle, and S. P. Wood, Proceedings of the 6th American Peptide Symposium, 1979, ed. E. Gross, p. 1011.

antiparallel in dimers with two-fold symmetry. The zinc is co-ordinated to 34-His$^{mol\,1}$, 1-Gly$^{mol\,2}$, and 23-Asn$^{mol\,3}$.[158] Studies on solutions of avian, bovine, and canine PP show an increase in Stokes radius on going from pH 4 to pH 8, without noticeable changes in the c.d. characteristics or frictional ratios.[159] This suggests that PPs retain their conformation on disaggregation, and that the association, as studied by integral boundary analysis of large zone chromatographic runs, depends on ionizable groups with pK_a 4.5—5.5.[160]

Human PP has been synthesized in *ca.* 6% yield by solid-phase methods.[161] Amino-acids were coupled as their *N*-tBoc-protected derivatives using di-isopropylcarbodi-imide (with 1-hydroxybenzotriazole for Boc-Gln and Boc-Asn). Aliphatic hydroxyfunctions were protected with benzyl groups, aromatic hydroxyls with 2-BrZ-, side-chain carboxyls with 4-ClBzl-, and arginine with toluene sulphonyl groups. The complete peptide was recovered from the 1% crosslinked benzydrylamine support with anhydrous HF, and purified by Sephadex G25, CM-cellulose chromatography, and finally by partition chromatography. The material was homogeneous by h.p.l.c. in three different systems. It produced a potent dose-related inhibition of pancreatic secretion in dogs.

The search for PP-binding tissues will be facilitated by an improved method for preparing and purifying a stable iodinated derivative,[162] using an electrophoretic method that is also applicable to other hormones.

5 Gastrointestinal Peptides*

Contributed by D. Voskamp and H. C. Beyerman

This review is a selection of the pertinent, mainly chemical, literature that appeared in a part of 1979, in 1980, and occasionally in 1981. *Chemical Abstracts* (1980 until May 1981) were used as the primary source. Papers concerning peptides that were originally discovered in and isolated from tissues of the gastrointestinal (g.i.) tract have been considered especially. Papers concerning peptides now known to be present in the g.i. tract, but originally discovered in and isolated from other organs, have in general been excluded.

During the reporting period several books have been published that deal with different research related to g.i. peptides.[1-3] The proceedings of some symposia also have appeared.[4-8]

* The amino-acid residues are referred to by the one letter symbols, recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.*, 1968, **5**, 151). The symbol □ indicates that the *C*-terminal amino-acid is in the amide form. <E denotes a pyroglutamic acid residue.

[158] J. E. Pitts, S. P. Wood, R. Horuk, S. Bedekar, and T. L. Blundell, 1980, ref. 2, p. 671.
[159] M. E. Noelken, P. J. Chang, and J. R. Kimmel, *Biochemistry*, 1980, **19**, 1838.
[160] P. J. Chang, M. E. Noelken, and J. R. Kimmel, *Biochemistry*, 1980, **19**, 1944.
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Synthetic efforts in the field of g.i. peptides have been described.[9] The occurrence, chemistry, and (patho)physiology of g.i. peptides has been reviewed [10-14] and structure–function relations have been discussed.[15] The classification of gut endocrine cells,[16] the histochemistry of gut peptides in the human digestive tract,[17] and the use of immunohistochemistry for the study of gastroenterology and (neuro)endocrinology have been described.[18-20] Papers concerning the scope and limitations of the radioimmunoassay (RIA) of g.i. peptides[21, 22] and discussing the heterogeneity of g.i. peptides have appeared.[23, 24] Evolutionary aspects of peptides from gut endocrine cells and nerves have been considered.[13, 25] The effects of g.i. peptides on g.i. sphincters,[26] on g.i. motor functions,[27] on gastric, pancreatic, biliary, and intestinal secretions,[28] on growth of g.i. tissue,[29] and on electrolyte secretion in the g.i. tract[30] have been discussed. The g.i. peptide–receptor interactions in the pancreas have been reviewed.[31] A review

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dealing with vasoactive peptides, present in the g.i. tract and autonomic nervous system, has appeared.[32] The satiety effect of g.i. hormones,[33] the influence of several peptides on the pathogenesis of obesity,[34] and the role of peptides in the enteroinsular axis have been discussed.[35, 36] The intraluminal secretion of g.i. peptides has been the subject of some papers.[22, 37] Some aspects of peptides with dual function (neuroregulators and gut hormones) have been discussed.[38, 39] The clinical significance of g.i. peptides has been discussed.[40] The growing realization that biologically active polypeptides can be grouped in families, the members of which show structural and functional relatedness, was illustrated by the families that are represented by some pancreas hormones: insulin, glucagon, pancreatic polypeptide (PP), and somatostatin.[41] The classification of gut hormones in peptide families is further discussed in ref. 12.

Gastrin (Figure 1).— The occurrence, chemistry, and (patho)physiology of gastrin have been reviewed,[42] and its occurrence in nerve tissues has been discussed.[24, 43] The previously assigned structure of human gastrin 34 (h-G34) has been revised as a result of sequencing and immunological studies on synthetic peptides.[44] Segments of the originally and newly proposed sequence of the *N*-terminal region of h-G34, and analogues thereof, were synthesized by a segment condensation approach. The newly proposed sequence is presented in Figure 1.

In a series of articles the syntheses of [H^7,S^9]-h-G34-I, the originally proposed sequence of h-G34-I, and [H^7,S^9,L^{32}]-h-G34-I have been described in detail.[47] Recently the synthesis of h-G34-I according to the revised sequence has been described.[45] The immunochemical behaviour of [H^7,S^9]-h-G34-I revealed that it differed from natural h-G34-I in the *N*-terminal region.[44] Synthetic h-G34-I had the same immunochemical behaviour as natural h-G34-I.[45] Sequences h-G17-I, G-K-[Q^1]-h-G17-I, and [H^7,S^9]-h-G34(1—15) were synthesized by a segment

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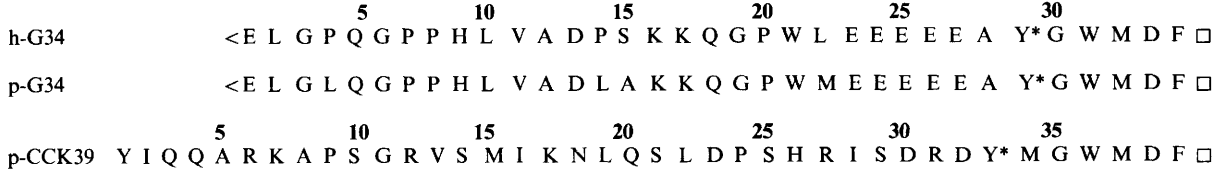

Figure 1 *The sequences of human (h) and porcine (p) gastrin (G) and porcine (p) cholecystokinin (CCK)*[44-46]

<E: pyroglutamyl; Y*: unsulphated tyrosine in gastrin I, sulphated in gastrin II and CCK; □ denotes an amide group

condensation approach.[48] h-G17-I was assembled on a Boc-Phe-Merrifield resin, using Nps-amino-acid derivatives,[49] and the 15-leucine analogue on a polyamide resin using Fmoc-amino-acid derivatives.[50] The synthesis of h-G17-I on both polystyrene and polyamide supports was studied.[51] Analogues of G5 were synthesized that contained an aminosuccinimide moiety.[52] A patent describes the synthesis of G4.[53] A procedure to obtain monoiodinated h-G16 has been developed.[54] A process for putting into industrial production injectable medicinal forms of Ac-C and G5 has been described.[55] The conformation of several peptides related to human gastrin was studied using c.d. measurements,[56] as was the interaction of Ca^{2+} ions to [Nle^{11}]-h-G13-I in trifluoroethanol.[57] The structure of gastrin mRNA was studied.[58] Methods were described for obtaining gastrin antisera specific for distinct regions of the molecule.[59] Several reviews discussing the scope and limitations of the radioimmunoassay (RIA) of gastrin have appeared.[59, 60] An enzymatic procedure was developed to quantitate large molecular forms of gastrin and cholecystokinin.[61] This procedure can, in principle, be applied to all polypeptides for which quantitation of precursor concentrations is desirable. Gastrin was found to occur in various molecular forms, *e.g.* extended at the *C*-terminal end.[62]

Cholecystokinin (CCK), *formerly called pancreozymin (PZ)* (Figure 1).—The chemistry, distribution, and (patho)physiology of CCK have been reviewed.[46] The role of CCK as a neuropeptide was discussed.[24, 63, 64] The RIA of CCK was discussed.[64, 65] The role of CCK in the control of hunger was the subject of papers.[66] [T^{28},Nle^{31}]-CCK(25—33) was prepared.[67] A patent describes a CCK

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octapeptide amide sulphate ester.[68] Analogues of CCK8, altered in the Trp[30]-residue, were prepared and tested for their biological activity.[69] Several *C*-terminal fragments of CCK and analogues thereof were synthesized and studied.[70, 71] H.p.l.c. systems were developed for separation of CCK-peptides.[72]

Vasoactive Intestinal Peptide (VIP) (Figure 2).—Several reviews appeared dealing with the chemistry, distribution, and (patho)physiology of VIP,[76] and the role of VIP as a neural peptide.[77] The RIA of VIP was discussed.[78, 79] VIP was found to occur in several molecular forms.[80] Immunoreactive VIP was found in various tissues.[81] The synthesis of chicken (c)-VIP was described: six segments were prepared and assembled by the azide procedure.[82] Porcine (p) VIP[83] and its [E[8]]-analogue[84] were synthesized analogously. [Nle[17]]-p-VIP was synthesized by a segment condensation approach.[85] As an example for chain lengthening with the aid of Fmoc-amino-acid active esters, the preparation of *C*-terminal segments of c-VIP was performed.[86] p-VIP was synthesized on a benzhydrylamine resin.[87]

Secretin (Figure 2).—The chemistry, distribution, and (patho)physiology of secretin have been reviewed[88–90] and the RIA of secretin has been discussed.[78, 91]

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Protected and unprotected p-secretin(16—27) were synthesized by solid phase syntheses using a classical Merrifield resin or a benzhydrylamine resin.[92] The repetitive excess mixed anhydride (REMA) method for the synthesis of p-secretin has been improved.[93] Several analogues of p-secretin were synthesized and studied using synthetic methods developed for the synthesis of p-secretin.[94] Reverse-phase h.p.l.c. of p-secretin, analogues thereof, and other peptides, using perfluoro-alkanoic acids as lipophilic counter-ions, has been studied.[95] The isolation and structure elucidation of chicken (c) secretin have been described (Figure 2),[73] and the isolation of bovine (b) secretin has been reported.[89]

The stability of p-secretin has been studied by biological, enzymic, and chromatographic methods. It transpires that the rapid loss of biological potency in dilute secretin solutions, reported previously, can be explained by a *reversible* adsorption to glass or plastic materials (see also ref. 90 and ref. cited herein). This phenomenon can be prohibited by the addition of suitable additives, *e.g.* serum albumin. A slow loss of potency occurs because of chemical transformations in solution, such as $Asp^3(\alpha \rightarrow \beta)$-Gly- and $Asp^{15}(\alpha \rightarrow \beta)$-Ser- rearrangements, or Asp^3-Gly chain splitting.[96]

Gastric Inhibitory Peptide = Glucose-dependent Insulinotropic Peptide (GIP) (Figure 2).—The chemistry, distribution, and (patho)physiology of GIP have been reviewed.[97] The RIA of GIP has been described,[98] and the role of the enteroinsular axis, *e.g.* in obesity, discussed.[36, 99] The sequence of porcine (p) GIP was revised as a result of sequencing studies.[74] In the previously proposed 43-peptide the Gln^{30}-residue had been omitted (see Figure 2). The natural p-GIP preparations proved to contain some contaminants, the most important one being GIP(3—42).[74] These findings make new extensive studies on GIP necessary.

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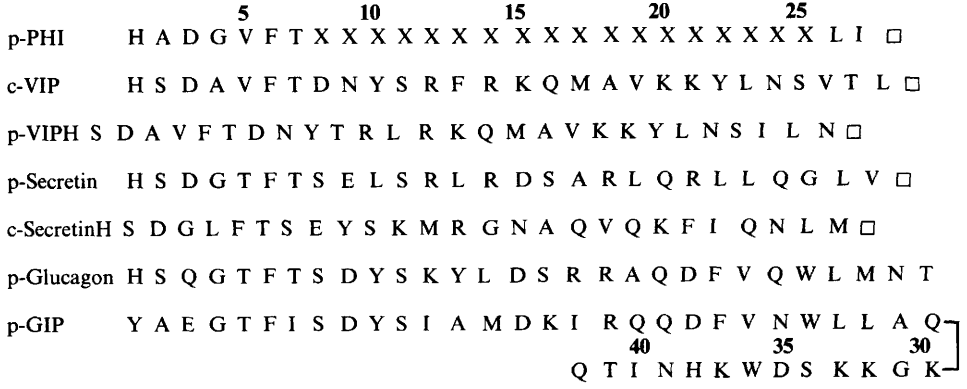

Figure 2 *The secretin family* [73–75]
□ denotes an amide group

Motilin (Figure 3).—Several papers, reviewing the occurrence, chemistry, and (patho)physiology of motilin, have appeared.[11, 101] Partial sequences and analogues of porcine (p) motilin were synthesized and studied.[102] The protected segments motilin(1—8) and motilin(9—22) were synthesized by the repetitive excess mixed anhydride (REMA) method. After coupling, deprotection, and purification a highly potent material was obtained.[100] The RIA of motilin was described.[78, 103] Motilin was found to occur in various molecular forms.[104]

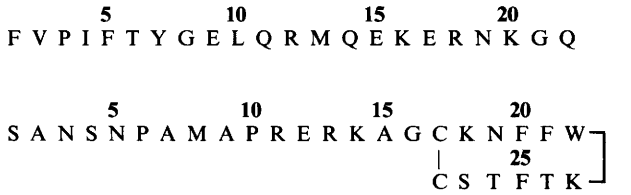

Figure 3 *The sequence of porcine motilin*[100]

Other Gastrointestinal Peptides.—The (possible) occurrence of several (peptide) factors in the gastrointestinal tract and their effects have been discussed,[12, 105] *e.g.* chymodenin,[106] pancreatic polypeptide (PP),[11, 107] substance P,[108, 109] neurotensin,[109] bombesin,[110] gut glucagon,[111] villikinin,[112] bulbogastrone,[113] anthral chalon,[114] a new secretin-like peptide,[115] a new CCK-like peptide,[115] gastrozymin,[116] entero-oxyntin,[117] enkephalin,[118] endorphin,[118] and somatostatin.[118, 119] *N*-Terminally extended somatostatin was isolated from porcine intestinal tissue. It proved to be a 28-peptide[120] (see Figure 4). This

[100] E. Izeboud and H. C. Beyerman, *Recl. Trav. Chim. Pays-Bas*, 1980, **99**, 124; H. C. Beyerman, E. Izeboud, P. Kranenburg, and D. Voskamp, ref. 5, p. 333; E. Izeboud, Thesis, Delft, 1980.

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[103] N. Yanaihara, C. Yanaihara, K. Nagai, H. Sato, F. Shimizu, K. Yamaguchi, and K. Abe, *Biomed. Res.*, 1980, **1**, 76.

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[105] V. Mutt, ref. 1, p. 971.

[106] J. W. Adelson, M. E. Nelback, R. Chang, C. B. Glaser, and G. B. Yates, ref. 1, p. 387.

[107] T. M. Lin, ref. 1, p. 275; J. C. Floyd, ref. 2, p. 657.

[108] G. Bertaccini, ref. 1, p. 315.

[109] V. Erspamer, ref. 1, p. 343.

[110] P. Melchiorri, ref. 1, p. 717.

[111] F. Sundby and A. J. Moody, ref. 1, p. 307; A. J. Moody and F. Sundby, ref. 1, p. 831; A. J. Moody, ref. 2, p. 699.

[112] E. Kokas, J. J. Pisano, and B. Crepps, ref. 1, p. 899.

[113] G. Nilsson, ref. 1, p. 911.

[114] G. B. J. Glass, ref. 1, p. 929.

[115] K. Tatemoto, ref. 1, p. 975.

[116] M. Vagne, ref. 1, p. 978.

[117] M. Vagne, ref. 1, p. 980.

[118] C. A. Meyers and D. H. Coy, ref. 1, p. 363.

[119] S. J. Konturek, ref. 1, p. 693; R. Arnold and P. G. Lankisch, ref. 2, p. 733.

[120] L. Pradayrol, H. Jörnvall, V. Mutt, and A. Ribet, *FEBS Lett.*, 1980, **109**, 55.

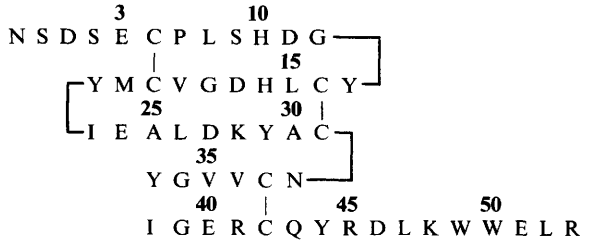
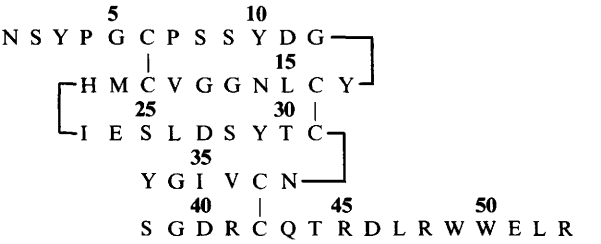


Figure 4 *The sequence of porcine (p) somatostatin-28* [120]

sequence was synthesized, using a segment condensation approach.[121] The occurrence of 'g.i. peptides' in other tissues was discussed in various papers.[122]

Urogastrone. The isolation, structure, origin, and actions of human (h) urogastrone, the RIA of this peptide, and its relationships to mouse (m) epidermal growth factor (m-EGF) have been discussed.[123] The sequences of h-β-urogastrone and m-EGF are presented in Figure 5.

h-β-Urogastrone



m-Epidermal growth factor

Figure 5 *The sequences of human (h) β-urogastrone and mouse (m) epidermal growth factor* [123]

PHI and PYY. The isolation and partial characterization of two new peptides (PHI and PYY) from the upper small intestinal tissues of hogs have been described.[75] Strong indications point to the presence of both PHI and PYY in brain tissue of hogs.

PHI consists of 27 amino-acid residues. The name has been derived from peptide (P) starting with histidine (H) and terminating with isoleucine (I). The sequence has been partially elucidated. It can be considered as a member of the secretin family (Figure 2). This is supported by the finding that PHI has both VIP- and secretin-like pharmacological properties.[124]

[121] L. Moroder, M. Gemeiner, W. Goehring, E. Jaeger, and E. Wünsch, ref. 6, p. 121; E. Wünsch, L. Moroder, M. Gemeiner, E. Jaeger, A. Ribet, L. Pradayrol, and N. Vaysse, *Z. Naturforsch.*, 1980, **35**, 911.

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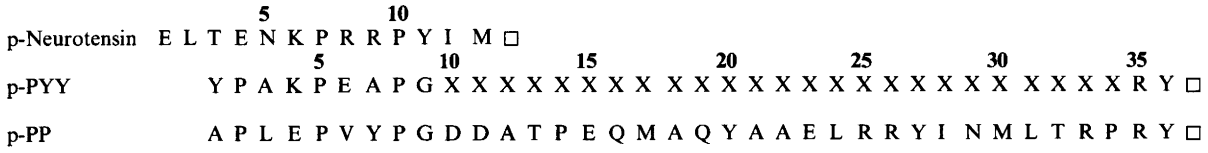

Figure 6 *The sequences of porcine (p) neurotensin, porcine (p) PYY, and porcine (p) pancreatic polypeptide (PP)*[12,41,75]
□ denotes an amide group

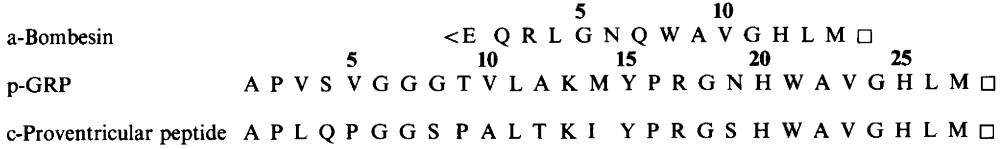

Figure 7 *The sequences of amphibian (a) bombesin, porcine (p) gastric gastrin releasing peptide (GRP), and chicken (c) proventricular peptide*[126]
<E: pyroglutamyl; □ denotes an amide group

PYY consists of 36 amino-acid residues. Its sequence has also been partially elucidated. The peptide (P) possesses on both ends tyrosine (Y). It can be considered to be a member of the neurotensin–pancreatic polypeptide family (Figure 6).

Gastrin Releasing Peptide (GRP). The recently proposed sequence of porcine (p) GRP has been synthesized by a segment condensation approach.[125] The synthetic product exhibited a high gastrin releasing potency. A 27-peptide amide has been isolated from chicken proventricular tissue and characterized.[126] This peptide shows much homology with frog bombesin and p-GRP. Figure 7 shows the sequences of some members of this bombesin family.

6 Vasoactive Peptides

Contributed by P. D. Roy

Bradykinin.—Reviews have covered a number of aspects of the kallikrein-kinin system, including its role in hypertension and renal disease,[1] pharmacological prospects in the system,[2] and current developments in research in the system in relation to hypertension.[3]

The Table lists new bradykinin analogues with available biological data, reported mainly during 1980. The search for an effective *in vivo* inhibitor of bradykinin continues. Mazur and co-workers have studied the effect of N^{α}-methyl amino-acid substitution in the 1, 4, 5, 8, or 9 position of bradykinin [analogues (9), (21), (22), (29), and (33), respectively]. A similar approach in the angiotensin series has led to the antagonist [MePhe^8]-angiotesin II. The N^{α}-methyl bradykinin analogues, however, failed to show interesting antagonist activity, and moreover the [MeArg^1]-bradykinin analogue (9) retained 61% agonist activity in isolated guinea-pig ileum.[4]

Biological activities of a series of bradykinin and des-[Arg^9]-bradykinin analogues, in which the boron-containing amino-acid L-*o*-carboranylalanine (Car) is used to replace phenylalanine residues, have been reported,[5] following earlier details of synthesis.[6] The bradykinin and [Car^8]-des-[Arg^9]-bradykinin analogues were essentially inactive, whereas the analogues [Car^5]-des-[Arg^9]-bradykinin (partial agonist) and lysyl-[Car^5, Leu^8]-des-[Arg^9]-bradykinin (potent antagonist) showed a prolonged duration of action on the B_1 kinin receptor in rabbit aorta.

An extensive study into bradykinin receptor-like binding in uterine preparations has been carried out using iodinated analogues of [Tyr^5]-, [Tyr^8]-, and tyrosyl-bradykinin. The monoiodinated derivatives (31) and (35) were better agonists than [monoiodo-Tyr^5]-bradykinin (24), and therefore more suitable as receptor probes.

[125] H. Yajima, K. Akaji, N. Fujii, M. Moriga, M. Aono, and A. Takagi, *Chem. Pharm. Bull.*, 1980, **28**, 2276.
[126] T. J. McDonald, H. Jörnvall, M. Ghatei, S. R. Bloom, and V. Mutt, *FEBS Lett.*, 1980, **122**, 45.
[1] O. A. Carretero and A. G. Scicli, *Am. J. Physiol.*, 1980, **238**, F247.
[2] H. S. Margolius, *Trends Pharmacol. Sci.*, 1980, **1**, 293.
[3] A. Roeckel and A. Heidland, *Contrib. Nephrol.*, 1980, **23**, 105.
[4] R. H. Mazur, P. A. James, D. A. Tyner, E. A. Hallinan, J. H. Sanner, and R. Schulze, *J. Med. Chem.*, 1980, **23**, 758.
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[6] O. Leukart, E. Escher, and D. Regoli, *Helv. Chim. Acta*, 1979, **62**, 546.

The radioactive analogue [^{125}I]tyrosyl-bradykinin was used to demonstrate the presence of bradykinin binding sites with properties expected of receptors.[7]

The synthesis of bradykinin derivatives containing pipecolic acid (2-piperidine carboxylic acid) residues in place of proline has been reported. Biological data on this series of analogues, (10), (13), (14), (17), (26), and (27), await publication.[8] Other analogues with proline substitution by α-aminoisobutyric acid have also recently been described.[9]

Table *Analogues of bradykinin*

1 5 9
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Bradykinin

Compound number	*Structure*[a]	*Biological activity*[b]	*Test*[b]	*Ref.*
1	(5—7)-bradykinin	132.0%	E	10
2	D-Phe^5-(5—7)-bradykinin	124.0%		
3	(4—7)-bradykinin	132.0%		
4	D-Phe^5-(4—7)-bradykinin	114.1%		
5	Ala^5-(4—7)-bradykinin	103.6%		
6	D-Ala^5-(4—7)-bradykinin	99.6%		
7	Gly^5-(4—7)-bradykinin	113.7%		
8	β-Ala^5-(4—7)-bradykinin	123.7%		
9	MeArg^1	61.0%	A	4
10	D-Pip^2			8
11	Aib^2			9
12	Aib^{2,3}	inactive	A,B,E	9
13	Pip^{2,3}			8
14	Pip^{2,3,7}			8
15	Aib^{2,3,7}	inactive	A,B,E	9
16	Aib^{2,7}	inactive	A,B,E	9
17	Pip^{2,7}			8
18	Aib^3			9
19	Aib^{3,7}			9
20	Pip^{3,7}			8
21	Sar^4	0.4%	A	4
22	MePhe^5	1.0%	A	4
23	Tyr^5	0.6%	B	7
24	monoiodo-Tyr^5	0.1%	B,C	7
25	di-iodo-Tyr^5	0	B	7
26	Pip^7			8
27	D-Pip^7			8
28	Aib^7			9
29	DL-MePhe^8	1.5%	A	4
30	Tyr^8	24.0%	B	7
31	monoiodo-Tyr^8	20.0%	B	7
		15.0%	C	7
32	di-iodo-Tyr^8	0.1%	B	7
33	MeArg^9	0.5%	A	4
34	tyrosyl-bradykinin	102.0%	B	7

[7] C. E. Odya, T. L. Goodfriend, and C. Péna, *Biochem. Pharmacol.*, 1980, **29**, 175.
[8] L. Balaśpiri, Gy. Papp, M. Tóth, F. Sirokmán, and K. Kovacs, *Acta Phys. Chem.*, 1979, **25**, 179.
[9] R. J. Vavrek and J. M. Stewart, *Peptides (Fayetteville, NY)*, 1980, **1**, 231.
[10] Y. Okada, Y. Tsuda, and M. Yagyu, *Chem. Pharm. Bull.*, 1980, **28**, 310.

Table *(cont.)*

Compound number	*Structure*[a]	*Biological activity*[b]	*Test*[b]	*Ref.*
35	monoiodotyrosyl-bradykinin	88.0%	B	7
		69.0%	C	7
36	di-iodotyrosyl-bradykinin	62.0%	B	7
37	*cyclo*(lysyl-bradykinin)[x]	*cf.* bradykinin	D	11
38	*cyclo*(lysyl-[Gly[6]]-bradykinin)[x]			

[a] Pip = pipecolic acid (2-piperidine carboxylic acid); Aib = α-aminoisobutyric acid; x,N^{ε}-amino-group of lysine coupled to *C*-terminal carboxyl-group. [b] A, guinea-pig ileum [activities relative to bradykinin (100%)]; B and C, rat and bovine uterus, respectively [activities relative to bradykinin (100%)]; D, rat blood pressure; E, mouse brain [activities indicate prolongation of the pentobarbital-induced sleeping time relative to saline control (100%)].

Conformational analysis of the bradykinin molecule has revealed close proximity of the *C*-terminal carboxy-group and the guanidino-function in the arginine[1] residue, and this has led to synthesis of cyclic analogues where this configuration is stabilized by covalent bonding. The cyclic compounds, (37) and (38), elicited depressor effects in rats similar to bradykinin but in addition over a prolonged period.[11] It may be interesting to examine the corresponding retro-all-D cyclic analogues for inhibitory properties.

Angiotensin.—Brunner and Gavras have reviewed the renin–angiotensin system in health and disease states.[12]

Structure–activity relationship studies have examined the effect of variations in the 1-, 4-, and 8-positions of angiotensin II.

1 4 8
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

Angiotensin II

[Sar[1],Cys(Me)[8]]angiotensin II, prepared by solid phase synthesis, is reported to be an extremely potent antagonist of angiotensin II on vascular smooth muscle both *in vitro* (rabbit aorta) and *in vivo* (rat blood pressure), but a considerably weaker antagonist on visceral smooth muscle (guinea-pig ileum and rat uterus).[13] The corresponding [Sar[1],Cys(Me)[8]]angiotensin I analogue likewise displays organ selective antagonism, being a potent non-competitive inhibitor of angiotensin II in the rat blood pressure assay, but only moderately active in rabbit aorta and guinea-pig ileum or inactive in the rat uterus assay.[14]

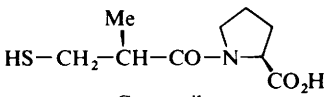
The activity of two analogues of [Sar[1],Val[5]]angiotensin II, containing carboranylalanine (Car) in place of Tyr^4 or Phe^8, has been reported. The [Sar[1],Car[4],Val[5]]angiotensin II analogue was inactive in the rabbit aorta assay, but [Sar[1],Val[5],Car[8]]angiotensin II retained 15% intrinsic activity, with a prolonged duration of action.[15]

[11] G. I. Cipens, F. Mutulis, O. Lando, and N. V. Myshlyakova, *Ger. Offen.*, **2**, 939 522, 17 April, 1980.
[12] H. R. Brunner and H. Gavras, *Am. J. Med.*, 1980, **69**, 739.
[13] R. J. Freer, J. C. Sutherland, jun., and A. R. Day, *Circ. Res.*, 1980, **46**, 720.
[14] R. J. Freer, J. C. Sutherland, and A. R. Day, *Eur. J. Pharmacol.*, 1980, **65**, 349.
[15] E. Escher, G. Guillemette, O. Leukart, and R. Regoli, *Eur. J. Pharmacol.*, 1980, **66**, 267.

Substitution of asparagine in [Asn^1]angiotensin II by the bulkier, more hydrophobic residues, Asp(*N*-dipropyl) and Asp(*N*-di-isopropyl), yields analogues with reduced potency and affinity, but again increased duration of action. These effects are greater with corresponding increase in size and hydrophobicity of the alkyl substituent in the side chain of position 1. As has previously been observed in the case of [Asp(*N*-dimethyl)^1]angiotensin II, dialkylation of the amide group does not alter intrinsic activity relative to [Asn^1]angiotensin II as determined in rabbit aorta strips. In the latter assay, the relative potencies of the series [Asn^1]angiotensin II, the corresponding dimethyl, dipropyl, and di-isopropyl analogues, were 100, 46, 16, and 9%, respectively. In the rat blood pressure assay they were 100, 30, 9, and 7%, respectively.[16]

The action of four known angiotensin antagonists, [Sar^1,Leu^8]-, [Leu^8]-, [Ala^8]-, and [Gly^8]-angiotensin, on the myotropic action of prostaglandins E_2 and $F_{2\alpha}$ in rat colon has been studied. The pattern of inhibition observed differed from angiotensin-induced contraction, in respect of the potency of antagonists, which was similar, and the absence of a dose–response relationship. Partial inhibition only was obtained and this varied from 40—59% for PGE_2 and from 31—49% for $PGF_{2\alpha}$ over a wide range of antagonist concentrations.[17] The conformation in dilute aqueous solution of the analogues, [Tyr^1,Phe^4,Val^5,Trp^8]- and [Trp^1,Phe^4,Val^5,Tyr^8]-angiotensin II, with transposition of the terminal residues, has been studied by fluorescence techniques. Results indicate that the conformations of both peptides are not as compact as some of the models that have been proposed for angiotensin II.[18]

Potentiating Peptides and Enzyme Inhibitors.—Cushman and Ondetti have presented an authoritative account on the development of inhibitors of angiotensin converting enzyme for treatment of hypertension, with special reference to Captopril or SQ 14 225 (D-3-mercapto-2-methylpropanoyl-L-proline).[19]



Captopril

Captopril, an orally active inhibitor of angiotensin converting enzyme, is reported from clinical studies to be a suitable antihypertensive compound for long term use in patients with essential hypertension.[20] The major component of its action seems to be the beneficial reduction of angiotensin II levels, though as with all the main groups of antihypertensive drugs, unwanted symptomatic or biochemical side-effects have also been attributed to it. It appears, nonetheless, to be a welcome addition to the present range of antihypertensive drugs offering a new approach to the treatment of several forms of hypertension.[21,22]

[16] P. Cordopatis and D. Theodoropoulos, *J. Med. Chem.*, 1981, **24**, 209.
[17] P. Sirois and D. J. Gagnon, *J. Pharm. Pharmacol.*, 1980, **32**, 232.
[18] P. W. Schiller, *Int. J. Pept. Protein Res.*, 1980, **16**, 259.
[19] D. W. Cushman and M. A. Ondetti, *Biochem. Pharmacol.*, 1980, **29**, 1871.
[20] C. I. Johnston, J. A. Millar, B. P. McGrath, and P. G. Matthews, *Lancet*, 1979, ii, 493.
[21] A. B. Atkinson and J. I. S. Robertson, *Lancet*, 1979, ii, 836.
[22] *Drugs Fut.*, 1980, **5**, 576 (*Squibb*).

An analogue of another inhibitor of angiotensin converting enzyme, benzyl-Phe-Gly-Pro, has been synthesized, in which the Phe-Gly amide bond is replaced by the enzyme resistant ketomethylene group (—CO—CH_2—). This analogue was found to be four-fold more potent than Captopril in inhibiting converting enzyme *in vitro*. Kinetic studies indicated non-competitive inhibition using hippuryl-His-Leu as substrate, but competitive inhibition when angiotensin I was used as substrate. Possible reasons for this mixed type of enzyme kinetics are discussed in relation to the active-site model for angiotensin converting enzyme. *In vivo* experiments to investigate the oral activity of the ketomethylene tripeptide and its analogues are planned.[23]

Studies on renin-inhibitory peptides have led to a potent decapeptide analogue, Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys, with specific *in vivo* activity in monkey. The terminal residues of the decapeptide are important for conferring adequate solubility (Pro,Lys) and increasing *in vivo* half-life (Lys), whilst inclusion of phenylalanine residues improves significantly the binding of the decapeptide to renin.[24]

Structure–activity relationship studies on the bradykinin potentiating pentapeptide, BPP5a, Glp-Lys-Trp-Ala-Pro, indicate that the native sequence is almost optimal for activity in terms of chain length, hydrophobic–hydrophilic balance, and the presence of the L-configuraton of tryptophan in position 3. The residues in the first two positions are important for enhancing activity.[25]

The results of conformational analysis of BPP5a, a synthetic analogue (Phe^3,Pro^4-BPP5a), and BPP9a (Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro), have been reported. The semi-empirical method used predicted stable compact conformations for BPP5a and BPP9a, in which ion-pair formation between the side-chain of Lys^2 or Arg^4, respectively, and the *C*-terminal carboxy-group, was a stabilizing interaction. In contrast, the only possible conformation for the biologically inactive synthetic analogue was one with an unfolded backbone.[26]

Tachykinins.—For a classification of peptides in this category the reader is referred to an excellent prior article.[27] In the following review the literature that appeared in 1980 has been covered.

The methods of synthesis and the effects on activity of variations in the structure of substance P have been summarized. In the latter report is also described a complete series of alanine analogues of substance P, in which each residue of the undecapeptide is systematically replaced.

1 **5** **10**
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Substance P

The results indicate that substitution with alanine at positions 1 to 6, or 9, of substance P does not alter hypotensive or myotropic activity (rat *in vivo*, guinea-pig

[23] R. G. Almquist, W.-R. Chao, M. E. Ellis, and H. L. Johnson, *J. Med. Chem.*, 1980, **23**, 1392.
[24] J. Burton, R. J. Cody, jun., J. A. Herd, and E. Haber, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5476.
[25] G. Heuver, C. van der Meer, J. G. R. Ufkes, and B. J. Visser, *Br. J. Pharmacol.*, 1980, **68**, 156P.
[26] N. N. Sevast'Yanova and E. M. Popov, *J. Mol. Struct.*, 1980, **65**, 125.
[27] V. Ersparmer, G. F. Ersparmer, and L. Negri, in 'Fogarty International Center Proceedings, No. 27', ed. J. J. Pisons and K. F. Austen, U.S. Government Printing Office, Washington, 1976, p. 153.

ileum, rabbit mesenteric vein, and *vas deferens* of rat *in vitro*, respectively), whereas substitution at the other positions reduces affinity. Alanine substitution at position 7 or 8, or removal of the *C*-terminal amide, gave analogues with 10—20% hypotensive activity at high doses. None of the less active analogues displayed antagonist activity.[28] Analogues of Glp^6-(6—11) substance P, in which the peptide bond between Phe^7 and Phe^8, or Phe^8 and Gly^9, has been replaced by a *trans*-olefinic bond, have been described. The novel derivatives obtained either retained full or 24% activity, respectively, relative to the parent hexapeptide in isolated guinea-pig ileum. Saturation of the olefinic bonds gave less active analogues.[29]

Replacement of either Phe^7 or Phe^8 in (4—11) substance P by L-carboranylalanine yields analogues which are essentially inactive as agonists or antagonists. The analogues exhibited a marked reduction in affinity (guinea-pig ileum, rabbit mesenteric vein) and it appears, therefore, that the larger more hydrophobic carboranyl ring can not be accommodated into the receptor site for the phenyl ring in the side chain of position 7 or 8.[5]

The effect of dimethylation of the amide group in the side chain of Gln^5 or Gln^6 of (5—11) substance P has been examined. The two analogues synthesized, Glu(*N*-Me_2)^5- and Glu(*N*-Me_2)^6-(5—11) substance P, displayed different activity profiles in guinea-pig ileum. The latter had 37% contractile activity suggesting no steric inhibition to interaction with the receptor, wheras earlier studies on the position-5 analogue had shown it to be a weak antagonist.[30]

Stimulation of phagocytosis is a newly found activity of substance P residing in the *N*-terminal tetrapeptide sequence. The latter resembles that of the well known phagocytosis-stimulating peptide, tuftsin (Thr-Lys-Pro-Arg), in containing a proline residue between two basic amino-acid residues. This finding suggests a role for substance P in inflammatory processes of neural origin.[31]

A good system for the separation of substance P from its sulphoxide ([Met(O)NH_2]^{11}substance P) on reverse phase h.p.l.c. has been described. The biological activity of the sulphoxide was shown to be 40% less than substance P in the rat blood pressure assay, whilst its immunoreactivity was reduced to 40% or 80% depending on the type of antiserum used.[32] In the latter connection, the preparation of a novel *N*-terminal directed substance P antiserum is reported and will complement the present range of *C*-terminal directed antisera.[33]

Circular dichroism studies on substance P and its *C*-terminal sequences did not indicate the occurrence of ordered conformations in dilute aqueous solution.[34]

A number of new peptides isolated from the amphibian skin have been reported. Methanol extracts of the skin of the Australian frog, *Uperoleia rugosa*, contain

[28] A. Fournier, R. Couture, J. Magnan, M. Gendreau, D. Regoli, and S. St-Pierre, *Can. J. Biochem.*, 1980, **58**, 272.
[29] M. T. Cox, J. J. Gormley, C. F. Hayward, and N. N. Petter, *J. Chem. Soc., Chem. Commun.*, 1980, 800.
[30] C. P. Poulos, N. Pinas, and D. Theodoropoulos, *Experientia*, 1980, **36**, 1104.
[31] Z. Bar-Shavit, R. Goldman, Y. Stabinsky, P. Gottlieb, M. Fridkin, V. I. Teichberg, and S. Blumberg, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 1445.
[32] E. Floor and S. E. Leeman, *Anal. Biochem.*, 1980, **101**, 498.
[33] C. M. Lee, P. C. Emson, and L. L. Iversen, *Life Sci.*, 1980, **27**, 535.
[34] B. Mehlis, M. Rueger, M. Becker, M. Bienert, H. Niedrich, and P. Oehme, *Int. J. Pept. Protein Res.*, 1980, **15**, 20.

Glu(OEt)^{2}litorin and [Lys^5,Thr^6]physalaemin, which appear to have very similar activities to the respective parent compounds, litorin and physalaemin.[35]

Glp-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Litorin

Glp-Ala-Asp-Pro-Asn-Lys-Phe-Try-Gly-Leu-Met-NH_2
Physalaemin

The methanol extract of the skin of the South American frog, *Phyllomedusa sauvagei*, contains an active polypeptide, sauvagine, which has been isolated in purified form and is currently being sequenced. Its biological activity in rat and dog awaits publication.[36]

The results of a comparative bioassay on litorin and its natural analogue, Glu(OMe)^{2}litorin, show that in the majority of the smooth muscle preparations examined the latter analogue was considerably less potent. However, Glu(OMe)^{2}litorin, was equipotent in rat uterus and urinary bladder, and three to six times more active than litorin on the isolated longitudinal muscle–myenteric plexus preparation of the guinea-pig ileum.[37]

The role of the tryptophyl^6 residue in xenopsin, Glp-Gly-Lys-Arg-Pro-Trp-Ile-Leu, isolated from the skin of *Xenopus laevis*, and an analogue [Lys^2,Gly^3]xenopsin, has been investigated. Application of a novel technique for selective reduction at the 2,3-position of the indole ring of Trp^6, to indoline, (pyridine–borane–trifluoroacetic acid), yielded corresponding [dihydro-Trp^6]xenopsin analogues. The latter proved to be essentially inactive on rat stomach strip, clearly demonstrating the importance of the Trp^6 residue for contractile activity.[38]

7 Enkephalins, Endorphins, and Related Peptides

Contributed by G. W. Hardy

Introduction.—The growth of the effort devoted to research on all aspects of the endogenous opiates has continued unabated in 1980. The use of an increasing number of assay systems and the discovery of many more opioid peptides have combined to aggravate the problems of those involved in attempts to delineate the physiological role of these molecules. Much of this work is beyond the scope of the present review, which concentrates on the more chemical aspects of the field and, in particular, on structure–activity relationships of the enkephalins and endorphins. 'Current Contents, Life Sciences' (Institute for Scientific Information, Philadelphia, U.S.A.), the Derwent 'Ringdoc' abstracts, and a 'Medlars' profile

[35] T. Nakajima, T. Yasuhara, V. Erspamer, G. F. Erspamer, L. Negri, and R. Endean, *Chem. Pharm. Bull.*, 1980, **28**, 689.
[36] P. C. Montecucchi, A. Anastasi, R. Castiglione, and V. Erspamer, *Int. J. Pept. Protein Res.*, 1980, **16**, 191.
[37] G. F. Erspamer and D. Piccinelli, *J. Pharm. Pharmacol.*, 1980, **32**, 497.
[38] S. Tachibana, K. Araki, and Y. Kikugawa, *Life Sci.*, 1980, **26**, 1013.

(National Library of Medicine, Bethesda, U.S.A.) were the initial sources of reference.

Review articles covering most areas of the field have appeared during the year and many have dealt with the relationship between the opiate peptides and their receptors. Included among these are a comprehensive review by Childers,[1] two shorter articles by Kosterlitz,[2,3] and a summary of cellular aspects of the receptors by Miller and Dawson.[4] A brief review by Hughes[5] summarizes the current knowledge of peripheral opiate receptor mechanisms while Miller and Pickel[6] have discussed the distribution and function of the enkephalins. Miller has also reviewed the role of opiate peptides as neurotransmitters.[7] A mini-review by Yeung *et al.* covers much of the recent work on the opioid peptides found in the adrenal gland.[8] Progress in studies on the biosynthesis, function, and pharmacology of the endorphins was detailed in a review by Kobylecki and Morgan,[9] which also included recent advances in the development of non-peptide opiates. Structure–activity relationships of the enkephalins have been discussed by Beddell *et al.*[10] and by Morley.[11] Possible involvement of the endorphins in anaesthesiology was reviewed by Stoelting[12] while Parksepp[13] has analysed their relevance to social behaviour.

Research on the opiate peptides was a major topic at several meetings held recently.[2,3,14–16]

New Opioid Peptides.—During 1980 several groups devoted a significant effort to the isolation and characterization of new opioid peptides. The details of the structures and sources of these peptides are presented in Table 1. The majority of the peptides release enkephalins upon treatment with proteases and hence may be regarded as potential precursor molecules. Two brief reviews of this area have appeared.[8,17]

The efficient fractionation of peptides by h.p.l.c. as developed by the Roche group has been applied to extracts of bovine adrenal medulla with the resultant

[1] S. R. Childers, in 'Receptors and Recognition', Ser. B, Vol. 9, Neurotransmitter Receptors, Part 1, ed. S. J. Enna and H. I. Yamamura, Chapman and Hall, London, 1980, p. 105.
[2] H. W. Kosterlitz, in 'Endogenous Peptides and Centrally Acting Drugs', Progress in Biochemical Pharmacology, Vol. 16, ed. A. Levy, E. Heldman, Z. Vogel, and Y. Gutman, S. Karger A.G., Basel, 1980, p. 3.
[3] H. W. Kosterlitz, in 'Neuropeptides and Neural Transmission', International Brain Research Organisation Monograph Series, Vol. 7, ed. C. A. Marsan and W. Z. Traczyk, Raven Press, New York, 1980, p.191.
[4] R. J. Miller and G. Dawson, *Cell Surface Reviews*, 1980, **6**, 351.
[5] J. Hughes, *Trends Pharmacol. Sci.*, 1981, 21.
[6] R. J. Miller and V. M. Pickel, *J. Histochem. Cytochem.*, 1980, **28**, 903.
[7] R. J. Miller, *Pharmacol. Therapeut.*, 1980, **12**, 73.
[8] H.-Y. T. Yeung, T. Hexum, and E. Costa, *Life Sci.*, 1980, **27**, 1119.
[9] R. J. Kobylecki and B. A. Morgan, *Annu. Rep. Med. Chem.*, 1980, **15**, 32.
[10] C. R. Beddell, L. A. Lowe, and S. Wilkinson, *Progr. Med. Chem.*, 1980, **17**, 1.
[11] J. S. Morley, *Ann. Rev. Pharmacol. Toxicol.*, 1980, **20**, 81.
[12] R. K. Stoelting, *Anesth. Analg.*, 1980, **59**, 874.
[13] J. B. Parksepp, *Neurosci. Behav. Rev.*, 1980, **4**, 473.
[14] 'Neural Peptides and Neuronal Communication', Advances in Biochemical Psychopharmacology, Vol. 22, ed. E. Costa and M. Trabucchi, Raven Press, New York, 1980.
[15] Proceedings of the 16th European Peptide Symposium, ed. K. Brunfeldt, Scriptor, Copenhagen, 1981.
[16] 'Exogenous and Endogenous Opiate Agonists and Antagonists', ed. E. Leong Way, Pergamon Press, New York, 1980.
[17] M. J. Brownstein, *Nature*, 1980, **287**, 678.

discovery of a family of polypeptides containing enkephalin sequences.[18-23] The largest of these is a 50 000 dalton protein, which contains one Leu-enkephalin and seven Met-enkephalin sequences.[21] A 14 000 dalton protein was found to contain two internal and one *C*-terminal enkephalin sequences.[24] Similarly, a single enkephalin sequence was located at the *C*-terminus of an 8000 dalton peptide.[24] The complete primary structure of a 34-peptide and the larger part of that of a 39-peptide have been determined; both contain two enkephalin sequences.[22,23] The heptapeptide, [Arg^6, Phe^7]-Met-enkephalin, originally isolated from bovine adrenal chromaffin granules,[18] has also been found in large amounts in bovine, rat, and human brain.[20] Its pharmacological profile was shown to be similar to that of the enkephalins. Analgesically, the molar potency of the peptide was approximately $8\times$ that of Met-enkephalin, $1.7 \cdot 10^{-2}\times$ that of morphine, and $8.7 \cdot 10^{-4}\times$ that of β_c-endorphin by the i.c.v. route.[19] As with the enkephalins, substitution of D-Ala for Gly^2 increased the potency to $130\times$ that of the natural compound.[20]

With the development of a highly sensitive radioimmunoassay for [Arg^6]-Leu-enkephalin by a Japanese group[25] the detection and purification of a second family of enkephalin-containing peptides have progressed rapidly. [Arg^6]-Leu-enkephalin was itself isolated from porcine pituitaries, whereas [Arg^{6,7}, Ile^8]-Leu-enkephalin [PH-8P, dynorphin (1—8)] was found in porcine hypothalamus.[26] Subsequent investigation of extracts of bovine adrenal medulla led to the isolation and sequencing of the peptides BAM-12P, BAM-20P, and BAM-22P.[27,28] The sequence of BAM-12P, which is contained in the two larger peptides, is also found in the 39-peptide partially sequenced by Jones *et al.*[22] (see above). However, as the 39-peptide lacks tryptophan, the homology with the BAM peptides apparently ceases at this point.

The identification of dynorphin,[29] α-neoendorphin,[30] and [Met(O)^5, Arg^6]-enkephalin[31] has been discussed previously in these reports (Volume 12).

Met-enkephalin and two proposed precursors of mol. wt. 2000 were isolated

[18] C. E. Insurrisi, J. G. Umans, D. Wolff, A. S. Stern, R. V. Lewis, S. Stein, and S. Udenfriend, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5512.
[19] A. S. Stern, R. V. Lewis, S. Kimura, J. Rossier, L. D. Gerber, L. Brink, S. Stein, and S. Udenfriend, *Proc. Natl. Acad. Sci. USA*, 1980, **76**, 6680.
[20] J. Rossier, Y. Audigier, N. Ling, J. Cros, and S. Udenfriend, *Nature*, 1980, **288**, 88.
[21] R. V. Lewis, A. S. Stern, S. Kimura, J. Rossier, S. Stein, and S. Udenfriend, *Science*, 1980, **208**, 1459.
[22] B. N. Jones, A. S. Stern, R. V. Lewis, S. Kimura, S. Stein, S. Udenfriend, and J. E. Shively, *Arch. Biochem. Biophys.*, 1980, **204**, 392.
[23] S. Kimura, R. V. Lewis, A. S. Stern, J. Rossier, S. Stein, and S. Udenfriend, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1681.
[24] R. V. Lewis, A. S. Stern, S. Kimura, S. Stein, and S. Udenfriend, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5018.
[25] K. Kangawa, K. Mizuno, N. Minamino, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1467.
[26] N. Minamino, K. Kangawa, A. Fukuda, H. Matsuo, and M. Igarashi, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1475.
[27] K. Mizuno, N. Minamino, K. Kangawa, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1482.
[28] K. Mizuno, N. Minamino, K. Kangawa, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1283.
[29] A. Goldstein, S. Tachibana, L. I. Lowney, M. Hunkapiller, and L. Hood, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 6666.
[30] K. Kangawa and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1979, **86**, 153.
[31] W.-Y. Yang, R. C. C. Chang, A. J. Kastin, D. H. Coy, and A. V. Schally, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 6177.

from an adrenal medullary tumour.[32] The biosynthesis of opiate-like peptides in the adrenal medulla and their secretion and regulation have been studied.[33-35]

Of particular interest and significance in the field of new endogenous opioid peptides was the isolation of the dermorphins.[36-40] These unique heptapeptides were isolated from the skin of the South American hylid frogs *Phyllomedusa sauvagei* and *Ph. rhodei*. The presence of D-Ala in position 2 provides the first observation of a D-amino-acid in a peptide isolated from a vertebrate and also serves to underline the frequent observation that natural systems anticipate the best tricks of the medicinal chemist! In the hot plate analgesia test the potency (i.c.v.) of demorphin is *ca.* $10^3\times$ morphine and $10^4\times$ dynorphin. [L-Ala^2]-dermorphin has only 0.1% of the activity of the parent; however, the addition of the protease inhibitor bacitracin raises this activity.[37] Apparently the dermorphins are only the first representatives of a group of opioid peptides that have been detected in the skins of various amphibians.[36]

Table 1 *New opioid peptides*

*Structure**	*Source†*	*Trivial name*	*Ref.*
YGGFMRF	A		18—20
Protein 50 000 daltons	A		21
YGGFMKKMDELYPLEVEE EANGGEVLGKRYGGFM	A		22, 23
SP(T)LEDEHKELQKRYG GFMRRVGRPE(B, Z, P_2, M, Y, K, R)YGGFL	A		22, 23
Protein 8000 daltons	A		24
Protein 14 000 daltons	A		24
YGGFLRRI	B	PH-8P	25
YGGFMRRVGRPE	A	BAM-12P	26
YGGFMRRVGRPEWWM(B, Y, Z)KR	A	BAM-20P	27
YGGFMRRVGRPEWWMDY QKRYG	A	BAM-20P	27
YGGFLRRIRPKLK	C	Dynorphin	28
YGGFLR(P, G, Y_2, K_2, R)	B	α-Neoendorphin	29
YGGFM(O)R	B		30
YAFGYPS-NH_2 (D)	D	Dermorphin	36—40
YAFGYXS-NH_2 (X = Hyp) (D)	D	Hyp^6-dermorphin	36—40

* Single letter code is used to denote amino-acid sequence. † A, bovine adrenal medulla; B, porcine hypothalamus; C, porcine pituitary; D, *Phyllomedusa sauvagei* skin.

[32] V. Clemment-Jones, R. Corder, and P. J. Lowry, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 665.
[33] O. H. Viveros, S. P. Wilson, E. J. Dilberto, jun., E. Hazum, and K.-J. Chang, in 'Proceedings XXVIII International Congress of Physiological Sciences', ed. J. Meites, The Publishing House of the Hungarian Academy of Sciences, Budapest, 1980.
[34] S. P. Wilson, K.-J. Chang, and O. H. Viveros, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 4364.
[35] L. Tan and P. H. Yu, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 1901.
[36] V. Erspamer and P. Melchiorri, *Trends Pharmacol. Sci.*, 1980, 391.
[37] R. de Castiglione, F. Faoro, G. Perseo, and S. Piani in ref. 15.
[38] R. de Castiglione, F. Faoro, G. Perseo, and S. Piani, *Int. J. Pept. Protein Res.*, 1981, **17**, 263.
[39] P. C. Montecucchi, R. de Castiglione, S. Piani, L. Gozzini, and V. Erspamer, *Int. J. Pept. Protein Res.*, 1981, **17**, 275.
[40] P. C. Montecucchi, R. de Castiglione, and V. Erspamer, *Int. J. Pept. Protein Res.*, 1981, **17**, 316.

Degradation of Opioid Peptides.—Considerable progress has been made recently on the isolation and characterization of the specific enzymes involved in enkephalin degradation. Investigations by several groups have confirmed the two major points of cleavage as the Tyr^1-Gly^2 bond and the Gly^3-Phe^4 bond.[41-48] An amino-peptidase specific for aromatic residues has been identified as the enzyme responsible for the rapid inactivation of enkephalin in blood.[41] Traficante *et al.*[42] have thoroughly characterized a specific amino-peptidase in homogenates of corpora striata from human brain.

A number of groups have partially characterized 'enkephalinase', a membrane bound dipeptidylcarboxypeptidase, which is readily distinguished from angiotensin converting enzyme (kininase II).[43-48] Gorenstein and Snyder[47,48] described the most complete characterization of this enzyme. The activity was obtained in two fractions (enkephalinase A_1 and A_2) separated from an amino-peptidase and a novel dipeptidylaminopeptidase (enkephalinase B), which cleaves the Gly^2-Gly^3 bond. The correlation between the distribution of enkephalinase A with that of opiate receptors in the brain lends support to the hypothesis that the enzymes may be responsible for inactivation of enkephalins involved in 'enkephalinergic' neurotransmission, and has stimulated interest in the development of specific inhibitors of enkephalinase A.

A study of peptide inhibitors[45] was considerably extended by Llorens *et al.*[49] to delineate the requirements for efficient inhibition by dipeptides. Essentially, the most potent dipeptides combined an *N*-terminal aromatic residue with a small side-chain on the second residue. These observations coupled with the rationale for the design of the kininase II inhibitor, Captopril, led to the synthesis of the extremely potent inhibitor Thiorphan, (DL-3-mercapto-2-benzylpropanoyl)glycine (K_i 4 nm).[50,51] Thiorphan increases the duration of analgesia of enzyme susceptible enkephalin analogues. The inhibition of enkephalinase by amino-acid hydroxamates has been described.[52]

^{13}C n.m.r. has been applied to the study of the degradation of enkephalins by neuroblastoma × glioma cells.[53] Graf and Hollosi[54] have demonstrated that β-lipotropin is cleaved by trypsin to yield β-endorphin almost exclusively when in an environment which promotes secondary structure.[54] The degradation of the

[41] M.-A. Coletti-Previero, H. Mattras, B. Descomps, and A. Previero, *Biochem. Biophys. Acta*, 1981, **657**, 122.
[42] L. J. Traficante, J. Rotosen, J. Siekierski, H. Tracer, and S. Gershon, *Life Sci.*, 1980, **26**, 1697.
[43] S. Sullivan, H. Akil, D. Blacker, and J. D. Barchas, ref. 14, p. 357.
[44] Z. Vogel and M. Alstein, ref. 14, p. 353.
[45] S. Sullivan, H. Akil, D. Blacker, and J. D. Barchas, *Peptides*, 1980, **1**, 31.
[46] M. Benuck and N. Marks, *Biochem. Biophys. Res. Commun.*, 1980, **95**, 822.
[47] C. Gorenstein and S. H. Snyder, *Proc. R. Soc. London, Ser. B*, 1980, **210**, 123.
[48] C. Gorenstein and S. H. Snyder, ref. 14, p. 345.
[49] G. Llorens, G. Gacel, J. P. Swerts, R. Perdrisot, M.-C. Fournie-Zaluski, J.-C. Schwartz, and B. P. Roques, *Biochem. Biophys. Res. Commun.*, 1980, **96**, 1710.
[50] B. P. Roques, M.-C. Fournie-Zaluski, E. Soroca, J.-M. Lecomte, B. Malfroy, C. Llorens, and J.-C. Schwartz, *Nature*, 1980, **288**, 286.
[51] M.-C. Fournie-Zaluski, C. Llorens, G. Gacel, B. Malfroy, J.-P. Swerts, J.-M. Lecomte, J.-C. Schwartz, and B. P. Roques in ref. 15, p. 476.
[52] S. Blumberg, Z. Vogel, and M. Alstein, *Life Sci.*, 1980, **28**, 301.
[53] R. Deslauriers, H. C. Jarrell, D. W. Griffith, W. H. McGreggor, and I. P. Smith, *Int. J. Pept. Protein Res.*, 1980, **16**, 487.
[54] L. Graf and M. Hollosi, *Biochem. Biophys. Res. Commun.*, 1980, **93**, 1089.

neuroleptic peptide des-Tyr^1-γ-endorphin by brain extract was studied by h.p.l.c.[55] The major degradation products retained neuroleptic activity, β-LPH(66—77) being the minimum sequence required for activity.

Structure–Activity Relationships.—The multitude of new analogues of the opiate peptides described during 1980 have been examined for an ever increasing range of biological activities both *in vitro* and *in vivo*. The novel effects described are far too numerous to review here, only major innovations will be mentioned. The original tests based on the inhibition of the contractions of the electrically stimulated guinea-pig ileum (GPI) or mouse vas deferens (MVD) remain the most popular *in vitro* bioassay. The determination of opiate receptor binding activity is becoming more common with the greater availability of radiolabelled opiate agonists and antagonists. In contrast to previous years, both the tail-flick and the hot plate test in either mice or rats have been used for the determination of *in vivo* analgesic potency.

The new analogues have been tabulated as usual according to the methods of assay and are described in the separate sections which follow. Frequently, a known analogue has been included within a group to allow easy comparison. Examples where compounds have not been examined in the standard assays are detailed at the end of the individual sections.

Isolated Tissue Assays. Table 2 lists the new analogues together with their GPI- or MVD-assay activities. Several more or less successful attempts have been made to develop analogues having δ-(MVD) or μ-(GPI) opiate receptor selectivity.

Table 2 *Isolated tissue activities*

Compound number	*Structure**	*MVD*	*Ref.*	*GPI*	*Ref.*
(a) Enkephalin analogues					
1	D-Ala^2, D-Ala^3, Met^5-NH$_2$			1000[a]	56
2	Tyr-D-Ala-Gly-NH(CH$_2$)$_2$Ph			146[a]	56
3	Tyr-D-Ala-Gly-NH(CH$_2$)$_3$-C$_6$H$_4$(o+p-N$_3$)			2.4[a]	56
4	Tyr-D-Ala-Gly-NH(CH$_2$)$_3$-C$_6$H$_4$(p-N$_3$)			0.44[a]	56
5	D-Ala^2, des-Met^5, NH(CH$_2$)$_3$-C$_6$H$_4$(o+p-N$_3$)			428[a]	56
6	D-Ala^2, des-Met^5, NH(CH$_2$)$_3$-C$_6$H$_4$(p-N$_3$)			428[a]	56
7	D-Ala^2, DL-Phe(m-N$_3$)^4, Leu^5-NH$_2$			546[a]	56
8	Tyr-D-Ala-Gly-Phe-NH-(isoamyl)	44[b]	57	940[b]	57
9	*N*-MeTyr-D-Ala-Gly-Phe-NH(isoamyl)	35[b]	57	520[b]	57
10	D-Ala, des-Met, N(Me)-(isoamyl)	15[b]	57	800[b]	57

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Table 2 *(cont.)*

Compound number	*Structure**	*MVD*	*Ref.*	*GPI*	*Ref.*
11	*N*-MeTyr-D-Ala-Gly-Phe-N(Me)(isoamyl)	26[b]	57	1000[b]	57
12	Tyr-D-Ala-Gly-*N*-MePhe-NH(isoamyl)	14[b]	57	500[b]	57
13	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-NH(isoamyl)	5[b]	57	235[b]	57
14	Tyr-D-Ala-Gly-*N*-MePhe-N(Me)(isoamyl)	3[b]	57	65[b]	57
15	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-N(Me)(isoamyl)	4[b]	57	71[b]	57
16	Tyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$	1.8[b]	57	850[b]	57
17	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$	4[b]	57	435[b]	57
18	Tyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$ ↓ O	18[b]	57	650[b]	57
19	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$ ↓ O	3[b]	57	790[b]	57
20	D-Ala^2, Phe(αMe)^4, des-Met^5			16	58
21	D-Ala^2, Phe(αMe)^4, Leu^5			48[c]	58
22	D-Ala^2, Phe(αMe)^4, Val^5			29[c]	58
23	D-Ala^2, Phe(αMe)^4, des-Met^5			16[c]	58
24	Tyr(αMe)^1, D-Ala^2, D-Leu^5			<0.75[c]	58
25	Aib^2, Met^5-NH_2			117[c]	58
26	D-Ala^2, des(Phe^{4}Met^5)-NH-(phenethyl)			19[c]	58
27	D-Ala^2, des(Phe^{4}Met^5)-NH-(1-indanyl)			47[c]	58
28	des(Phe^{4}Met^5)-NH-(1-indanyl)			3.3[c]	58
29	Aib^2, des(Phe^4, Met^5)-NH-(1-indanyl)			2.5[c]	58
30	Aib^3, des(Phe^4, Met^5)-NH-(1-indanyl)			<0.8[c]	58
31	D-Ala^2, des(Phe^4, Met^5)-NH-(2-indanyl)			<0.9[c]	58
32	D-Ala^2, des(Phe^4, Met^5)-NH-(benzyl)			14[c]	58
33	D-Ala^2, des(Phe^4, Met^5)-NH-(cyclobutylmethyl)			6.3[c]	58
34	D-Ala^2, des(Phe^4, Met^5)-NH-(cyclopropylmethyl)			3.7[c]	58
35	D-Ala^2, des(Phe^4, Met^5)-OBzl			2.5[c]	58
36	D-Ala^2, des(Phe^4, Met^5)			<0.08[c]	58
37	D-Ala^2, Cha^4, D-Leu^5			25[c]	58
38	Glu(OEt)^1, D-Ala^2, D-Leu^5			<0.06[c]	58
39	D-Ala^2, Bug^5-NH_2	<100[d]	59	*ca.*250[b] 80[b]	59 60
40	Nle^5	100[e]	61	100[e]	61

Table 2 *(cont.)*

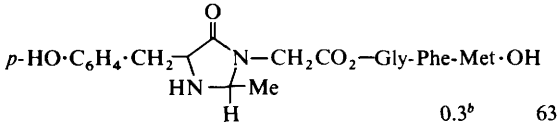
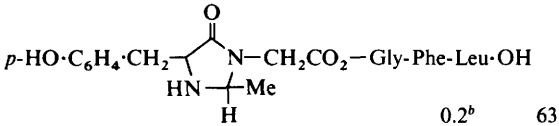
Compound number	*Structure**	*MVD*	*Ref.*	*GPI*	*Ref.*
41	(descarboxy-Nle)^5-SO_3H	490[e]	61	620[e]	61
42	(descarboxy-Nle)^5-PO_3H_2	890[e]	61	290[e]	61
43	(descarboxy-D-Nle)^5-SO_3H	11[e]	61	10[e]	61
44	D-Ala^2, Nle^5	2060[e]	61	560[e]	61
45	D-Ala^2, (descarboxy-Nle)^5-SO_3H	3680[e]	61	1560[e]	61
46	D-Ala^2, (descarboxy-Nle)^5-PO_3H_2	10 600[e]	61	2230[e]	61
47	D-Ala^2, (descarboxy-D-Nle)^5-SO_3H	120[e]	61	558[e]	61
48	D-Nle^2, Nle^5	2220[e]	61	612[e]	61
49	D-Nle^2, (descarboxy-Nle)^5-SO_3H	9820[e]	61	4290[e]	61
50	D-Nle^2, (descarboxy-Nle)^5-PO_3H_2	8700[e]	61	980[e]	61
51	D-Nle^2, (descarboxy-D-Nle)^5-SO_3H	21[e]	61	310[e]	61
52	D-Met^2, Nle^5	3440[e]	61	950[e]	61
53	D-Met^2, (descarboxy-Nle)^5-SO_3H	4210[e]	61	1990[e]	61
54	D-Met^2, (descarboxy-Nle)^5-PO_3H_2	1280[e]	61	340[e]	61
55	D-Met^2, (descarboxy-D-Nle)^5-SO_3H	11[e]	61	380[e]	61
56	D-Met^2, (descarboxy-D-Nle)^5-PO_3H_2	16[e]	61	390[e]	61
57	D-Ser^2, Leu^5, Thr^6	4.5[b]	62	180[b]	62
58		0.3[b]	63	1.1[b]	63
59		0.2[b]	63	0.5[b]	63
60	*N*-EtTyr^1	0.9[b]	63	6.7[b]	63
61	D-Ala^2, Met^5-NHEt			718[b]	64
62	D-Ala^2, Met-NH-(n-propyl)			131[b]	64
63	D-Ala^2, Met^5-NH-(isopropyl)			162[b]	64
64	D-Ala^2, Met^5-NH-(n-butyl)			59[b]	64
65	D-Ala^2, Met^5-NH-(n-hexyl)			141[b]	64
66	(2-aminoindan-2-oyl)^1, Leu^5-OMe	0.007[b]	65	0.4[b]	65
67	(2-amino-5-hydroxyindan-2-oyl)^1, Leu^5-OMe	0.03[b]	65	2.7[b]	65
68	(2-aminotetralin-2-oyl)^1, Leu^5-OMe	0.007[b]	65	inactive	65
69	(2-amino-5-hydroxytetralin-2-oyl)^1, Leu^5-OMe	5.2[b]	65	132[b]	65

Table 2 *(cont.)*

Compound number	*Structure**	*MVD*	*Ref.*	*GPI*	*Ref.*
70	D-Ala^2, β-Ala^3, des(Phe^4, Met^5)-NH(CH$_2$)$_2$Ph	<1[b]	66	<1[b]	66
71	D-Ala^2, des(Phe^4, Met^5)-NHCH(Me)CHCHMe$_2$	<1[b]	66	32[b]	66
72	D-Met^2, des(Phe^4, Met^5)-NHCH(Me)CH$_2$CHMe$_2$	<1[b]	66	62[b]	66
73	Tyr-NHCH$_2$CH=CHCH$_2$CO-Phe-Pro-NH$_2$			0.1[f]	67
74	*p*-HO.C$_6$H$_4$.CH$_2$CHCH(NH$_2$)CHCH$_2$CO-Gly-Phe-Leu-OMe	24[g]	67	300[g]	67
75	D-Met^2, Pro^5-NH$_2$	1986[h]	68	564[h]	68
76	D-Met^2, Leu^5-NH$_2$	1226[h]	68	244[h]	68
77	D-Met^2, Met^5-NH$_2$	1489[h]	68	461[h]	68
78	D-Met^2, Cha^4, Met^5-NH$_2$	851[h]	68	154[h]	68
79	D-Met^2, Cha^4, Leu^5-NH$_2$	60[h]	68	211[h]	68
80	D-Met^2, Cha^4, Pro^5-NH$_2$	117[h]	68	284[h]	68
81	D-Ala^2, Cha^4, Pro^5-NH$_2$	31[h]	68	78[h]	68
82	D-Ala^2, Ada^5-NH$_2$			270[b]	60
83	D-Ala^2, Ada^5-OH			520[b]	60
84	D-Ala^2, D-Ada^5-NH$_2$			150[b]	60
85	D-Ala^2, D-Ada^5-OH			360[b]	60
86	D-Ala^2, Leu^5, Lys^6-(N^{ε}Ac)-NH$_2$			84[b]	60
87	[D-Ala^2, Leu^5, Lys^6-NH$_2$ (COCH$_2$S-]$_n$-TMV	$n=7$ 369[b]	60	294[b]	60
		$n=263$ 445[b]	60	798[b]	60
88	des-Met^5-NHCHCH$_2$CHMe$_2$ (COCH$_2$Cl)			46[h]	69
89	D-Ala^2, des-Met^5-NHCHCH$_2$CHMe$_2$ (COCH$_2$Cl)			535[h]	69
90	Phe(*p*-NO$_2$)^1			<0.37[i]	70
91	Phe(*p*-NH$_2$)^1			<0.37[i]	70
92	Tyr(3,3,5,5-F$_4$)^1, D-Ala^2, Met^5-NH$_2$			<0.37[i]	70
93	Tyr(3-F)^1, D-Ala^2, Met^5-NH$_2$			54[i]	70
94	D-Tyr(3-F)^1, D-Ala^2, Met^5-NH$_2$			<3.7[i]	70
95	Tyr(3-Me)^1, D-Ala^2, Met^5-NH$_2$			9.3[i]	70
96	D-Tyr(3-Me)^1, D-Ala^2, Met^5-NH$_2$			<3.7[i]	70
97	Tyr(3,5-Me$_2$)^1, D-Ala^2, Met^5-NH$_2$			<3.7[i]	70
98	D-Tyr(3,5-Me$_2$)^1, D-Ala^2, Met^5-NH$_2$			<3.7[i]	70

Table 2 (*contd.*)

Compound number	*Structure**	*MVD*	*Ref.*	*GPI*	*Ref.*
99	Phe(3-OH), D-Ala^2, Met^5-NH_2			<3.7[i]	70
100	D-Phe(3-OH)^1, D-Ala^2, Met^5-NH_2			<3.7[i]	70
101	Tyr(αMe)^1, D-Ala^2, Met^5-NH_2			<0.37[i]	70
102	D-Tyr(αMe)^1, D-Ala^2, Met^5-NH_2			<0.37[i]	70
103	Arg^0, D-Ala^2, Met^5-NH_2			42[i]	70
104	Lys^0, D-Ala^2, Met^5-NH_2			10.5[i]	70
105	Gly^0, D-Ala^2, Met^5-NH_2			51.4[i]	70
106	β-Ala^0, D-Ala^2, Met^5-NH_2			<3.7[i]	70
107	D-Ala^0, D-Ala^2, Met^5-NH_2			<3.7[i]	70
(b) Endorphin analogues					
I	β_p-EP-(1—31)	100[j]	81	100[j]	81
II	β_p-EP-(1—29)	105[j]	81	136[j]	81
III	β_p-EP-(1—27)	99[j]	81	69[j]	81
IV	β_p-EP-(1—25)	55[j]	81	51[j]	81
V	β_p-EP-(1—23)	92[j]	81	60[j]	81
VI	β_p-EP-(1—21)	119[j]	81	53[j]	81
VII	β_p-EP-(1—19)	91[j]	81	27[j]	81
VIII	D-Ala^2-β_h-EP			47[k]	82
IX	Met(O)^5-β_h-EP			23[k]	82
X	Leu^5-β_h-EP			16[k]	82
XI	Aib^2-β_h-EP			2[k]	82
XII	Arg^0-β_h-EP			14[k]	82
XIII	D-Arg^0-β_h-EP			1.4[k]	82
XIV	Ac-Arg^0-β_h-EP			1.4[k]	82
XV	Met(O)^5-β_p-EP			25[j]	83
XVI	Met(O_2)^5-β_p-EP			22[j]	83
XVII	Met(CH_2CO_2H)^5-β_p-EP			4[j]	83
XVIII	Tyr(3,5-I_2)^1-β_h-EP			<1[k]	94
XIX	Tyr(3,5-I_2)^{27}-β_h-EP			37[k]	94
XX	Tyr(3,5-$^3H_2$)$_2^{1,27}$-β_h-EP			<1[k]	94
XXI	Tyr(3,5-$^3H_2$)^1-β_h-EP			97[k]	94
XXII	Tyr(3,5-$^3H_2$)^{27}-β_h-EP			100[k]	94
XXIII	Tyr(3,5-$^3H_2$)$_2^{1,27}$-β_h-EP			95[k]	94
XLIII	Phe^{27}, Gly^{31}-β_h-EP			143[k]	85
XLIV	D-Lys^9, Phe^{27}, Gly^{31}-β_h-EP			100[k]	85
XLV	D-Thr^2, D-Lys^9, Phe^{27}, Gly^{31}-β_h-EP			86[k]	85 85
XLVI	D-Phe^{18}, Phe^{27}, Gly^{31}-β_h-EP			104[k]	85
XLVII	D-Thr^2, D-Phe^{18}, Phe^{27}, Gly^{31}-β_h-EP			104[k]	85

* Except in cases where entire structural formulae are given [*e.g.* (11)—(19)], the amino-acid residues of enkephalin analogues are numbered according to the sequence of Met^5 enkephalin (H-Tyr-Gly^2-Gly^3-Phe^4-Met^5-OH). Endorphin analogues are related to the sequences of human (β_h), camel (β_c), or porcine (β_p) β-endorphin. [a]% Potency relative to normorphine. [b]% Potency relative to Met^5-enkephalin. [c]% Potency relative to D-Ala^2,D-Leu^5-enkephalin. [d]% Potency relative to Leu^5-enkephalin. [e]% Potency relative to Nle^5-enkephalin. [f]% Potency relative to Pro^5-enkephalinamide. [g]% Potency relative to Leu^5-enkephalin methyl ester. [h]% Potency relative to morphine. [i]% Potency relative to D-Ala^2, Met^5-enkephalinamide. [j]% Potency relative to β_p-endorphin. [k]% Potency relative to β_h-endorphin.

A series of truncated analogues [(3)—(7)] bearing an azido-substituted aryl amide have been prepared for receptor photoaffinity studies.[56] The tetrapeptide amide derivatives retain reasonable potency on the GPI and irreversible inactivation of opiate binding was observed upon irradiation. Morgan *et al.*[57] have described the systematic variation of the structure of a series of 4-peptide amides in order to optimize stability and activity both *in vitro* and *in vivo* [(8)—(19)]. A respectable separation of the GPI- and MVD-potencies was observed [*e.g., ca.* 450-fold for (16)]; those analogues most potent in these assays were not the most active *in vivo* (see below). Significant *in vitro* activity was found in a series of tripeptide amide derivatives [(26)—(34)] during a study aimed at delineating the requirements of the μ-receptor.[58] The stringent requirement for the functionalities of the first and fourth residues of the enkephalins was confirmed [(20)—(25); (35)—(38)]. Another group[66] found that Phe^4 plays a crucial role for differential recognition by the μ- and δ-receptors. GPI-activity was retained in two tripeptide analogues in which Phe^4 was substituted by an alkylamide whereas MVD activity was virtually abolished. The same workers have also described a hexapeptide (57), which is highly specific for the δ-receptor, having a selectivity of approximately 620-fold.[63]

Bajusz *et al.*[61, 71] have described a series of peptides bearing the sulphonic or phosphonic acid analogues of norleucine at the carboxy-terminus [(40)—(56)]. The rationale for these modifications is that preferential δ-receptor activity appears to be related to the presence of the terminal acidic group; the greatest selectivity was shown by the phosphonate analogues. Schwyzer[60] has described several analogues containing a 'fat'-residue at position 5 that have enhanced activity on the GPI [(39), (82)—(85)]. In addition, conjugates of [D-Ala^2, Lys^6]-Leu-enkephalin with tobacco mosaic virus [TMV, (86), (87)] exhibited 'superaffinity' for opiate receptors.

In general, apart from *N*-methylation, modification of Tyr^1 reduces potency. One exception is the conformationally restricted tetralin-analogue, (69), whose

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GPI activity is marginally increased.[65] Other analogues in the series were virtually inactive (66)—(68). Similary, the potencies of the acetaldehyde adducts (58) and (59) were determined to be 1% of that of Met-enkephalin.[63] Coy and Kastin[70] prepared a number of peptides containing ring-substituted analogues of Tyr at position 1, [(99)—(102)]; all showed markedly reduced potency.

Two groups have described the replacement of the peptide bond by an isosteric ethylenic linkage.[67, 72] Cox *et al.*[67] devised a general route to diastereomeric mixture of dipeptide isoteres and hence prepared enkephalin analogues bearing substitutions of the Gly^2-Gly^3 and Tyr^1-Gly^2 bond, respectively [(73), (74)]. Hann *et al.*[72] prepared the acid analogue of (74) by a stereospecific route. Both groups found that activity was preserved on replacement of the Tyr^1-Gly^2 bond; however, substitution of the Gly^2-Gly^3 bond drastically reduced potency on the GPI.[67]

In a series of pentapeptide alkylamides [(61)—(65)] greatest potency on the GPI was found with the NEt analogue but other members were more active *in vivo*.[64] Support for the hypothesis that μ-receptors mediate the analgesic response was gained from a comprehensive study of a number of Cha^4-pentapeptide amides [(75)—(81)].[68] Two enkephalin chloromethylketone derivatives were found to exhibit increased potency on the GPI (88), (89).[69] However, there was no indication of irreversible binding, an observation which was rationalized in terms of the work of Portoghese *et al.*[73, 74] on classical opiate chloromethylketone derivatives. Kiso and co-workers[75] have described the preparation and GPI-activity of a short series of Met-enkephalin analogues. Kosterlitz *et al.*[76] have reported a comprehensive study of the effects of changes in the structure of a series of known enkephalin analogues on their interaction with the μ- and δ-receptors.

The modification of both Met- and Leu-enkephalins with chlorosulphonic acid to produce analogues sulphonated at the 3-position of Tyr^1 has been described.[77, 78] Activity was retained in tests for epileptogenic potency and depression of respiratory neurones in the rat; the analogues were stable to serum proteolysis but were still degraded by brain proteases. The synthesis of [DL-β-ferrocenylalanyl^4]-Leu-enkephalin has been described but no activity was quoted.[79]

[D-Trp^2, Met^5]-enkephalinamide was found to act directly on the pituitary to release growth hormone specifically. A structure–activity study revealed that this activity was restricted to amidated analogues with D-Trp or D-Phe at position 2[80] and was not antagonized by naloxone.

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Graf *et al.*[81] examined the potencies of porcine β-endorphin *C*-terminal deletion analogues (I—VII). Successive dipeptide deletions from 31 to 19 residues had little effect on MVD-potency, whereas GPI-potency steadily decreased. Immunoreactivity fell to 48% at 23 residues then abruptly dropped to <1%. Some correlation between potency, immunoreactivity, and conformation was observed. The same group also described a reduction of α-helix potential and biological activity in response to modifications of Met[5] in porcine β-endorphin (XV—XVII).[83] A series of human β-endorphin analogues bearing single amino-acid extensions or substitutions in the 2 or 5 positions all had reduced GPI-activity (VIII—XIV).[82] Houghten *et al.*[84] observed reduced potencies for analogues of human β-endorphin iodinated at Tyr[1], Tyr[27], or both. Full activity was restored upon catalytic tritiation which yielded peptides of specific activity 50—100 Ci mmol^{-1} useful for receptor binding assays.

Yeung *et al.*[85] prepared a series of β_h-EP analogues (XLIII—XLVII) that were practically indistinguishable in the GPI-test but which showed reduced analgesic potency i.c.v. in the mouse tail-flick test (see below).

Structure–activity studies on a series of des-Tyr[1]-γ-endorphin fragments, β-LPH [(61)—(77)], led to the suggestion that β-LPH [(66)—(77)] may be an endogenous neuropeptide with neuroleptic activity.[86] Solution syntheses of human β- and γ-endorphins have been described.[87]

Opiate Receptor Activities. Receptor binding potencies of the new analogues of the enkephalins and endorphins are summarized in Table 3. The differing assay procedures in different laboratories, however, make direct comparisons difficult.

Table 3 *Opiate receptor affinities*

Compound number	*Structure*	*Affinity*	*Ref.*
(a) Enkephalin analogues			
2	Tyr-D-Ala-Gly-NH$(CH_2)_2$Ph	82[a]	56
3	Tyr-D-Ala-Gly-NH$(CH_2)_3C_6H_4$(o + p-N_3)	5.7[a]	56
4	Tyr-D-Ala-GlyNH$(CH_2)_3C_6H_4$(p-N_3)	0.12[a]	56
5	D-Ala[2], des-Met[5], NH$(CH_2)_3C_6H_4$(o + p-N_3)	114[a]	56
6	D-Ala[2], des-Met[5], NH$(CH_2)_3C_6H_4$(p-N_3)	114[a]	56
7	D-Ala[2], DL-Phe(p-N_3)[4], Leu[5]-NH_2	146[a]	56
20	D-Ala[2], Phe(αMe)[4], des-Met[5]	4.4[b]	58
21	D-Ala[2], Phe(αMe)[4], Leu[5]	19[b]	58
22	D-Ala[2], Phe(αMe)[4], Val[5]	19[b]	58
24	Tyr(αMe)[1], D-Ala[2], D-Leu[5]	<0.01[b]	58
25	Aib[2], Met[5]-NH_2	18[b]	58
26	D-Ala[2], des(Phe[4], Met[5])-NH-(phenethyl)	21[b]	58

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Table 3 *(cont.)*

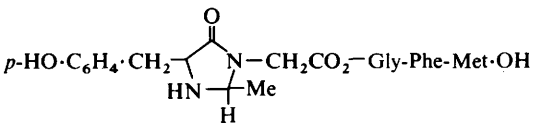
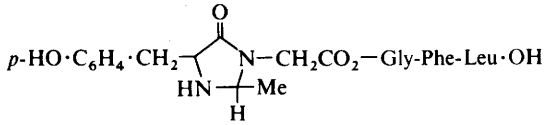
Compound number	*Structure*	*Affinity*	*Ref.*
27	D-Ala^2, des(Phe^4, Met^5)-NH-(1-indanyl)	23[b]	58
28	des(Phe^4, Met^5)-NH-(1-indanyl)	0.26[b]	58
30	Aib^{3}des(Phe^4, Met^5)-NH-(1-indanyl)	0.06[b]	58
31	D-Ala^2, des(Phe^4, Met^5)-NH-(2-indanyl)	0.47[b]	58
32	D-Ala^2, des(Phe^4, Met^5)-NH-benzyl	18[b]	58
33	D-Ala^2, des(Phe^4, Met^5)-NH-(cyclobutyl-methyl)	5.6[b]	58
34	D-Ala^2, des(Phe^4, Met^5)-NH-(cyclopropyl-methyl)	2[b]	58
35	D-Ala^2, des(Phe^4, Met^5)-OBzl	9.9[b]	58
36	D-Ala^2, des(Phe^4, Met^5)	0.01[b]	58
37	D-Ala^2, Cha^4, Leu^5	9.5[b]	58
38	Glu(OEt)^1, D-Ala^2, D-Leu^5	0.01[b]	58
75	D-Met^2, Pro^5-NH_2	207[c] 600[d]	88, 68
76	D-Met^2, Leu^5-NH_2	150[c] 500[d]	88, 68
77	D-Met^2, Met^5-NH_2	239[c] 400[d]	88, 68
78	D-Met^2, Cha^4, Met^5-NH_2	103[c]	88, 68
79	D-Met^2, Cha^4, Leu^5-NH_2	23[c] 44[d]	88, 68
80	D-Met^2, Cha^4, Pro^5-NH_2	23[c] 76[d]	88, 68
81	D-Ala, Cha^4, Pro^5-NH_2	11[c] 35[d]	88 68
108	Leu^5, Lys^6(*N*-(+)-biotinyl)-NH_2	10[e]	89
109	Leu^5, Lys^6(*N*-(+)-biotinyl:avidin)-NH_2	0.001[e]	89
110	Leu^5, Gly^6, Gly^7, Lys^8(*N*-(+)-biotinyl)-NH_2	10[e]	89
111	Leu^5, Gly^6, Gly^7, Lys^8(*N*-(+)-biotinyl: avidin)-NH_2	0.2[e]	89
112	D-Ala^6	67[f]	90
113	Ala^0	11[f]	90
114	D-Ala^0	0.003[f]	90
115	(Tyr-Gly-Gly)^0	0.09[f]	90
40	Nle^5	100[g]	91
116	Tyr-D-Ala-NHCH$_2$CH$_2$-Phe-Metol CH$_2$Ph	12[g]	91
117	Tyr-D-Ala-GlyNHCHCH$_2$-Metol CH$_2$Ph	286[g]	91
118	Tyr-Gly-Gly-NHCHCH$_2$-Metol	59[g]	91
119	*p*-HO.C$_6$H$_4$CH$_2$CHCH=CHCH$_2$CO-Gly- NH$_2$ Phe-LeuOH	29[h] 67[i]	72
58		1.2[j] 0.05[k]	63

Table 3 *(cont.)*

Compound number	*Structure*	*Affinity*	*Ref.*
59		1.8[j] 0.05[k]	63
60	*N*-EtTyr[1]	92[j] 0.06[k]	63
	(b) Endorphin analogues		
VIII	D-Ala[2]-β_h-EP	133[l]	82
XI	Aib[2]-β_h-EP	13[l]	82
XII	Arg[0]-β_h-EP	5[l]	82
XXIV	des-Gly[2]-β_c-EP	0.5[m]	92
XXV	des-Leu[14]-β_c-EP	58[m]	92
XXVI	des-Thr[6]-β_c-EP	45[m]	92
XXVII	des-Ser[10]-β_c-EP	90[m]	92
XXVIII	des-Thr[12]-β_c-EP	93[m]	92
XXIX	des-Met[5]-β_c-EP	2[m]	92
XXX	des-Val[15]-β_c-EP	64[m]	92
XXXI	des-Ile[22]-β_c-EP	139[m]	92
XXXII	des-Glu[11]-β_c-EP	120[m]	92
XXXIII	des-Pro[13]-β_c-EP	29[m]	92
XXXIV	des-Asn[20]-β_c-EP	47[m]	92
XXXV	des-Gln[11], Leu[14], Asn[20], Ile[22]-β_h-EP	41[m] 42[n]	92 92
XXXVI	β_h-EP-(1—27)	30[m]	93
XXXVII	Gln[8]-β_h-EP-(1—27)	90[m]	93
XXXVIII	AcTyr[1]-β_h-EP-(1—27)	0.04[m]	93
XXXIX	AcTyr[1], Gln[8]-β_h-EP-(1—27)	0.07[m]	93
XL	turkey-β-EP	94[m]	94
XLI	des-Ac-salmon-β-EP	169[m] 270[l]	94 95
XLII	Salmon-β-EP	0.005[l]	95
XLIII	Phe[27], Gly[31]-β_h-EP	126[n]	96
XLVIII	Cys[21]—Cys[26], Phe[27], Gly[31]-β_h-EP	358[n]	96
XLIX	Cys[14]—Cys[26], Phe[27], Gly[31]-β_h-EP	390[n]	96

[a]% Affinity relative to normorphine. Bovine caudate nucleus, [^{3}H]etorphine, 25 °C. [b]% Affinity relative to D-Ala^2,D-Leu^5-enkephalin. Rat brain particulate fraction, [^{3}H]naloxone, 0 °C. [c]% Affinity relative to morphine. Whole rat brain, [^{3}H]etorphine, 37 °C. [d]% Affinity relative to morphine. Whole rat brain, D-[^{3}H]Ala^2,Leu^5-enkephalinamide, 37 °C. [e]% Affinity relative to Leu^5-enkephalin. Rat brain synaptic membrane fraction, D-[^{3}H]Ala^2,Leu^5-enkephalin, 25 °C. [f]% Affinity relative to Met^5-enkephalin. Whole rat brain, [^{3}H]naloxone, 25 °C. [g]% Affinity relative to Nle^5-enkephalin. Whole rat brain, [^{3}H]naloxone, 25 °C. [h]% Affinity relative to [^{3}H]Leu^5-enkephalin at 0 °C. Whole rat brain, [^{3}H]naloxone at 30 °C. [i]% Affinity relative to Leu^5-enkephalin. Whole rat brain, [^{3}H]Leu^5-enkephalin, 0 °C. [j]% Affinity relative to morphine. Rat brain membranes, D-[^{3}H]Ala^2,D-Leu^5-enkephalin, 0 °C. [k]% Affinity relative to morphine. Rat brain membranes, [^{3}H]dihydromorphine, 0 °C. [l]% Affinity relative to β_h-EP. Rat brain membranes, [^{3}H]naloxone, 0 °C. [m]% Affinity relative to β_c-EP. Rat brain membranes, [$^3H_2$]Tyr^{27}-β_h-EP. [n]% Affinity relative to β_h-EP. Rat brain membranes, [$^3H_2$]Tyr^{27}-β_h-EP.

A good correlation between receptor affinity and GPI-potency (Table 2) was observed for the azido-affinity-label analogues (2)—(7)[56] and for the truncated analogues [(20)—(38)].[38] Hydrogenation of the Phe[4] residue of analogues [(75)—(77)] invariably reduced receptor binding affinity [(78)—(81)].[68, 88] However, a strong paired correlation was observed[68] between GPI-potency and competition for [^{3}H]etorphine receptor sites and between MVD-potency and [^{3}H]-[D-Ala^2, Leu^5]-enkephalin receptor sites, respectively. Consequently, the data were taken as further evidence for the distinction of μ- and δ-receptors. Proline in the fifth position enhanced all activities by the same degree.[68]

Receptor binding affinity was retained (10%) in two analogues substituted with biotinyl groups [(108), (110)] but was much reduced on binding to avidin [(109), (111)], which may impair their use as affinity ligands.[89] The effect of extending the enkephalin molecule at the *N*- or *C*-terminus was examined by Simon *et al.*[90] [(112)—(115)]. The acetaldehyde adducts of the enkephalins [(58), (59)] were found to have drastically reduced potencies in accord with their GPI- and MVD-activities (Table 2).[63] The potency of (60) in competition with [^{3}H]-[D-Ala^2, Leu^5]-enkephalin is hardly impaired but against [^{3}H]dihydromorphine the obverse is true.[63] Potency was retained in a series of analogues bearing isosteric replacements of peptide bonds [(116)—(118)], in particular replacement of the carbonyl-group of the Phe[4] residue with a methylene group enhanced activity (117).[91] Substitution of the Tyr[1]-Gly[2] amide bond by an ethylenic linkage caused only a minor loss of binding affinity (119).[72]

The receptor binding potencies of the endorphin analogues (XXIV) to (XXXV) were found to correlate closely with their GPI activities (Vol. 12 of these reports) but opiate activity, in general, was easily dissociated from immunoreactivity.[92] Acetylation of Tyr[1] of human β-endorphin [(1)—(27)] was shown to abolish opiate activity but to increase immunoreactivity by 3.8—8.8-fold [(XXXVI)—(XXXIX)].[93] Analogues [(VII)—(XII)] were of reduced potency in accord with their ileal activities,[83] whereas synthetic turkey-[94] and des-acetyl salmon-endorphins[94, 95] [(XL) and (XLI)] demonstrated high activity in the mammalian assay system. Blake *et al.*[96] prepared two further analogues of β_h-endorphin containing cystine bridges between residues 21 and 26 or 14 and 26; both showed markedly enhanced receptor binding [(XLVIII) and (XLIX)].

In vivo *Activities*. The relative analgesic potencies of the new analogues are summarized in Table 4. Four routes of administration have been investigated: intracerebroventricular (i.c.v.), intravenous (i.v.), intra-peritoneal (i.p.), and subcutaneous (s.c.); effective oral activity remains the elusive goal of many groups.

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Table 4 In vivo *activities*

Compound number	*Structure*	*Activity*	*Ref.*
(a) Enkephalin analogues			
8	Tyr-D-Ala-Gly-Phe-NH(isoamyl)	$>30^a$ $>100^b$	57
9	*N*-MeTyr-D-Ala-Gly-Phe-NH(isoamyl)	$>30^a$ 0.2^b	57
10	Tyr-D-Ala-Gly-Phe-N(Me)(isoamyl)	$>10^a$ 0.35^b	57
11	*N*-MeTyr-D-Ala-Gly-Phe-N(Me)(isoamyl)	$>30^a$ 0.4^b	57
12	Tyr-D-Ala-Gly-*N*-MePhe-NH(isoamyl)	$>30^a$ 0.2^b	57
16	Tyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$	1.4^a 0.003^b	57
17	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2$-NMe_2	2.3^a 0.07^b	57
18	Tyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$ ↓ O	0.32^a $<0.8^b$	57
19	*N*-MeTyr-D-Ala-Gly-*N*-MePhe-$NH(CH_2)_2NMe_2$ ↓ O	0.48^a 0.002^b	57
41	(Descarboxy-Nle)$^5SO_3H$	2^c 2^d	61
42	(Descarboxy-Nle)$^5PO_3H_2$	1^c 1^d	61
43	(Descarboxy-D-Nle)$^5SO_3H$	2^c 2^d	61
45	D-Ala^2, (descarboxy-Nle)$^5SO_3H$	2^c 2^d	61, 71
46	D-Ala^2, (descarboxy-Nle)$^5PO_3H_2$	15^c 2^d	61
47	D-Ala^2, (descarboxy-D-Nle)$^5SO_3H$	9.8^c 2^d	61, 71
49	D-Nle^2, (descarboxy-Nle)$^5SO_3H$	2^c 2^d	61, 71
50	D-Nle^2, (descarboxy-Nle)$^5PO_3H_2$	4.5^c 2^d	61
51	D-Nle^2, (descarboxy-D-Nle)$^5SO_3H$	2^c 2^d	61, 71
53	D-Met^2, (descarboxy-Nle)$^5SO_3H$	9.5^c 2^d	61, 71
54	D-Met^2, (descarboxy-Nle)$^5PO_3H_2$	25^c 2^d	61, 71
55	D-Met^2, (descarboxy-D-Nle)$^5SO_3H$	6.4^c 2^d	61, 71
56	D-Met^2, (descarboxy-D-Nle)$^5PO_3H_2$	52^c 16.8^d	61, 71
61	D-Ala^2-Met^5-NHEt	38.4^e 12.7^f	64
62	D-Ala^2-Met^5-NH(n-propyl)	142.8^e 209.8^f	64
63	D-Ala^2-Met^5-NH(isopropyl)	500^e 10.7^f	64

Table 4 *(cont.)*

Compound number	*Structure*	*Affinity*	*Ref.*
64	D-Ala^2-Met^5-NH(n-butyl)	0.6[e]	64
65	D-Ala^2-Met^5-NH(n-hexyl)	26.3[e]	64
		7[f]	
75	D-Met^2, Pro^5-NH$_2$	5923[g]	68, 88
76	D-Met^2, Leu^5-NH$_2$	125[g]	68, 88
77	D-Met^2, Met^5-NH$_2$	297[g]	68, 88
78	D-Met^2, L-Cha^4, Met^5-NH$_2$	82[g]	68, 88
79	D-Met^2, L-Cha^4, Leu^5-NH$_2$	51[g]	68, 88
80	D-Met^2, L-Cha^4, Pro^5-NH$_2$	1540[g]	68, 88
81	D-Ala^2, L-Cha^4, Pro^5-NH$_2$	358[g]	68, 88
120	D-Ala^2, Pro^5-NH$_2$	2960[g]	97
121	D-Ala^2, Nva^5-NH$_2$	132[g]	97
122	D-Met^2, Pro^5-NH$_2$	5920[g]	97
123	D-Met^2, Nva^5-NH$_2$	248[g]	97
124	L-Arg^2	670[h]	98
125	L-Arg^2, Met^5-NH$_2$	138[h]	98
126	D-Arg^2	49 700[h]	98
127	D-Arg^2, Met^5-NH$_2$	40 000[h]	98
128	L-Arg^2, Leu^5	233[h]	98
129	L-Arg^2, Leu^5-NH$_2$	177[h]	98
130	D-Arg^2, Leu^5	2471[h]	98
131	D-Arg, Leu^5-NH$_2$	7000[h]	98
132	D-Ala^2, des-Met^5-NH$_2$	5[i]	99
		5[j]	
133	D-Ala^2, des-Met^5-OEt	5[i]	99
		5[j]	
134	D-Ala^2, des-Met^5-NHNH$_2$	10[i]	99
		10[j]	
135	D-Ala^2, des-Met^5-NHOMe	<1[j]	99
136	D-Ala^2, des-Met^5-NH(n-pentyl)	<5[j]	99
137	D-Ala^2, des-Met^5-NHNHMe	2.5[j]	99
138	D-Ala^2, des-Met^5-NHNMe$_2$	<5[j]	99
139	D-Ala^2, des-Met^5-NHNHPh	<5[j]	99
140	D-Ala^2, des-Met^5-NHNHCOMe	50[j]	99
141—153	D-Ala^2, des-Met^5-NHNHCOR		
141	R = Et	50[i]	99
		50[j]	
142	R = Pr	50[i]	99
		50[j]	
143	R = n-butyl	50[i]	99
		50[j]	
144	R = n-pentyl	50[i]	99
		25[j]	
145	R = n-hexyl	10[j]	99
146	R = isobutyl	50[i]	99
		25[j]	
147	R = OEt	50[i]	99
		50[j]	
148	R = CH_2SMe	50[j]	99
149	R = $CH_2CH_2NH_2$	10[i]	99
150	R = CH=CHMe	10[j]	99
151	R = CH_2Ph	10[i]	99
152	R = $CH_2C_6H_4$-*p*-Me	10[i]	99
153	R = cyclopentyl	10[j]	99

Table 4 (*contd.*)

Compound number	*Structure*	*Activity*	*Ref.*
154	*N*-MeTyr^1, D-Ala^2, des-Met^5-NHNHCOEt	50[j]	99
155	D-N-MeAla^2, des-Met^5-NHNHCOEt	2.5[j]	99
156	D-Ala^2, Sar^3, des-Met^5-NHNHCOEt	$<<50$[j]	99
157	D-Ala^2, *N*-MePhe^4, des-Met^5-NHNHCOEt	100[j]	99
158	D-Ala^2, des-Met^5-N(Me)NHCOEt	5[j]	99
159	*N*-MeTyr^1, D-Ala^2, *N*-MePhe^4, des-Met^5-NHNHCOEt	200[j]	99
160	D-Leu^2, des-Met^5-NHNHCOMe	10[j]	99
161	D-Nva^2, des-Met^5-NHNHCOMe	50[j]	99
162	D-Met^2, des-Met^5-NHNHCOEt	50[j]	99
163	D-Phe^2, des-Met^5-NHNHCOMe	25[j]	99
164	D-Ser^2, des-Met^5-NHNHCOMe	25[j]	99
165	D-Thr^2, des-Met^5-NHNHCOMe	100[j]	99
166	D-Lys^2, des-Met^5-NHNHCOMe	25[j]	99
167	D-Arg^2, des-Met^5-NHNHCOMe	50[j]	99
168	D-Gln^2, des-Met^5-NHNHCOMe	100[j]	99
169	D-Glu(NHMe)^2, des-Met^5-NHNHCOEt	50[j]	99
170	D-Met(O)^2, des-Met^5-NHNHCOEt	400[j]	99
171	D-Met(O)^2, des-Met^5-$NHNH_2$	100[j]	99
172	D-Met(O)^2, des-Met^5-NHNHCOMe	200[j]	99
173	D-Met(O)^2, des-Met^5-NHNHCOPr	100[j]	99
174	D-Met(O)^2, *N*-MePhe^4, des-Met^5-NHNHCOEt	400[j]	99
175	*N*-MeTyr^1, D-Met(O)^2, des-Met^5-NHNHCOEt	200[j]	99
176	D-Met(O_2)^2, des-Met^5-NHNHCOEt	50[j]	99
177	D-Met(Me^+)^2, des-Met^5-NHNHCOEt	<50[j]	99
(b) Endorphin analogues			
VII	D-Ala^2-β_h-EP	5[k]	82
IX	Met(O)^5-β_h-EP	3[k]	82
X	Leu^5-β_h-EP	1[k]	82
XI	Aib^2-β_h-EP	1[k]	82
XII	Arg^0-β_h-EP	10[k]	82
XXXVI	β_h-EP-(1—27)	<2[k]	93
XXXVII	Gln^8-β_h-EP-(1—27)	12[k]	93
XXXVIII	AcTyr^1-β_h-EP-(1—27)	<2[k]	93
XXXIX	AcTyr^1, Gln^8-β_h-EP-(1—27)	<2[l]	93
XLIII	Phe^{27}, Gly^{31}-β_h-EP	119[k]	85
XLIV	D-Lys^9, Phe^{27}, Gly^{31}-β_h-EP	21[k]	85
XLV	D-Thr^2, D-Lys^9, Phe^{27}, Gly^{31}-β_h-EP	42[k]	85
XLVI	D-Phe^{18}, Phe^{27}, Gly^{31}-β_h-EP	23[k]	85
XLVII	D-Thr^2, D-Phe^{18}, Phe^{27}, Gly^{31}-β_h-EP	21[k]	85
L	Phe^{27}, Gly^{31}-β_h-EP	100[l]	103
LI	Ala^{17}, Phe^{27}, Gly^{31}-β_h-EP	68[l]	103
LII	Ala^{18}, Phe^{27}, Gly^{31}-β_h-EP	15[l]	103
LIII	Ala^{19}, Phe^{27}, Gly^{31}-β_h-EP	15[l]	103

[a] i.v. Rat tail flick. ED_{50} mg kg^{-1}. [b] i.v. Mouse stretch. ED_{50} mg kg^{-1}. [c] i.c.v. Rat tail flick, % potency relative to morphine. [d] i.v. Rat tail flick, % potency relative to morphine. [e] i.c.v. Rat hot plate, % potency relative to morphine, HCl. [f] i.p. Rat hot plate, % potency relative to morphine, HCl. [g] i.c.v. Mouse tail flick, % potency relative to morphine. [h] i.c.v. Mouse tail pinch, % potency relative to Met-enkephalin. [i] i.v. Mouse hot plate, % potency relative to morphine. [j] s.c. Mouse hot plate, % potency relative to morphine. [k] i.c.v. Mouse tail flick, % potency relative to β_h-EP. [l] i.c.v. Mouse tail flick, % potency relative to Phe^{27}, Gly^{31}-β_h-EP.

In the series [(8)—(19)] of 4-peptide amides studied by Morgan *et al.*[57] metabolic stabilization by *N*-methylation at various points increased the activity in the mouse-stretch test; high activity in the tail-flick test appeared only upon substitution of a dimethylaminoethylamide for the isoamylamide of the parent compounds. The most potent compounds resulted from oxidation of this tertiary amine function to the *N*-oxide; however, the increased activity was not reflected in the *in vitro* assays.

In accordance with the suggestion that the *C*-terminal acid group is important for 'enkephalin-like' activity but detrimental to analgesia, all of the analogues bearing phosphonic acid or sulphonic acid groups, described by Bajusz *et al.*,[61] are much less potent than morphine [(41)—(56)]. Further support for this postulate is evident in a correlation diagram relating the analgesic potencies of almost fifty analogues with their GPI-activities.[71] The most potent analgesics were found to be amides whose ratio of GPI- to MVD-potency was >1. In a similar way a good correlation between analgesic activity and affinity for the μ-receptor was observed for [(75)—(81)][60,68-74,88-91,97-99] Also, the substitution of Pro-NH_2 in the fifth position enhanced the analgesic potencies of several analogues without concomitant increase in their μ-receptor affinity.[68] A further investigation of this effect led to the observation that although the Nva^5 [(121), (123)] and Pro^5 [(120), (122)] peptides were equi-active *in vitro* the latter have *ca.* 20-fold higher activity *in vivo*.[97]

Inconsistencies were observed in the relative activities of the alkylamide derivatives (61)—(65).[64] Whereas (61) had the highest *in vitro* activity, (62) was the most potent analgesic by the i.p. route but (63) by the i.c.v. route. Potent analgesic activity relative to Met-enkephalin was found in the series of analogues containing kyotorphin-like Arg^2 substitutions [(124)—(131)].[98] The same group also studied similar variations of the Tyr-Arg sequence alone.[100] In both cases the analogues with D-Arg^2 were found to have the highest anti-nociceptive activity.

In a report whose length totally belies the extent of the effort involved Fujino *et al.*[99] have described the structure–activity relationships for hot-plate analgesic potency in a series of 45 tetrapeptide hydrazide derivatives [(132)—(177)]. Initially, high activity was detected with the acyl-hydrazide, (140). Systematic variation eventually led to analogues (170) and (174), which were found to be 4-times as potent as morphine and apparently more potent than the Sandoz compound FK 33-824.

Kastin *et al.*[101] found that in the mouse tail-flick test, analogues containing a pentafluoro-Phe^4 residue were potent analgesics. Generally, compounds designed to be more lipophilic or to possess additional binding sites (dimerization) were shown to be highly active after peripheral administration. The pharmacological activities, after i.c.v. administration, of a series of enkephalin oligomers (n = 2 to 6) were investigated by Munekata *et al.*[102]

[97] Y. Audigier, R. Gout, H. Mazarguil, and J. Cros, *Eur. J. Pharmacol.*, 1980, **64**, 187.
[98] M. Kubota, O. Nagase, H. Amano, H. Tagaki, and H. Yajima, *Chem. Pharm. Bull.*, 1980, **28**, 2580.
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[100] H. Yajima, H. Ogawa, H. Ueda, and H. Takagi, *Chem. Pharm. Bull.*, 1980, **28**, 1935.
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[102] E. Munekata, H. Ishiyama, F. Higa, T. Ohtaki, and K. Izumi, *Pept. Chem.*, 1979, **17**, 209.

The few endorphin analogues tested for analgesic activity all showed markedly reduced potencies relative to human β-endorphin [(VIII), (IX)—(XII), (XXXVI)—(XXXIX), (XLIII)—(XLVII), (LI)—(LIII)].[83, 85, 93, 103] Li's group[104] have shown that the β-endorphin fragments β_c-EP(6—31) and β_c-EP(20—31) inhibit the analgesia induced by morphine or β_h-endorphin in the mouse tail-flick test. In contrast, β_h-EP(1—5)-(16—31) inhibited only morphine induced analgesia but showed marked behavioural and toxic effects at higher doses; the amino-terminal peptide, β_h-EP(1—15), showed no inhibitory effects. The results were discussed in terms of the postulate that the enkephalin sequence of β-endorphin occupies an enkephalin receptor whilst the carboxy-terminal sequence occupies the morphine receptor and elicits analgesia.

Other Biological Activities and Receptor Studies.—Reports of the isolation of opioid peptides from the adrenal medulla coupled with the observation that enkephalin analogues can affect ACTH and cortisol levels *in vivo* prompted studies by Racz *et al.*,[105] which demonstrated that the enkephalins inhibit adrenal corticosteroid biosynthesis. Wei and co-workers have suggested[106] that the cardiovascular effects elicited by enkephalins and β-casomorphin may provide a useful bioassay for the rapid detection and estimation of *in vivo* pharmacological activities of new opioid peptides. Studies of opiate-like peptides in bovine splenic nerve preparations led Wilson *et al.*[107] to suggest that these peptides may act as co-transmitters in noradrenergic vesicles of sympathetic nerves. Nicoll and colleagues[108] observed that a stable enkephalin analogue markedly attenuated various GABA-ergic inhibitory pathways in the CNS of vertebrates without affecting the action of GABA itself. It has been suggested that the naloxone-reversible, antihypertensive effects produced by α-adrenergic receptor activators, such as clonidine, may be mediated by release of β-endorphin.[109] Tolerance develops rapidly to the hypothermia induced by β-endorphin; cross-tolerance with the enkephalins was also demonstrated.[110]

Goldstein's group[111] have compared the behavioural effects initiated by administration of dynorphin, morphine, or β_c-endorphin directly to the brain of rats. High doses of dynorphin elicited 'bizarre postures with limb-rigidity and barrel-rolling'; these effects were not naloxone reversible. Katz[112] observed similar effects at high doses in mice and also found that lower doses increased the likelihood of feeding or grooming behaviour. Friedman *et al.*[113] have suggested that dynorphin may act as a modulator of the analgesia induced by morphine or β-

[103] J. Blake, L.-F. Tseng, and C. H. Li, *Int. J. Pept. Protein Res.*, 1980, **15**, 167.
[104] N. M. Lee, H. J. Friedman, L. Leybin, T. M. Cho, H. H. Loh, and C. H. Li, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 5525.
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[110] J. P. Huidobro and E. L. Way, *Eur. J. Pharmacol.*, 1980, **65**, 221.
[111] B. H. Herman, F. Leslie, and A. Goldstein, *Life Sci.*, 1980, **27**, 883.
[112] R. J. Katz, *Neuropharmacology*, 1980, **19**, 801.
[113] H. J. Friedman, M.-F. Jen, J. K. Chang, N. M. Lee, and H. H. Loh, *Eur. J. Pharmacol.*, 1981, **69**, 357.

endorphin in the rat. The behavioural and e.e.g. effects of the Sandoz enkephalin analogue FK 33-824 were investigated by Bo *et al.*[114] Bloom and co-workers[115] have commented upon the developmental, cellular, and behavioural effects of the endorphins. Fasting in the rat was reported to reduce β-endorphin levels in the hypothalamus but not in the pituitary.[116] Also the endorphins were found to alter the acquisition and consolidation of an inhibitory avoidance response in the rat.[117]

Morley and Wei[118] have shown that, in contrast to an earlier claim, [D-Met^2, Cha^4, Pro^5]-enkephalinamide does give rise to physical dependence. Wei[119,120] has also investigated structure–activity relationships with respect to the addictive liability of other synthetic enkephalin analogues. Bhargava[121] found that two enkephalin analogues were more potent than morphine in their effect on the abstinence responses of morphine-dependent mice.

Several groups are actively investigating the opiate receptor at the cellular level. Zukin *et al.*[122] have prepared labelled enkephalin–macromolecule complexes using [^{125}I]-FK 33-824 followed by crosslinking with dimethylsubermididate and also by direct crosslinking using [^{125}I]-[D-Ala^2, Leu^5, Lys^6]-enkephalin. SDS-Gel electrophoresis separated three major radioactive bands from the former complex but only one from the latter. In an extension of their work on neuroblastoma cell suspensions,[123] Hazum and colleagues[124] have reported that treatment of cells adhering to cover slips with either agonists or antagonists induces clustering of opiate receptors. The nature of the clusters induced by agonists differs from that induced by antagonists in that only the former may be dispersed with dithiothreitol. Pretreatment with thiol-reagents prevents clustering but does not alter the ability of agonists to inhibit adenylate cyclase. Further evidence that opiate receptor function may be modulated by a thiol–disulphide redox mechanism was adduced by Marzullo and Hine.[125] Intracerebroventricular administration of cupric ion induces naloxone-reversible analgesia in mice. The molar potency of Cu^{II} was similar to that of morphine and the effect of either agent was antagonized by dithiothreitol.

The use of an autoradiographic procedure developed by Young and Kuhar[126] enabled Goodman and co-workers[127] to distinguish μ- and δ-opiate receptors after labelling with [^{125}I]-FK 33-824 and [^{125}I]-[D-Ala^2,-D-Leu^5]-enkephalin, respectively. These studies led the authors to propose that discrete physiological effects may be induced by Met-enkephalin at μ-receptors and by Leu-enkephalin

[114] P. Bo, M. Maurelli, G. Nappi, and F. Savoldi, *Il Farmaco, Ed. Sc.*, 1980, **35**, 924.
[115] F. Bloom, A. Bayon, E. Battenberg, E. French, L. Koda, G. Koob, M. Lemoal, J. Rossier, and W. Shoemaker, ref. 16, p. 619.
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[127] R. R. Goodman, S. H. Snyder, M. J. Kuhar, and W. S. Young III, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 6329.

at δ-receptors. Kachur *et al.*[128] have reported a very clear differentiation of the effects mediated by opiate receptors in the guinea-pig intestine. Electrolyte secretion appears to be under the control of a δ-opiate receptor whereas opioid effects on the smooth muscle are mediated by a μ-receptor.

Conformation Studies.—Roques and colleagues[129] have carried out a comprehensive investigation of the solution conformations adopted by [D-Met^2, Pro^5]-enkephalin and [D-Met^2, D-Pro^5]-enkephalinamide. Theoretical calculations and a variety of n.m.r. techniques were applied and resulted in the proposal that, despite the absence of a classical β-turn, the peptides exhibit folded structures induced by their high content of hydrophobic residues. The relative populations of the conformers with *cis* and *trans* geometry at the Phe^4-Pro^5 bond were also determined. The data were found to be consistent with an earlier proposal of the active conformation at the μ-receptor. The conformations of the above compounds and some related analogues have also been studied by circular dichroism.[130, 131] The peptides were found to form stable complexes with divalent cations, a property which may have some bearing on receptor activity.[131] ^{15}N-n.m.r. spectroscopy has been applied to conformational analysis using the *N*-terminal tetrapeptide of enkephalin as a model compound.[132] Manavalan and Momany[133] have calculated the low energy conformers for a series of biologically active peptide analogues including two enkephalins. Of the many low energy conformers obtained one was found to be common for both of these peptides with respect to their side-chain orientations.

Clinical Studies with Enkephalins and Endorphins.—Most clinical studies of the effects of the enkephalins were carried out with the Sandoz analogue FK 33-824. Two separate investigations[134, 135] in human volunteers have shown that this peptide inhibits the secretion of vasopressin but stimulates free water clearance and prolactin secretion. Not all of these effects were antagonized by naloxone. Further studies of the endocrine effects of FK 33-824 confirmed these results and showed, in addition, that the release of growth hormone was enhanced while cortisol levels were significantly lowered.[136, 137] Plasma pituitary hormone responses to FK 33-824 in normal subjects and those with pituitary disease were investigated by Demura *et al.*[138] A possibly hopeful sign for the future development of the opiate peptides came from a report that heorin addicts when offered a choice of FK 33-824 or morphine chose the latter in preference[139] and asserted that the peptide evoked an 'intoxication without euphoria'. A second enkephalin analogue has now reached the clinical trial stage, in this case specifically as an

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analgesic agent with less addictive liability or tendency to cause respiratory depression than the classical opiates.[140]

Studies of the possible antipsychotic properties of des-Tyr^1-γ-endorphin have continued.[141, 142] It is apparent that responses to the peptide vary markedly and are dependent upon the specific subtype of schizophrenic psychosis involved. Pickar and colleagues[143] have reported on the behavioural and other effects of acute β-endorphin injections in subjects suffering from schizophrenia or depression. The varied responses of the patients rendered the overall study somewhat inconclusive.

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[142] H. M. Emrich, M. Zandig, D. van Zerssen, A. Herz, and W. Kissling, *Lancet*, 1980, 1364.
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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 in the area during the years 1979 and 1980. As in our previous reviews we have tried to satisfy the requirements of both organic and inorganic chemists with interests in this field. Biological inorganic chemistry is a substantial growth area, and more and more inorganic chemists are becoming interested in the interaction of metal ions with biologically important molecules such as amino-acids, peptides, proteins, and nucleic acids.

A variety of books and review articles have appeared: 'Synthetic Oxygen Carriers Related to Biological Systems',[1] 'The Chemistry of Biological Manganese'[2] (including a discussion of amino-acid complexes), 'Blue-Copper Proteins: Nuclear Magnetic Resonance Investigations',[3] 'Coordination Catalysis: The Activation of Coordinated Amino Acids and Related Ligands',[4] a book 'Biological Aspects of Inorganic Chemistry'[5] which contains a number of valuable review articles. A useful book on 'Molybdenum and Molybdenum Containing Enzymes' has also appeared.[6]

The first issue of the Specialist Periodical Report on Inorganic Biochemistry has been published,[7] which contains a chapter on metal complexes of amino-acids and peptides. A book 'Metal Complexes in Organic Chemistry'[8] also contains much material relevant to the amino-acid and peptide area. A further book 'New Trends in Bio-Inorganic Chemistry'[9] contains much valuable material. A number of other useful texts have appeared.[10-17, 18]

Volumes 8 and 9 of Sigel's 'Metal Ions in Biological Systems' have been

[1] R. D. Jones, D. A. Summerville, and F. Basolo, *Chem. Rev.*, 1979, **79**, 139.
[2] G. D. Lawrance and D. T. Sawyer, *Coord. Chem. Rev.*, 1978, **27**, 173.
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[5] 'Biological Aspects of Inorganic Chemistry', ed. A. W. Addison, W. R. Cullen, D. Dolphin, and B. R. James, Wiley-Interscience, 1977.
[6] 'Molybdenum and Molybdenum Containing Enzymes', ed. M. P. Coughlan, Pergamon Press, New York, 1980.
[7] 'Inorganic Biochemistry', ed. H. A. O. Hill (Specialist Periodical Reports), The Chemical Society, London, 1979, Vol. 1.
[8] 'Metal Complexes in Organic Chemistry', R. P. Houghton, Cambridge University Press, London, 1979.
[9] 'New Trends in Bio-Inorganic Chemistry', ed. R. J. P. Williams and J. R. F. F. Da Silva, Academic Press, London, 1979.
[10] 'Superoxide and Superoxide Dismutases', ed. A. M. Michelson, J. M. McCord, and I. Fridovich, Academic Press, London, 1977.
[11] 'Progress in Inorganic Chemistry', Vol. 25, ed. S. J. Lippert, John Wiley, Chichester, 1979.

published.[19] Volume 9 is concerned with amino-acids and derivatives as ambivalent ligands. The new 'Advances in Inorganic Biochemistry'[14] is the first in a series of volumes designed to update and supplement the original two-volume treatise, 'Inorganic Biochemistry' (edited by G. L. Eichhorn) which was published in 1973.

Other review articles of interest are 'Caeruloplasmin: The Enigmatic Copper Protein',[20] 'Structure and Function of Copper Proteins',[21] 'Iron–Sulphur Centres of the Chloroplast Membrane',[22] and 'Some Aspects of the Bioinorganic Chemistry of Molybdenum'.[23] Vallee and Wacker[24] have produced a detailed listing of all the characterized metalloproteins and metalloenzymes with information regarding their molecular weight, metal stoicheiometry, source, and function with appropriate references.

2 Amino-acids

Equilibrium Studies.—Solution equilibrium studies between metal ions and amino-acid ligands continue to attract considerable attention. There have been a number of attempts to model physiological conditions. Thus equilibrium analysis of a model system for the *in vivo* reaction between penicillamine and Cu^{I}, the penicillamine–glutathione–Cu^{I} system, indicates that in a certain concentration range the use of penicillamine as a drug will not disturb the normal Cu^{I} metabolism.[25] Complex formation between D-penicillamine (Pen) and copper(II) has been studied under simulated physiological conditions in the presence and absence of the blood plasma constituents albumin, alanine, histidine, and zinc(II).[26] The major species formed at neutral pH and 0.15 mol dm^{-3} NaCl is shown to have the same stoicheiometry as the recently reported[27] solid-state complex $[Cu^{I}_{8}Cu^{II}_{6}(Pen)_{12}Cl]^{5-}$. A potentiometric investigation of the zinc(II)–histamine ternary complexes with Cys, His, Glu, Thr, and citrate has also appeared[28] as a model for histamine as a ligand in blood plasma.

[12] 'Organometals and Organometalloids, Occurrence and Fate in the Environment', ed. F. E. Brinckman and J. M. Bellama, A.C.S. Symposium Series 82, A.C.S. Washington, 1978. Contains a review article by A. J. Carty on mercury, lead, and cadmium complexation by sulphhydryl-containing amino-acids.

[13] 'Methods for Determining Metal Ion Environments in Proteins. Structure and Function of Metalloproteins', ed. D. W. Darnall and R. G. Wilkins, Elsevier–North Holland, New York, 1980.

[14] 'Advances in Inorganic Biochemistry', Vol. 1, ed. G. L. Eichhorn and L. G. Marzilli, Elsevier–North Holland, New York, 1980.

[15] 'Zinc and Copper in Clinical Medicine', ed. K. M. Hambidge and B. L. Nichols, SP Medical and Scientific Books, London, 1978.

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[19] 'Metal Ions in Biological Systems', ed. H. Sigel, Vols. 8 and 9, Marcel Dekker, New York, 1979.

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L-Cysteinatogold(I) has been prepared by the reaction of L-Cys with $KAuBr_4$ in acidic solution. The solubility at pH 7.4 and 37 °C is 1 μmol dm^{-3}, but the solubility increases in the presence of excess cysteine due to the formation of bis(L-cysteinato)gold(I).[29] There has been increasing interest in the use of gold drugs and the topic has been reviewed.[30]

Binary and ternary complexes of D-Pen and L-Cys with nickel(II) and zinc(II) have been studied[31] and polynuclear species detected. Potentiometric and spectrophotometric investigations of the nickel(II)–D-penicillamine have been published.[32] Formation of mixed valence complexes of copper with L-Cys and its derivatives (L-CysOMe, *N*-acetyl-L-Cys, and glutathione) has been investigated.[33]

The solution structure and equilibria of vanadium(V), molybdenum(VI), and tungsten(VI) complexes of EDTA, ethylenediamine-*NN'*-diacetic acid, and nitrilotriacetic and iminodiacetic acids have been studied potentiometrically and spectrophotometrically.[34] Polarographic investigations[35] of uranyl complexes with potentially bidentate α-, β-, and γ-amino-acids indicate unidentate carboxylate co-ordination in the complexes. The amino-group is not involved in co-ordination.

Metal complexes from plants have been identified in a number of cases. Recently iron has been found as the citrate,[36] nickel(II) in malic and malonic complexes,[37] and zinc as the galacturonate.[38] Evidence for the possible occurrence of a copper–proline complex from the roots of *Armeria maritima* has now been presented.[39]

Since lead(II) is of current environmental interest a number of potentiometric studies of lead(II) complexes of amino-acids have appeared.[40,41]

Many studies of ternary complexes are now being published; these include the ternary copper(II)–L-histidine–diglycyl-L-histidine system,[42] copper(II)–amino-acids with thiodicarboxylic and pyridine dicarboxylic acids,[43] copper(II)–bipyridyl with histamine and glycine,[44] copper(II)–glycylsarcosine–amino-acids,[45] binary and ternary complexes of copper(II) involving imidazole, histamine, and L-histidine,[46] copper(II) or zinc(II) with bipyridyl or phenanthroline with amino-acids,[47] and copper(II) with some amino-acids and malonate.[48] Other similar

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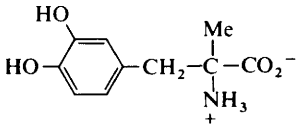
studies include the copper(II)–histidine–amino-acid system in 20 v/v% dioxane–water,[49] binary and ternary complexes of various metal ions, nucleoside 5′-monophosphates and amino-acids,[50] and mixed ligand complexes of *O*-phospho-DL-serine.[51]

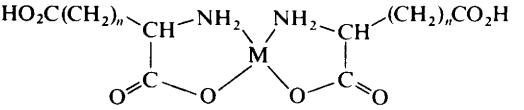
Heterobinuclear copper(II)–L-histidine complexes with nickel(II), zinc(II), and cadmium(II) in aqueous solution have been investigated potentiometrically;[52] $[CuNi(His)_2]^{2+}$ is the most stable species.

The interaction of cadmium(II)[53] and zinc(II)[54] with glycine, *N*-acetyl-, and *N*-benzoyl-glycine in aqueous and ethanolic solution has been studied polarographically. Potentiometric and spectrophotometric studies of copper(II) complexes of methyldopa (1), methyltyrosine, and catechol have been made,[55] and the equilibrium constants of the parent complexes of nickel(II), copper(II), and zinc(II) with dopamine and the mixed ligand complexes with alanine or pyrocatechol as the second ligand determined.[56] The interaction of some α-L-glutamic acid oligomers and polymers with copper(II) and aqueous solution has been studied in detail.[57]



(1)

Under appropriate conditions, α-amino-dicarboxylic acids act as bidentate ligands with platinum group cations. A variety of Pt^{II} and Pd^{II} complexes of type (2) have been prepared,[58] and the pK_a values for the carboxy-group ionization obtained. Approximate formation constants are also reported.



(2) M = Pt^{II} or Pd^{II}
$n = 1, 2, 3$, or 4

The species distribution and relevant formation constants for iron(III)–glycine hydroxamate have been determined by analytical potentiometry.[59] These results in conjunction with magnetic susceptibility measurements establish that polymeric species are absent at physiological pH values.

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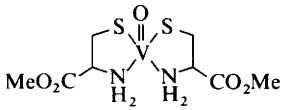
Synthetic and Spectroscopic Studies.—The synthesis of amino-acid complexes of various metals continues apace. There has been a marked change in emphasis to complexes of the first half of the first transition series, and to complexes of the second and third transition series.

A recent topic of interest in bioinorganic chemistry has been the chemistry of vanadium. Bis(methylcysteinato)oxovanadium(IV) (3) has now been characterized as a purple solid.[60] The i.r. and Raman spectra show two strong split absorptions at 956 and 945 cm^{-1} due to the vanadyl ($V = 0$) group suggesting the presence of both *cis*- and *trans*-isomers.



(3)

Tetrakis-μ-acetatodirhodium(II), $[Rh_2(O_2CMe)_4]$, exhibits anticancer activity against many types of tumour. The reaction of amino-acids containing free sulphydryl groups with $[Rh_2(O_2CMe)_4]$ gives monomeric square planar rhodium(II) (S,N) bonded complexes, but the *S*-acylated ligands lead to the formation of 1 : 2 adducts without breakdown of the acetato cage.[61]

The reaction of $Mo(OH)_3$ with $C_2O_4^{2-}$ and the amino-acids α-Ala, β-Phe, and 4-hydroxyproline gives the complexes $[Mo_2OC_2O_4(AA)_2(H_2O)_4].xH_2O$ (AA = amino-acid anion). The binuclear complexes contain the μ-oxo-μ-oxalato-dimolybdenum(III) group.[62] Mixed amino-acid–thiocyanato complexes of dimolybdenum(II) of composition $Mo_2[O_2CCH(R)NH_3]_2(NCS)_4.nH_2O$ have been isolated using glycinate and L-isoleucinate and their crystal structures determined.[63]

Dioxouranium(IV) complexes of L-arginine have been prepared and studied by i.r. and ^1H n.m.r.[64] N.m.r. and spectroscopic studies of the interaction of UO_2^{2+} with aspartic acid and asparagine have also been made.[65] Interactions of La^{III}, Nd^{III}, and Lu^{III} with aspartic acid and asparagine have also been investigated.[66]

There has been considerable interest in complexes of palladium(II) and platinum(II) with amino-acids. Papers in this area have dealt with rotational isomerism in palladium(II) complexes with *S*-methyl-L-cysteine and its derivatives,[67] n.m.r. and *X*-ray studies of Pd^{II} and Pt^{II} complexes with *S*-methyl-L-cysteine sulphoxide,[68] the reaction of the methyl cysteinate complex (4) with nucleosides,[69] and n.m.r.

[60] H. Sakurai, Y. Yamada, S. Shimomura, and S. Yamashita, *Inorg. Chim. Acta*, 1980, **46**, L119.
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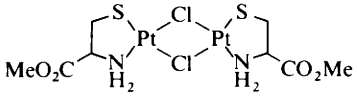
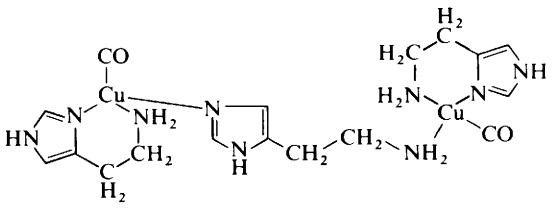
studies of ligand–ligand interactions in the ternary palladium(II)–L-cysteate–L-threoninate system in aqueous solution.[70] Chelate ring closure and cleavage reactions in bis- and tris(glycinato) complexes of *cis*-dimethylplatinum(IV)[71] and the preparation, isomerization, exchange, and substitution reactions of mono(glycinato) complexes of *cis*-dimethylplatinum(IV)[72] have also been investigated.



(4)

The antibacterial, antiviral, and antitumoural effects of platinum and palladium compounds are well documented; however, exposure to these compounds may lead to 'platinosis' which manifests itself in the form of dermatitis and asthmoid conditions, as a result of the histamine-liberating action of platinum compounds. The histamine-liberating and histamine-binding action of platinum and palladium compounds has now been studied.[73]

The copper(I)–histamine system has been found to act as a reversible CO carrier.[74] *X*-Ray analysis of $[Cu_2(\text{histamine})_3(CO)_3](BPh_4)_2$ establishes the structure (5). A detailed account of this work has now been published.[75]



(5)

A variety of studies have appeared dealing with metal complexes of *N*-acetyl amino-acids; these include complexes of cobalt(II), nickel(II), and zinc(II) with *N*-acetyl-DL-leucine,[76] copper(II) complexes of *N*-acetyl-DL-valine and their amine adducts,[77] bis(*N*-acetyl-DL-leucinato)copper(II) complexes and their amine adducts,[78] and bis(*N*-acetyl-DL-tryptophanato)copper(II) and its amine adducts.[79] This latter paper also described the crystal structure of diaquabis(*N*-acetyl-DL-tryptophanato)bis(pyridine)copper(II).

[70] A. Odani and O. Yamamauchi, *Inorg. Chim. Acta*, 1980, **46**, L63.
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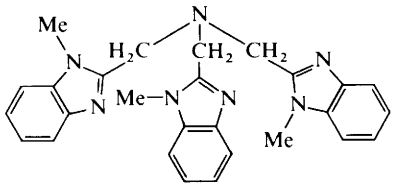
Evidence for an imidazolate bridged Fe^{II}–Cu^{II} complex in solution has been obtained[80] and a binuclear ruthenium(III)–histidine complex has been prepared in which the two nitrogens of the imidazole ring bridge the two metal ions (6).[81]

(6)

The charge transfer absorptions of Cu^{II}–imidazole and Cu^{II}–imidazolate chromophores have been studied in detail.[82] New copper(II) dimers bridged by the imidazolato ligand have been described.[83] The bridged Cu–Im–Zn unit is believed to occur in bovine superoxide dismutase and the Fe–Im–Cu unit in cytochrome *c* oxidase[84, 85] (ImH = imidazole; Im^- = imidazolate).

The copper(II) bridged dimers were prepared[83] using the ligand tris[2(*N*-methyl)benzimidazolymethyl]amine [L = (7)].

Addition of the ligand to $Cu(ClO_4)_2 . 6H_2O$ in methanol followed by sodium imidazolate gives the complexes $[Cu_2(Im)L_2](X)_3$ (X = ClO_4,BF_4, or NO_3). New synthetic procedures have been described to prepare Pt^{II} and Pd^{II} complexes with imidazole as a ligand.[86]



(7)

Ternary copper(II) complexes [Cu(His)(AA)] where His is L- or D-histidine and AA is L- or D-Asn, L-Gln, L-Ser, L-homoserine, or L-citrulline have been crystallized and their properties studied, including the determination of formation constants.[87]

The complex $[Co(D\text{-}Pyala)_2](NO_3) . 0.5H_2O$ has been prepared by reaction of Co^{II} or Co^{III} with D-$NH_2CH(CH_2C_5H_4N)CO_2^-$ (D-$Pyala^-$), the tridentate analogue of histidine.[88] *X*-Ray work established that of the three possible isomeric forms, the most stable was that in which the carboxylate groups of the two ligands are mutually *trans*. This structure contrasts with the predominant isomer of $Co(L\text{-}His)_2^+$, in which the imidazole groups are mutually *trans*. The complex $[Ni(D\text{-}Pyala)_2] . 2H_2O$ has also been characterized[89] and in this case the amino-nitrogens are mutually *trans*.

[80] D. Kovacs and R. E. Shepherd, *J. Inorg. Biochem.*, 1979, **10**, 67.
[81] R. Gulka and S. S. Isied, *Inorg. Chem.*, 1980, **19**, 2842.
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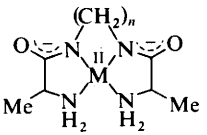
[88] S. R. Ebner, R. A. Jacobsen, and R. J. Angelici, *Inorg. Chem.*, 1979, **18**, 765.
[89] S. R. Ebner, B. J. Helland, R. A. Jacobsen, and R. J. Angelici, *Inorg. Chem.*, 1980, **19**, 175.

A number of Pd^{II} and Pt^{II} complexes of amino-acids and dipeptides have been isolated and characterized and their chiroptical properties studied.[90] A variety of complexes of Pd^{II} and Pt^{II} with cysteine and methyl cysteinate have also been prepared;[91] the co-ordination sites depend strongly on the pH of the reaction mixture and the metal to ligand ratio employed.

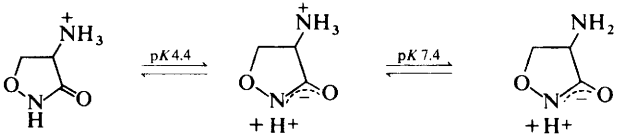
Trimethylplatinum(IV) complexes of Ala, Val, Phe, and α-aminoisobutyric acid, $PtMe_3(AA)L$, L = MeOH, 3,5-lutidine, and $[PtMe_3(AA)_2]^-$ have been prepared and their ^1H n.m.r. studied.[92] ^1H and ^{13}C n.m.r. and i.r. studies of Pd^{II} and Pt^{II} complexes with *S*-methyl-L-cysteine have also been reported.[93] ^1H, ^{13}C, and ^{195}Pt n.m.r. studies on diastereoisomeric Pt^{II} complexes of prochiral olefins containing a chiral amino-acid ligand have been published.[94]

Interesting square planar complexes of the ML type where M = Pd^{II}, Ni^{II}, or Cu^{II} and L = *N,N'*-bis(L-alanyl)-propane-1,3-diaminate dianion or the *N,N'*-bis(L-alanyl)-ethane-1,2-diaminate anion (8) have been synthesized by Komorita and Shimura[95] and their c.d. spectra studied. Dianionic ligands of this type (similar to deprotonated peptides) are expected to stabilize Cu^{III} and Ni^{III}.



(8) $n = 2$ or 3

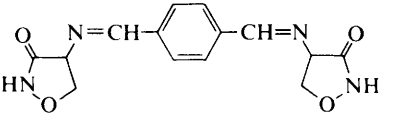
The antibiotics are known to interact with metal ions and the suggestion that the antibiotic action of the tetracyclines may be related to their metal binding ability has stimulated much activity.[96] Copper(II) is known to promote the hydrolysis of the β-lactam ring of penicillins.[96] The antibiotic cycloserine, (4-amino-3-isoxazolidone) (9), forms complexes with Pd^{II}, Pt^{II}, and Cu^{II}, which have now been



(9)

crystallized and their physical properties studied.[96] The ligand is always monodentate but through different donor sites; nickel(II), copper(II), zinc(II), and cadmium(II) halide complexes of the cycloserine derivative 1,4-bis(3-oxy-4-isoxazolidinyliminomethyl)benzene ('Terizidone') (10) have also been studied.[97]

[90] E. A. Sullivan, *Can. J. Chem.*, 1979, **57**, 62.
[91] G. Pneumatakis and H. Hadjiliadis, *J. Inorg. Nucl. Chem.*, 1979, **41**, 429.
[92] T. G. Appleton, J. R. Hall, and T. G. Jones, *Inorg. Chim. Acta*, 1979, **32**, 127.
[93] B. Jezowska-Trzebiatowska, A. Allain, and H. Kozlowski, *Inorg. Nucl. Chem. Lett.*, 1979, **15**, 279.
[94] S. Shinoda, Y. Yamaguchi, and Y. Saito, *Inorg. Chem.*, 1979, **18**, 673.
[95] T. Komorita and Y. Shimura, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 1832.
[96] C. Preti and G. Tosi, *J. Coord. Chem.*, 1979, **9**, 125 and references therein.
[97] C. Preti and G. Tosi, *J. Coord. Chem.*, 1980, **10**, 209.

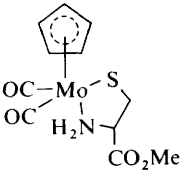


(10)

The interaction of copper(II) with methionine methyl ester in solution has been investigated by a variety of spectroscopic techniques and evidence for Cu–SMe bonding found from ^{1}H n.m.r. measurements.[98]

The co-ordination of the thioether sulphur of methionine to iron in cytochrome *c*[99] and to copper in plastocyanin[100] has been established. ^{13}C n.m.r. studies of Hg^{II} binding to *S*-carboxymethyl-L-cysteine and *S*-methyl-L-cysteine have been carried out as a model system for thioether co-ordination in metalloproteins.[101] Reactions of triorganotin(IV) compounds with L-cysteine, L-cysteine ethyl ester, *N*-acetyl L-cysteine, and reduced glutathione have been studied since triorganotin(IV) compounds are potent inhibitors of mitochondrial oxidative phosphorylation.[102]

The synthesis and stereochemistry of $C_5H_5(CO)_2Mo$ complexes of cysteine methyl ester (11) have also been described.[103]



(11)

Mixed valence complexes of copper with L-cysteine and its derivatives (L-cysteine methyl ester and *N*-acetyl-L-cysteine) have been characterized.[104]

The *X*-ray photoelectron spectra of copper complexes considered as models for metalloproteins containing copper–sulphur bonds have been measured.[105]

The synthesis of salts of Λ- and Δ-*N,S*-[$Co(en)_2$(*R*)cysteinato]$^{n+}$ and [$Co(en)_2$(*S*)penicillaminato]$^{n+}$ has been described, and *X*-ray analysis of the cysteinato-derivatives carried out.[106] A further paper[107] deals with the synthesis and *X*-ray crystal structure of Λ-*N,S*-[$Co(en)_2$(*S*)*S*-methyl(*R*)cysteinato](NCS)$_2$ (13) prepared by reaction of (12) with MeI. Sulphur bonding of the sulphide is confirmed and the chiral *S*-methyl centre is stereospecifically orientated. Rearrangement of the *N,S* to the *N,O* bond does not occur under acidic conditions.

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[99] N. Mandel, G. Mandel, B. L. Trus, J. Rosenberg, G. Carlson, and R. E. Dickerson, *J. Biol. Chem.*, 1977, **252**, 4619.
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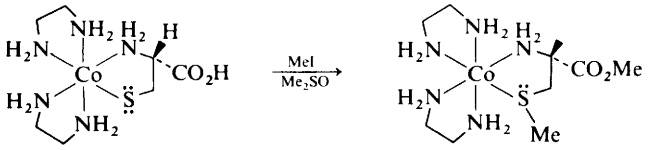
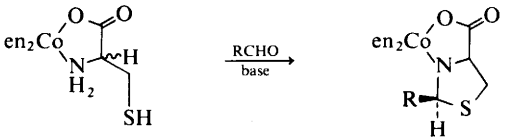
[104] I. Sóvágó, B. Harman, and A. Gergely, *Inorg. Chim. Acta*, 1980, **46**, L107.
[105] R. A. Walton, *Inorg. Chem.*, 1980, **19**, 1100.
[106] H. C. Freeman, C. J. Moore, W. G. Jackson, and A. M. Sargeson, *Inorg. Chem.*, 1978, **17**, 3513.
[107] G. J. Gainsford, W. G. Jackson, and A. M. Sargeson, *J. Chem. Soc., Chem. Commun.*, 1979, 802.



(12) (13)

The co-ordinated sulphur in the thiolato complexes $[Co(en)_2(SCH_2CH_2NH_2)]^{2+}$ and $[Co(en)_2(SCH_2CO_2)]^{+}$ is capable of ligating soft metal centres such as Ag^{I} and $MeHg^{+}$ so that inner sphere electron transfer can occur between appropriate metal centres.[108]

Ring closure reactions of the type shown in (14) → (15) may be used to prepare chiral thiazolidines. The *N,O* mode of bonding of the thiazolidine-4-carboxylate ligand has now been confirmed by *X*-ray analysis.[109] The synthesis of a 2-aminothiazolinecobalt(III) complex derived from (*R*)-cysteine has also been described.[110]



(14) (15)

Threonine can be synthesized in >95% yields using the base catalysed condensation of acetaldeyde with copper(II) glycinate,[111] and the formation of glycine in the hydrolysis of co-ordinated cyanogen has been observed.[112] The heterogeneous photosynthetic production of amino-acids from CH_4–NH_3–H_2O in the presence of Pt–TiO_2 has been described.[113] The results demonstrate the heterogeneous photosynthesis of amino-acids under irradiation with visible and near u.v. light.

Silver(I) complexes of *N*-acetyl and *N*-benzoyl derivatives of variety of amino-acids have been characterized,[114] and cobalt(II), nickell(II), and zinc(II) complexes of *N*-acetyl-DL-tryptophane (and their amine adducts) prepared.[115] A large number of complexes of (2,2′-bipyridyl)copper(II) and (1,10-phenanthroline)copper(II) with amino-acids have been prepared,[116] as have (2,2′-bipyridyl)copper(II) complexes of iminodiacetate and pyridine-2,6-dicarboxylate.[117]

Other synthetic papers include: the preparation and characterization of some mixed ligand complexes of chromium(III) nitrilotriacetate with amino-acids;[118] the

[108] M. J. Heeg, R. C. Elder, and E. Deutsch, *Inorg. Chem.*, 1979, **18**, 2036.
[109] G. J. Gainsford, W. G. Jackson, A. M. Sargeson, and A. D. Watson, *Aust. J. Chem.*, 1980, **33**, 1213.
[110] G. J. Gainsford, W. G. Jackson, and A. M. Sargeson, *J. Am. Chem. Soc.*, 1979, **101**, 3966.
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[113] H. Reiche and A. J. Baird, *J. Am. Chem. Soc.*, 1979, **101**, 3127.
[114] L. Antolini, L. Menabue, M. Saladini, and P. Morini, *Inorg. Chim. Acta*, 1980, **46**, L77.
[115] G. Marcotrigiano, L. Antolini, L. Menabue, and G. C. Pellacani, *Inorg. Chim. Acta*, 1979, **35**, 177.
[116] W. L. Kwik, K. P. Ang, and G. Chen, *J. Inorg. Nucl. Chem.*, 1980, **42**, 303.
[117] G. Nardin, L. Randaccio, R. P. Bonomo, and E. Rizzarelli, *J. Chem. Soc., Dalton Trans.*, 1980, 369.
[118] C. L. Sharma, P. K. Jain, and T. K. De, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1681.

synthesis and structure of diaquo-tetra-μ,β-alaninatodirhodium(II) tetraperchlorate dihydrate;[119] synthesis and i.r. spectra of metal complexes with pyridoxamine and pyridoxine;[120] synthesis, Mössbauer, and i.r. studies of inorganic tin derivatives of amino-acids;[121] polymorphs of tetrakis(glycine)tetrachlorodimolybdenum(II);[122] amino-acid dicarbonylrhodium(I) complexes;[123] the *cis* and *trans* isomers of bis(glycinato)copper(II) and their thermal isomerization;[124] ethylenediamine-*N,N'*-diacetato complexes of chromium(III);[125] the preparation and hydrolysis reactions of *trans*-$[Co(en)_2Cl\{O_2CCH(R)NH_2\}]^+$ complexes containing the carboxylato-bonded amino-acids glycine, DL-alanine, and DL-aminobutyric acid;[126] the preparation and characterization of *uns-cis*-trimethylenediamine-*N,N'*-diacetatocobalt(III) complexes with several amino-acids;[127] mixed cobalt(III) complexes with L-methioninate or *S*-methyl-L-cysteinate and L- or D-aspartate;[128] the preparation and isomerization of isomers of the L- or D-aspartato(L-histidinato)cobalt(III) complex;[129] the preparation and ^{13}C n.m.r. spectra of the cobalt(III) complexes containing β-alanine and glycine;[130] and the formation of $Ti(O_2)(edta)^{2-}$ by the addition of O_2 to $Ti(edta)(H_2O)^-$.[131] The crystal structure of $[Cr(edda)(OH_2)]_2 . 4H_2O$ containing a di-μ-hydroxo-bridge has been determined.[132]

Additional spectroscopic studies include the following: magnetic studies of two new copper hippurate dimers;[133] circularly polarized luminescence studies of the ternary complexes formed between terbium(III) pyridine-2,6-dicarboxylate and amino-acids;[134] triplet state properties of the methylmercury(II)–tyrosine complex;[135] the proton n.m.r. of nickel(II) and cobalt(II) complexes with potentially tridentate amino-acids;[136] and an investigation of the parameters affecting the stability of nitrosyl cobalt complexes with amino-acids.[137] Difficulties in detecting nickel amino-acid complexes in plants by chromatographic techniques have been the subject of comment.[138]

Diffraction Studies.—D-Penicillamine promotes the urinary excretion of copper in patients with Wilson's disease. It has previously been claimed that under simulated physiological conditions D-penicillamine and copper form a polynuclear, anionic,

[119] A. M. Dennis, R. A. Howard, J. L. Bear, J. D. Korp, and I. Bernal, *Inorg. Chim. Acta*, 1979, **37**, L561.
[120] T. A. Franklin and M. F. Richardson, *Inorg. Chim. Acta*, 1980, **46**, 191.
[121] P. A. Cusack, P. J. Smith, and J. D. Donaldson, *Inorg. Chim. Acta*, 1980, **46**, L73.
[122] A. Bino, F. A. Cotton, and P. E. Fanwick, *Inorg. Chem.*, 1979, **18**, 1719.
[123] Z. Nagy-Magos, P. Kvintovics, and L. Marko, *Transition Met. Chem.*, 1980, **5**, 186.
[124] B. W. Delf, R. D. Gillard, and P. O'Brien, *J. Chem. Soc., Dalton Trans.*, 1979, 1301.
[125] D. S. Veselinovic, D. J. Radanovic, and S. A. Grujic, *Inorg. Nucl. Chem. Lett.*, 1980, **16**, 211.
[126] K. B. Nolan and A. A. Soudi, *J. Chem. Soc., Dalton Trans.*, 1979, 1419.
[127] M. Okaybayashi, K. Igi, and J. Hidaka, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 753.
[128] T. Isago, K. Igi, and J. Hidaka, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 407.
[129] M. Watabe, H. Yano, and S. Yoshikawa, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 61.
[130] T. Ama and T. Yasui, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 79.
[131] F. J. Kristine and R. E. Shepherd, *J. Chem. Soc., Chem. Commun.*, 1980, 132.
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[135] M. V. Hershberger and A. H. Maki, *J. Inorg. Biochem.*, 1980, **13**, 273.
[136] B. Jezowska-Trzebiatowska and L. Latos-Grazynski, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1079.
[137] B. Jezowska-Trzebiatowska, K. Geraga, and G. Formicka-Kozlowska, *Inorg. Nucl. Chem. Lett.*, 1980, **16**, 563.
[138] M. E. Farago, I. Mahmood, and A. J. Clark, *Inorg. Nucl. Chem. Lett.*, 1980, **16**, 481.

mixed valence $Cu^{I}Cu^{II}$ complex. Similar complexes of D-penicillamine with metals other than copper and with combinations of different metals have now been characterized.[139] Diamagnetic cluster compounds of composition $[M^{I}_{8}M^{II}_{6}\{SC(Me)_2CH(NH_2)CO_2\}_{12}Cl]^{5-}$ with $M^{I}_{8}M^{II}_{6} = Cu^{I}_{8}Ni^{II}_{6}$, $Ag^{I}_{8}Ni^{II}_{6}$, or $Ag^{I}_{8}Pd^{II}_{6}$ have been shown to be structurally related to the well characterized $Cu^{I}_{8}Cu^{II}_{6}$ compound so that the M^{I} atoms are trigonally co-ordinated by three sulphur atoms and the M^{III} atoms are planar four-co-ordinate by two sulphur and two nitrogen atoms.

The crystal structure of silver(I) imidazole perchlorate[140] establishes the presence of a planar Ag^{I}_{6} cluster, each silver ion carrying two linearly co-ordinated imidazole ligands. The novel polymeric glycine complex $\{[Cu^{II}_{2}(\overset{+}{N}H_3\text{-}CH_2CO_2^{-})_4][Cu^{I}_{2}Cl_6]\}_{12}$ has been obtained from $CuCl_2 . 2H_2O$ and glycine (1 : 2 mole ratio) at pH ~ 2, and its crystal structure determined.[141]

The crystal structures of $Cu(L\text{-}Leu)_2$ and bis(DL-2-aminobutyrato)copper(II) have been established.[142] They contain tetragonally co-ordinated copper(II) arranged in isolated sheets. Equatorial N_2O_2 ligation is provided by *trans* co-ordination of two amino-acids, and axial Cu—O ligation by two neighbouring amino-acids.

The anti-tumour activity of *cis*-$[Pt(NH_3)_2Cl_2]$ and some related complexes has stimulated interest in amino-acid complexes. The reaction of *cis*-$[PtL_2]$ (where L = Gly or Ala) gives, on reaction with oxalic acid in water, crystals of stoicheiometry $Pt_2L_4(H_2C_2O_4).2H_2O$, which in the case of glycine have been shown to be $[Pt(NH_2CH_2CO_2)].2H_2C_2O_4.2H_2O$ by *X*-ray work.[143] The structure consists of *cis*-$[Pt(Gly)_2]$ and $H_2C_2O_4.2H_2O$ linked by H-bonds, and explains why the compound has a similar activity against L-1210 leukaemia to *cis*-$[Pt(Gly)_2]$.

X-Ray evidence has been presented for a metal–aromatic ring interaction in bis(L-tyrosinato)palladium(II).[144] The crystal structure of binuclear copper(II)–*N*-acetylglycinate monohydrate, $[Cu(O_2CCH_2NHCOMe)_2(H_2O)]_2$, establishes that the two copper atoms are held together in a dimeric unit by the four carboxylate groups and that each copper atom is further bonded to a water molecule resulting in square pyramidal geometry.[145] L-Asparaginato-L-histidinatocopper(II) and L-asparaginato-L-histidinatoaquacopper(II) trihydrate have been crystallized and their crystal structures determined.[146]

Sekizaka[147] has established the crystal structure *fac*(*N*)-Δ-tris(L-asparaginato)cobalt(III) trihydrate.

Stereochemistry and Stereoselectivity.—Stereoselective binding of D- or L-α-amino-acids by copper(II) complexes of *N*-benzenesulphonyl-L-α-phenylalanine

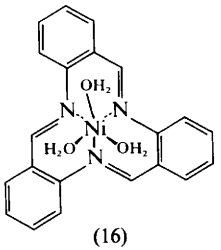
[139] P. J. M. W. L. Birker, *J. Chem. Soc., Chem. Commun.*, 1980, 946.
[140] G. W. Eastland, M. A. Mazid, D. R. Russell, and M. C. R. Symons, *J. Chem. Soc., Dalton Trans.*, 1980, 1682.
[141] T. Glowiak and H. Kozlowski, *Inorg. Chim. Acta*, 1980, **46**, L65.
[142] T. G. Fawcett, M. Ushay, J. P. Rose, R. A. Lalancette, J. A. Potenza, and H. J. Schugar, *Inorg. Chem.*, 1979, **18**, 327.
[143] M. A. A. F. de C. T. Carrondo, D. M. L. Goodgame, C. R. Hadjioannou, and A. C. Skapsie, *Inorg. Chim. Acta*, 1980, **46**, L32.
[144] M. Sabat, M. Jezowska, and H. Kozlowski, *Inorg. Chim. Acta*, 1979, **37**, L511.
[145] M. R. Udupa and B. Krebs, *Inorg. Chim. Acta*, 1979, **37**, 1.
[146] T. Ono, H. Shimanouchi, Y. Sasada, T. Sakurai, O. Yamauchi, and A. Nakahara, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2229.
[147] M. Sekizaki, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 403.

has been studied potentiometrically[148] and related to the previously reported optical resolution of DL-α-amino-acids by ligand exchange chromatography. Resolution of DL-valine, DL-phenylalanine, and DL-proline by ligand exchange chromatography has been achieved by using polyacrylamide resins containing the L-proline–copper(II) complex as a stationary phase.[149] Chromatographic elutions of DL-phenylalanine, DL-proline, and DL-histidine have been made on a copper-loaded polystyrene resin containing L-proline groups.[150] Enantiomers of DL-Phe were not separated, but DL-Pro and DL-His were completely resolved. Formation constants of the ternary complexes copper(II)–*N*-benzyl-L-proline and the D- or L-amino-acid were determined and used to rationalize the chromatographic results.

The reversed phase chromatographic resolution of amino-acids using copper(II) and zinc(II) complexes of the dipeptide L-aspartyl-L-phenylalanine methyl ester ('aspartame') as eluants has been described.[151] In this case a chiral reagent is introduced into the mobile phase rather than into the stationary phase. The diastereoisomeric ternary complexes so formed elute at different rates.

The *fac*-isomer of $[Co(\beta\text{-Ala})_3]$ has been completely resolved into its enantiomers on a column of the Na form of CM-Sephadex cation exchanger using Na_2-*d*-tartrate as eluant; Yamazaki and Yoneda[152] have now described the chromatographic resolution of the *mer*-isomer.

It has been observed that the tridentate Schiff base complex of nickel(II), triaquotribenzo[*b*,*f*,*j*][1.5.9]triazacyclodecine nickle(II), $[Ni(TRI)(OH_2)_3]^{2+}$ (16), displays substantial stereoselectivity on complexing with several amino-acids.[153]



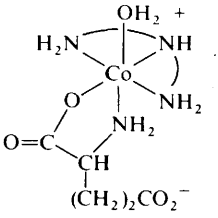
(16)

This stereoselectivity provides a convenient method for the resolution of $[Ni(TRI)(OH_2)_3]^{2+}$ using histidine as a resolving agent and either ion exchange or perchlorate salt crystallization techniques. The resolved $[Ni(TRI)(OH_2)_3]^{2+}$ may then be used to resolve other amino-acids or as a sensitive test of the stereochemistry of an amino-acid. The test can be carried out with milligram quantities. The amino-acid is readily released by adjusting the pH to *ca.* 2, and the general procedure has been tested with histidine, tyrosine, methionine, and phenylglycine.

When glutamic acid co-ordinates in $[Co(dien)(S)\text{-Glu}]^+$ as a tridentate ligand forming five- and seven-membered chelate rings the dien ligand must adopt a *fac* configuration, which is less preferred than the *mer* configuration. Recent re-

[148] D. Muller, J. Jozefonvicz, and M. A. Petit, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1665.
[149] D. Muller, J. Jozefonvicz, and M. A. Petit, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1083.
[150] J. Jozefonvicz, D. Muller, and M. A. Petit, *J. Chem. Soc., Dalton Trans.*, 1980, 76.
[151] C. Gilon, R. Leshem, Y. Tapuhi, and E. Grushka, *J. Am. Chem. Soc.*, 1979, **101**, 7612.
[152] S. Yamazaki and H. Yoneda, *Inorg. Nucl. Chem. Lett.*, 1979, **15**, 195.
[153] B. Erno and R. B. Jordan, *Can. J. Chem.*, 1979, **57**, 883.

sults[154] establish that (*S*)-Glu behaves as a bidentate ligand with a pendant $-(CH_2)_2CO_2^-$ group giving the structure (17) in which the axial site is occupied by water and the dien ligand is *mer*.



(17)

The [(2*S*,2*S*'-1,1'-trimethylenedipyrrolidine-2,2'-dicarboxylato)](α-aminocarboxylato)cobalt(III) complexes have been prepared and characterized using (*R*)- or (*S*)-Ala, (*R*)- or (*S*)-Val, sarcosinate, and a variety of other ligands. The Λ-*asym-mer* configuration is preferred.[156]

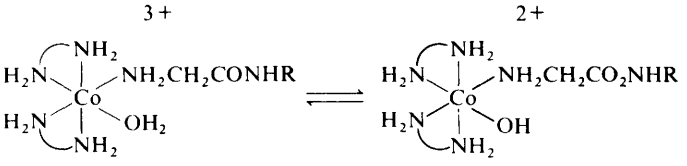
N-Methylalaninatocobalt(III) complexes with tetramines (2,3,2-tet and trien) have been prepared and their detailed stereochemistry studied.[156] Some dimeric molybdenum(V) complexes containing optically active amino-acid ligands have been prepared and their chiroptical properties investigated.[157]

Intermolecular energy transfer from terbium(III) to europium(III) complexes of aspartic acid appears to be subject to stereoselectivity.[158] Complexes of the racemic ligand are more efficient than those of the resolved ligands in energy transfer.[158]

A conformational study of double carboxylic bridges in bis(DL-α-alaninate)manganese(II) dibromide dihydrate and some related complexes has been published.[159]

Reactivity and Kinetics.—A number of interesting papers have appeared in this area. Buckingham and co-workers[160] have continued their work on the intramolecular hydrolysis of glycinamide and glycine dipeptides co-ordinated to cobalt(III). The intramolecular addition of cobalt(III) bound water or hydroxide ion to glycinamide, glycylglycine isopropyl ester, and glycylglycine in the complexes (18) and (19) has been studied in detail. For the dipeptide complex ($R = CH_2CO_2C_3H_7$) both the aqua and hydroxo-species form $[Co(en)_2(Gly)]^{2+}$. Oxygen exchange and glycinate ring opening in $[Co(en)_2(Gly)]^{2+}$ have also been investigated.[161] Although ester hydrolysis of $[Co(NH_3)_5NH_2CH_2CO_2Et]^{3+}$ has a half life in 1M CF_3SO_3H at 25 °C in excess of 1 month, the reaction for the corresponding Ru^{III} complex under the same conditions is complete in 1 h.[162] The

[154] F. Jursik, B. Hájek, and M. S. Abdel-Moez, *Inorg. Chim. Acta*, 1979, **33**, L123.
[155] M. Okabayashi, K. Okamoto, and J. Hidaka, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2257.
[156] M. Yamaguchi, S. Yano, M. Saburi, and S. Yoshikawa, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 691.
[157] K. Z. Suzuki, Y. Sasaki, S. Ooi, and K. Saito, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 1288.
[158] H. G. Brittain, *Inorg. Chem.*, 1979, **18**, 1740.
[159] Z. Ciunik and T. Glowiak, *Inorg. Chim. Acta*, 1980, **44**, L249.
[160] C. J. Boreham, D. A. Buckingham, and F. R. Keene, *J. Am. Chem. Soc.*, 1979, **101**, 1409.
[161] C. J. Boreham and D. A. Buckingham, *Aust. J. Chem.*, 1980, **33**, 27.
[162] A. Yeh and H. Taube, *J. Am. Chem. Soc.*, 1980, **102**, 4725.



(18) (19)

R = H, $CH_2CO_2C_3H_7$, or $CH_2CO_2^-$

products in the latter case are ($[(NH_3)_5RuOH_2]^{3+} + \overset{+}{N}H_3CH_2CO_2Et$) (30%) and ($[(NH_3)_5RuO_2CCH_2NH_3]^{3+}$ + EtOH) (70%).

Amino-acid esters interact with [glycylglycinato(2−)]copper(II) to give ternary complexes. Base hydrolysis of the ester ligands (GlyOMe, GlyOEt, L-α-AlaOEt, or L-PheOEt) is some 50-times faster than for the free esters.[163] Hydrolysis of α-amino-acid esters in ternary complexes with copper(II)–ethylenediaminemonoacetate has also been studied.[164] Nucleophilic attack by both water and hydroxide ion was observed and base hydrolysis is some 10^3-times faster than for the free esters. The kinetics of base hydrolysis of the ester ligand in bis(ethylcysteinato)palladium(II) have also been studied in detail.[165]

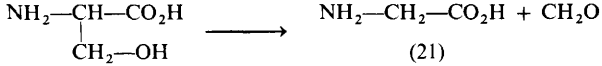
The reaction of mercury(II) acetate with polypeptides has been found to result in the selective binding of two atoms of mercury to each tyrosine and histidine residue. For a polymer containing tyrosine the mercuration kinetics follow the scheme:

$$Hg(OAc)_2 + \text{tyrosine} \xrightarrow{k_1} \text{Hg.tyrosine}$$

$$Hg(OAc)_2 + \text{Hg.tyrosine} \xrightarrow{k_2} Hg_2\text{.tyrosine}$$

At 60 °C, $k_1k_2^{-1} = 5.5$.[166] Chemical analysis of the products of the reactions of $Hg(OAc)_2$ with tyrosine amide, L(−)-histidine, and the methyl ester of L-(−)-histidine has established that mercuration results in the formation of a Hg—C bond at the C-3 and C-5 sites of the phenolic ring of tyrosine and the C-4 site in the imidazole ring of histidine. The site of the second mercury retained by histidine is uncertain but does not involve the amine functions of the imidazole ring.

Bis(L-serinato)copper(II) has been shown to exhibit serine aldolase reactivity.[167] Heating copper(II) and L-serine (1 : 2) at pH ~ 11 gave some glycine indicating the dealdolization reaction (20) → (21). Formation of ammonia and considerable racemization of the recovered serine established that oxidative deamination and racemization competed with dealdolization.



(20) (21)

The second-order rate constants (rate = k[complex][OD^-]) have been determined for the deuterium exchange of α-methylene or α-methine protons in a

[163] R. W. Hay and P. Banerjee, *J. Chem. Soc., Dalton Trans.*, 1980, 2385.
[164] R. W. Hay and P. Banerjee, *J. Chem. Soc., Dalton Trans.*, 1980, 2452.
[165] R. W. Hay and P. Banerjee, *Inorg. Chim. Acta*, 1980, **44**, L205.
[166] A. P. Korn, F. P. Ottensmeyer, and T. R. Jack, *J. Inorg. Biochem.*, 1979, **10**, 235.
[167] L. Casella, *Inorg. Chim. Acta*, 1981, **55**, L9.

variety of cobalt(III) complexes containing amino-acid ligands.[168] The major factors determining the deuteriation rate of α-hydrogens in the aminocarboxylato-chelate are the geometry and charge of the complex, and the nature of the substituent bonded to the chelate ring to be deuteriated.

The kinetics of complexation of a number of metal ions with amino-acid ligands have been studied; these studies include zinc(II) with the glycine zwitterion,[169] nickel(II) and cobalt(II) with *O*-phospho-DL-serine,[170] and histidine derivatives and glycine by a tridentate nickel(II) complex.[171]

The reaction of $[Cr(ox)_2(Gly)]^{2+}$ with $C_2O_4^{2-}$ has been studied kinetically[172] and the importance of ion pairing in the base hydrolysis of the $(+)_{589}$-*cis*-$[Co(en)_2X(Gly)]^+$ ions (X = Cl or Br) discussed in detail.[173]

The acid catalysed decarboxylation of β-$(+)_{436}$-β_1-[(2*S*,9*S*)-2,9-diamino-4,7-diazadecanecobalt(III)-*S*-aminomethylmalonate]$ClO_4.H_2O$ and Λ-$(-)_{436}$-β_2-[triethylenetetraminecobalt(III)-(*R*)-aminomethylmalonate]ClO_4 each lead to unequal amounts of (*R*)- and (*S*)-alanine products.[174] A systematic dependence on steric bulk is evident since decarboxylation of a series of 2,9-dimethyl substituted triethylenetetraminecobalt(III)aminomethylmalonate complexes leads to an excess of (*S*)-alanine over (*R*)-alanine, which varies from 10 to 30%.

The kinetics of formation of $[(NH_3)_5RhO_2CCH_2NH_3]^{3+}$ from $[Rh(NH_3)_5OH_2]^{3+}$ and glycine in weakly acidic media have been investigated over a temperature range ($\Delta H^{\ddagger} = 22.2\,kcal\,mol^{-1}$, $\Delta S^{\ddagger} = -9.2\,e.u.$).[175]

The bonding of nitric oxide to bis(histidinato)cobalt(II) was observed as early as 1961. These reactions can be summarized by the reaction (22) → (23) where AA is the amino-acid anion.

$$\underset{(22)}{[Co^{II}(AA)_2(H_2O)_n]} + NO \rightleftharpoons \underset{(23)}{[Co^{III}(AA)_2NO(H_2O)_{n-1}]} + H_2O$$

The effects of various amino-acids and of amino-acids and histidine on the above equilibrium have now been studied in detail.[176]

While at least four metals are known to be involved in various superoxide dismutases, the mammalian enzyme, which has a copper atom at the active site, and a zinc atom that is apparently not required for activity, has been most widely studied. The mechanism for catalytic dismutation is considered to be:

$$\text{enzyme–}Cu^{II} + O_2^- \longrightarrow \text{enzyme–}Cu^{I} + O_2$$

$$\text{enzyme–}Cu^{I} + O_2^- + 2H^+ \longrightarrow \text{enzyme–}Cu^{II} + H_2O_2$$

The complex $[CuHis_2H]^{3+}$ catalyses the disproportionation of O_2^- in the pH range 1—10, with $k = 3.4 \times 10^8\,M^{-1}\,s^{-1}$ in the pH range 2—7.[177] Two possible

[168] T. Ama, H. Kawaguchi, M. Kanekiyo, and T. Yasui, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 956.
[169] S. Harada, Y. Uchida, M. Hiraishi, H. L. Kuo, and T. Yasunaga, *Inorg. Chem.*, 1978, **17**, 3371.
[170] T. M. Che and K. Kustin, *J. Inorg. Biochem.*, 1980, **13**, 267.
[171] R. B. Jordon and B. E. Erno, *Inorg. Chem.*, 1979, **18**, 2895.
[172] T. W. Kallen and R. E. Hamm, *Inorg. Chem.*, 1979, **18**, 2151.
[173] C. J. Boreham, D. A. Buckingham, and C. R. Clark, *Inorg. Chem.*, 1979, **18**, 1990.
[174] R. Job, *Inorg. Chim. Acta*, 1980, **40**, 59.
[175] C. Chatterjee and A. K. Basak, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2710.
[176] B. Jezowska-Trzebiatowska, K. Geraga, and G. Formicka-Kozlowska, *Inorg. Chim. Acta*, 1980, **40**, 187.
[177] J. Weinstein and B. H. J. Bielski, *J. Am. Chem. Soc.*, 1980, **102**, 4916.

mechanisms are considered, one involving a transient superoxide complex of $[CuHis_2H]^{3+}$.

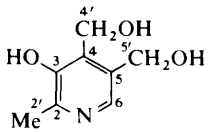
The reaction of di-μ-oxo-bis{oxo[L-(+)-cysteinato]molybdate(v)}$^{2-}$ with O_2 has been studied in detail[178] and the kinetics of dissociation of histamine from pentacyano(histamine)ferrate(II) have been investigated.[179]

Schiff Bases.—Currently there seems to be a marked resurgence of interest in this area. The glycine residue in *N*-salicylideneglycyl-L-valinatocopper(II) reacts with formaldehyde in aqueous solution at pH 8.5. Decomposition of the complex with H_2S at pH 2 gives seryl-L-valine containing optically active serine.[180] Little work has been reported on the formation of Schiff base complexes of thiol-containing amino-acids; some copper(II) and zinc(II) complexes of Schiff bases derived from penicillamine and salicylaldehyde or 2-hydroxy-l-naphthaldehyde have now been characterized.[181]

X-Ray structural analyses of two metal complexes of *O*-phospho-DL-threonine-pyridoxal Schiff base $[Ni(C_{12}H_{15}N_2O_8P)(H_2O)_3].2H_2O$ and $[Cu(C_{12}H_{15}N_2O_8P)(H_2O)].H_2O$ establish that the nickel(II) complex is a monomer with the octahedral nickel ion bonded to the tridentate Schiff base and three water molecules.[182] The copper(II) complex is a dimer with square pyramidal copper bonded to the tetradentate Schiff base involving a phosphate oxygen atom from a neighbouring molecule, and one water molecule.

A number of papers have appeared dealing with complexes of vitamin B_6. These include the interaction of pyridoxal with pyridoxamine in the presence of copper(II),[183] the kinetics and mechanism of ternary complex formation of iron(III) with picolinic acid and pyridoxal,[184] and a potentiometric study of ternary complexes of cobalt(II), nickel(II), and copper(II) involving pyridoxamine and the amino-acids Gly, DL-Ala, DL-Val, and β-Phe.[185]

A ^{13}C n.m.r. study confirms that co-ordination of metal ions by pyridoxine (24) is through the C-3 and C-4′ oxygens in aqueous solution. Nitrogen appears to become a more effective donor site in water–dimethylsulphoxamide mixtures.[186]



(24)

Differential reactivity of the α-methylene protons of bis(pyridoxylideneglycinato)cobalt(III) towards deuterium exchange has been observed.[187] Dunathan[188] has previously suggested that selective catalysis of cleavage of a bond of

[178] T. M. Tam and J. H. Swinehart, *Inorg. Chem.*, 1979, **18**, 975.
[179] N. E. Katz, M. E. Garcia Posse, and M. A. Martinez, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1782.
[180] S. Suzuki, H. Narita, and K. Harada, *J. Chem. Soc., Chem. Commun.*, 1979, 29.
[181] L. Macdonald, D. H. Brown, and W. E. Smith, *Inorg. Chim. Acta*, 1979, **33**, L183.
[182] K. Aoki and H. Yamazaki, *J. Chem. Soc., Chem. Commun.*, 1980, 363.
[183] M. S. El-Ezaby and N. El-Shatti, *J. Inorg. Biochem.*, 1979, **10**, 169.
[184] M. A. El-Dessouky, M. S. El-Ezaby, and N. M. Shuaib, *Inorg. Chim. Acta*, 1980, **46**, 7.
[185] M. S. El-Ezaby, H. M. Marafie, and S. Fareed, *J. Inorg. Biochem.*, 1979, **11**, 317.
[186] J. S. Hartman and E. C. Kelusky, *Can. J. Chem.*, 1979, **57**, 2118.
[187] J. R. Fischer and E. H. Abbott, *J. Am. Chem. Soc.*, 1979, **101**, 2781.
[188] H. C. Dunathan, *Proc. Natl. Acad. Sci. U.S.A.*, 1966, **55**, 712.

an amino-acid α-carbon atom can be accomplished by correctly orientating that bond with respect to the π-system of the azomethine group, and the above results are consistent with this view.

The syntheses of several iron(III) complexes of pyridoxylideneglycine (PLgly) and 5′-phosphopyridoxylideneglycine glycine (PLPgly) have been described.[189] The compounds Fe(PLgly)(H_2O)X (X = Cl, Br, or OAc) and Fe(PLPgly)(H_2O)(OAc) are five co-ordinate. Infrared spectra indicate that the ligands are co-ordinated to iron(III) *via* their azomethine nitrogen, phenolic oxygen, and carboxylic oxygen donor atoms. The *X*-ray structure of chloro-(*N*-pyridoxylidene-*N*′-salicycloylhydrazinato)copper(II) monohydrate has been determined.[190] Tautomeric and carbanion mechanisms for the racemization of L-alanine induced by pyruvate and zinc(II) have been considered.[191]

Copper(II) complexes with tridentate Schiff bases derived by the condensation of (+)-(hydroxymethylene)camphor or (+)-(hydroxymethylene)menthone with a series of (*S*)- and (*R*)-amino-acids have been synthesized.[192] Little interaction between the various chiral centres was found and the conformation of the chelate rings depends mainly on the configuration of the α-carbon atom of the amino-acid.

Cobalt(III) complexes of Schiff bases derived from 1-menthyl-3-(*o*-hydroxybenzoyl)propionate or cholesteryl-3-(*o*-hydroxybenzoyl)propionate and the amino-acids Ala, Val, Leu, and Phe have been prepared in solution and their chiroptical properties studied.[193]

3 Peptides

Structural Aspects.—Currently there are more publications dealing with the structural aspects of metal peptides than with their reactivity. Copper(II)–peptide systems are still the most extensively studied.

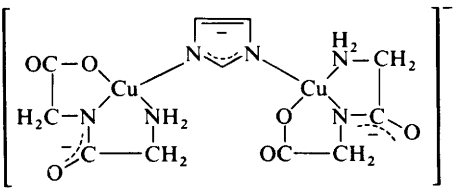
The polyaspartic acid–copper(II) system has been studied by c.d. and potentiometric techniques and a six-membered chelate ring shown to occur.[194] The magnetic properties of the unique nitrogen bridged dimer sodium(glycylglycylglycinato)cuprate(II) monohydrate has been investigated over a temperature range.[195] The synthesis, crystal structure, and electronic properties of (L-methionylglycinato)copper(II) have been reported.[196] The dipeptide acts as a pentadentate ligand and the copper is square pyramidal.

Bis[*cyclo*(-L-histidyl-L-histidyl-)]copper(II) perchlorate tetrahydrate has been prepared and its crystal structure determined.[197] In this case the copper(II) is not planar but distorted tetrahedral. N.m.r. and c.d. spectra of aqueous solutions of the complex indicate the presence of two 13-membered chelate rings.[198]

[189] J. T. Wrobleski and G. J. Long, *Inorg. Chim. Acta*, 1979, **36**, 155.
[190] P. Domiano, A. Musatti, M. Nardelli, C. Pelizzi, and G. Predieri, *Transition Met. Chem.*, 1979, **4**, 351.
[191] A. Dempsey and D. A. Phipps, *Inorg. Chim. Acta*, 1979, **36**, L425.
[192] L. Casella, M. Gullotti, A. Passini, and A. Rockenbauer, *Inorg. Chem.*, 1979, **18**, 2825.
[193] H. Okawa, Y. Numata, A. Mio, and S. Kida, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2248.
[194] A. Garnier, L. Musoni, and L. Tosi, *J. Inorg. Biochem.*, 1980, **13**, 23.
[195] W. E. Estes, C. Webster Andrews III, J. R. Wasson, and W. E. Hatfield, *Inorg. Chem.*, 1978, **17**, 3664.
[196] J. Dehand, J. Jordanov, F. Keck, A. Mosset, J. J. Bonnet, and J. Galy, *Inorg. Chem.*, 1979, **18**, 1543.
[197] F. Hori, Y. Kojima, K. Matsumoto, S. Ooi, and H. Juroya, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 1076.
[198] Y. Kojima, *Transition Met. Chem.*, 1979, **4**, 269.

The interaction of copper(II) with L-carnosine (β-alanyl-L-histidine) has been studied by e.s.r. techniques as a function of pH, temperature, and metal to ligand stoicheiometry.[199] Four different complexes were found to occur.

The ternary systems formed by copper(II) glycylsarcosine and amino-acids have been studied and formation constants obtained.[200] A biologically active peptide known as the 'throtropin releasing factor' (in fact a synthetic tripeptide of L-pyroglutamyl-L-histidyl-L-prolinamide and one of its dipeptide analogues) has been treated with copper(II) and the equilibria have been studied by spectrophotometry and circular dichroism.[201] The crystal and molecular structure of tetra-μ-*N*-acetylglycinatodiaquodicopper(II) has been determined by three-dimensional *X*-ray diffraction. It is reported that the two copper atoms in the molecule are held together in a dimeric unit by the four carboxylate groups and that each copper atom is further bonded to a water molecule resulting in a square pyramidal geometry.[202] Another important paper discusses imidazolate-bridged binuclear copper(II) complexes of tripeptides.[203] These complexes are useful models for the active site of bovine erythrocyte superoxide dismutase. This paper takes issue with the previously reported structure for this bridged copper complex, and a new structure (25) is proposed.



(25)

The absorption, c.d., and resonance Raman spectra of copper(II)–poly(L-glutamic, L-tyrosine) complexes have been used to provide evidence for phenolate co-ordination.[204] The contribution of even minor species to paramagnetic line broadening in copper(II)–peptide complexes has been discussed at length.[205] The preparation and characterization of glycyl-L-methioninatocopper(II) have been reported and crystallographic data recorded.[206] Polarographic studies of the oxidized glutathione–copper(II) system in basic solution have suggested a mechanism for the hydrolysis of the disulphide bonds, an important reaction in biological systems.[207]

The crystal structure and spectroscopic properties of a violet glutathione–copper(II) complex having axial sulphur co-ordination and two copper sites *via* a disulphide bridge have been reported in great detail.[208]

[199] C. E. Brown, W. E. Antholine, and W. Froncisz, *J. Chem. Soc., Dalton Trans.*, 1980, 590.
[200] B. R. Arbad, D. N. Shelke, and D. V. Jahagirdar, *Inorg. Chim. Acta*, 1980, **46**, L17.
[201] G. Formicka-Kozlowska, H. Kozlowski, B. Jezowska-Trzebiatowska, G. Kupryszewski, and J. Przybylski, *Inorg. Nucl. Chem. Lett.*, 1979, **15**, 387.
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[203] W. Mori, A. Nakahara, and Y. Nakao, *Inorg. Chim. Acta*, 1979, **37**, L507.
[204] J. M. Pastor, A. Garnier, and L. Tosi, *Inorg. Chim. Acta*, 1979, **37**, L549.
[205] Y. Kuroda and H. Aiba, *J. Am. Chem. Soc.*, 1979, **101**, 6837.
[206] L. Abello, A. Ensuque, A. Demaret, and G. Lapluye, *Transition Met. Chem.*, 1980, **5**, 120.
[207] M. Ostern, J. Pelczar, H. Kozlowski, and B. Jezowsak-Trzebiatowska, *Inorg. Nucl. Chem. Lett.*, 1980, **16**, 251.
[208] K. Miyoshi, Y. Sugiura, K. Ishizu, Y. Iitaka, and H. Nakamura, *J. Am. Chem. Soc.*, 1980, **12**, 6130.

Similarly, the detailed co-ordination chemistry of (glycylglycinato)(7,9-dimethylhypoxanthine)copper(II) tetrahydrate has been reported.[209] Mixed ligand complexes between copper(II) and imidazole and dipeptides derived from glycine, alanine, and leucine have been studied by potentiometric means and distribution diagrams for the mixed ligand hydroxy-complexes and ternary complexes obtained.[210] Copper(II) complexes of carnosine, glycylglycine, and glycylglycine–imidazole mixtures have also been studied by spectroscopic, potentiometric, and e.p.r. techniques[211] and the pH dependence of the spectroscopic properties of copper(II) complexes of carnosine has been established.[212] These results suggest that the imidazole ring of carnosine is the sole donor throughout the pH range 5—7, at reasonably high carnosine to copper ratios.[213] The potentiometric analysis of copper(I) complexes of penicillamine and glutathione have been reported, but readers should note the detailed list of corrections occurring in reference 214.

There have been three papers concerning nickel complexes: a ^{13}C n.m.r. spectral study of nickel complexes of tripeptides and dipeptide Schiff bases derived from salicylaldehyde,[215] potentiometric studies of the nickel(II)–glutathionate system,[216] and copper(III) and nickel(III) complexes of tripeptides and dipeptide Schiff bases studied by cyclic voltammetry.[217] The redox potentials of the copper(III)–copper(II) and nickel(III)–nickel(II) couples were related with e.s.r. and absorption spectral parameters. The zinc(II) and cobalt(II) binding of glycyl-L-tyrosine, a model for a carboxypeptidase substrate, has been compared using p.m.r. titrations.[218] No evidence could be found for direct metal-promoted phenolic dissociation and it was not entirely clear whether proton ionization in the zinc system occurred from the peptide linkage or from metal-bound water molecules. The preparations and n.m.r. spectra of cobalt(III) tripeptide complexes have been used to suggest the conformation of these species,[219] and, similarly, cobalt(II), nickel(II), and copper(II) complexes of di- and tetrapeptides containing tyrosine and glycine residues have been studied by pH titration.[220] A range of optically active complexes of bis(dipeptidato)cobalt(III) complexes have been resolved by ion-exchange chromatography and the c.d. spectra determined.[221] Knoevenagel reactions have been performed with the bis(glycylglycinato) complex of copper(II).

Metal complexing between peptides and the less common metal ions is becoming a popular field. The interaction of palladium(II) with glycyl-L-histidine

[209] L. G. Marzilli, K. Wilkowski, C. C. Chiang, and T. J. Kistenmacher, *J. Am. Chem. Soc.*, 1979, **101**, 7504.
[210] M. Sivasankaren-Nair, M. Santappa, and P. Natarajan, *Inorg. Chim. Acta*, 1980, **41**, 7.
[211] R. E. Viola, C. R. Hartzell, and J. J. Villafranca, *J. Inorg. Biochem.*, 1979, **10**, 293.
[212] R. E. Viola, C. R. Hartzell, and J. J. Villafranca, *J. Inorg. Biochem.*, 1979, **10**, 281.
[213] R. Österberg, R. Ligaarden, and D. Persson, *J. Inorg. Biochem.*, 1979, **10**, 341.
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[215] T. Sakurai, Y. Nakao, and A. Nakahara, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1673.
[216] G. Formicka-Kozlowska, P. M. May, and D. R. Williams, *Inorg. Chim. Acta*, 1980, **46**, L51.
[217] T. Sakurai, J.-I. Hongo, A. Nakahara, and Y. Nakao, *Inorg. Chim. Acta*, 1980, **46**, 205.
[218] D. W. Appleton, T. P. A. Kruck, and B. Sarkar, *J. Inorg. Biochem.*, 1979, **10**, 1.
[219] E. J. Evans, J. E. Grice, C. J. Hawkins, and M. R. Heard, *Inorg. Chem.*, 1980, **19**, 3496.
[220] M. S. El-Eazby, J. M. Al-Hassan, N. F. Eweiss, and F. Al-Massaad, *Can. J. Chem.*, 1979, **57**, 104.
[221] L. V. Boas, C. A. Evans, R. D. Gillard, P. R. Mitchell, and D. A. Phipps, *J. Chem. Soc., Dalton Trans.*, 1979, 582.

and with cytidine and GMP has been studied by ^{13}C n.m.r.[222] and the interaction of a range of transition-metal ions such as platinum(II), palladium(II), ruthenium(III), gold(III), *etc.* with cyclo(-L-methionyl-L-methionine) studied by ^{1}H n.m.r.[223] The n.q.r. spectra, crystal, and molecular structures of chloroglycylglycinato(imidazole)cadmium has led to an unusual coupling constant for the amide nitrogen.[224] The preparation and structural characterization of polypeptide complexes of dimolybdenum(II) and a tetrakis(glycylglycine) complex conform to the standard dimensions and conformation expected.[225] Models approximating to the molybdenum binding site in nitrogenase have been investigated using EXAFS[226] and ^{19}F n.m.r.,[227] and aspects of europium ion co-ordination with γ-carboxyglutamic acid-containing ligand systems monitored kinetically and by potentiometric titration.[228] Finally, the synthesis of complexes of rhenium(IV) with di- and tri-peptides,[229] and of palladium(II) with alanine-tyrosine and D-leucine-tyrosine have been reported.[230]

Bleomycin is a newly isolated peptide attracting a great deal of attention. Originally, it was obtained as a copper(II) complex from a culture of *Streptomyces verticillus*. Bleomycin is a histidine-containing glycopeptide antibiotic, and has been used in the treatment of selected human neoplastic diseases. The antibiotic has both metal-binding and DNA-binding sites and its biological activity is believed to be related to its bifunctionality. Although both the free ligand and the copper complex function as anti-tumour agents, it is not surprising that co-ordination chemists have focused upon the metal complexing aspects of this peptide. Bleomycin is believed to complex with iron(II) and molecular oxygen *in vivo* thus permitting the cleavage of DNA. Iron(III) cannot replace iron(II) in this degradation.

The nickel(III) oxidation state was considered to be relatively rare until pulse radiolysis was used to generate nickel(III) complexes of ethylenediamine and glycine in an aqueous solution. It has since been postulated by Margerum and others that nickel(III) complexes might be expected in biological systems. Thus, the nickel(III) complexes of a range of histidine-containing dipeptides and bleomycin have been investigated by e.s.r.[231] Similarly, e.s.r. spectroscopy has been used to study the iron complexes of bleomycin and to indicate the importance of the fifth axial nitrogen co-ordinating to iron and its role in oxygen activation.[232] Spectral investigation of the copper(II) complexes of bleomycin suggests a N_3O donor set in solution. Details and justifications as well as discussions of the limitations on the

[222] E. Matczak-Jon, B. Jezowska-Trzebiatowska, and H. Kozlowski, *J. Inorg. Biochem.*, 1980, **12**, 143.
[223] R. Ettorre, V. Guantieri, A. M. Tamburro, and L. Denardo, *Inorg. Chim. Acta*, 1979, **32**, L39.
[224] C. I. H. Ashby, W. F. Paton, and T. L. Brown, *J. Am. Chem. Soc.*, 1980, **102**, 2990.
[225] A. Bino and F. A. Cotton, *J. Am. Chem. Soc.*, 1980, **102**, 3014.
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[227] G. B. Wong, D. M. Kurtz, jun., R. H. Holm, L. E. Mortenson, and R. G. Upchurch, *J. Am. Chem. Soc.*, 1979, **101**, 3078.
[228] M. M. Sarasua, M. E. Scott, J. A. Halpern, P. B. W. Ten-Kortenaar, N. T. Boggs III, L. G. Pedersen, K. A. Koehler, and R. G. Hiskey, *J. Am. Chem. Soc.*, 1980, **102**, 3404.
[229] S. M. Basitova, A. B. Zegelman, F. Sh. Shodiev, T. Yu. Yusupov, and O. Kh. Khoshimova, *Dokl. Akad. Nauk Tadzh., SSR*, 1978, **21**, 22.
[230] H. Kozlowski, M. Jezowska, and H. Szyszuk, *J. Mol. Struct.*, 1978, **50**, 625.
[231] Y. Sugiura and Y. Mino, *Inorg. Chem.*, 1979, **18**, 1336.
[232] Y. Sugiura, *J. Am. Chem. Soc.*, 1980, **102**, 5208.

interpretation of these structures are reported.[233] Chromatographic analysis of cobalt–bleomycin on columns of CM Sephadex C25 separated the bleomycin into two forms. A reaction scheme is suggested in which a different occupation of the sixth binding site of cobalt is correlated with the different forms observed.[234] The ^{13}C n.m.r. and e.s.r. spectra of cobalt–bleomycin and its oxygenated form have shown that in the latter all the cobalt is present as cobalt(III). The donor atoms are the nitrogens of the pyrimidine and the imidazole rings and the primary and secondary amino-groups of the diaminopropanoic amide; all bind equatorially to the central metal ion.[235] Potentiometric and fluorometric titrations of bleomycin have disclosed three acidic groups with pK_a values of 7.50, 4.93, and 2.72. The conjugate nitrogen bases of these three groups comprise three of the binding sites to copper; the fourth donor is believed to be an acid with a pK_a value that is so large it cannot be measured by conventional techniques. Log stability constants for the copper–bleomycin interaction have been reported.[236] The e.s.r. spectrum of bleomycin Fe(II)–nitrosyl complex exhibits rhombic symmetry in the low spin state and a stable iron(III)–nitrosyl complex of bleomycin could not be formed.[237] No doubt we shall hear much more about this peptide in future publications.

Reactivity.—The bulk of papers concerned with models of metal peptide interactions *in vivo* have used glycine based peptides and metals such as copper, cobalt, or nickel. The reaction of copper(II) tetraglycine with a variety of aliphatic free radicals generated by pulse radiolysis have been investigated and three types of reaction observed, oxidation to copper(III), a rapid reduction to copper(I), and the formation of copper(III)–carbon bonded intermediates.[238] This work has been extended to other peptides.[239] The reactions of copper(II) triglycine with macrocyclic tetra-amine compounds have been studied kinetically.[240] The kinetics of base hydrolysis of α-amino-acid esters in mixed-ligand complexes with [glycylglycinato(2)−)]copper(II) have been studied.[241] The ester ligands in the ternary complexes undergo base hydrolysis some 50-times faster than the free esters. This result is consistent with the formation of mixed-ligand complexes involving a unidentate ester species. The base hydrolysis of the peptide bond in a variety of carbonyl bonded glycine peptides in the co-ordination sphere of $Co(dien)^{3+}$ has been studied and rate constants have been reported.[242] The rate of peptide bond hydrolysis by *cis*-β-$[Co(trien)(OH)(OH_2)]^{2+}$ has also been investigated for eleven dipeptides;[243] phosphate does not affect the rate and some other hydroxyaquo-complexes are catalytically inactive.

Equilibrium studies have been performed on zinc(II) and cobalt(II) binding to tripeptides, which are said to be analogues of the amino-terminus of human serum

[233] R. D. Bereman and M. E. Winkler, *J. Inorg. Biochem.*, 1980, **13**, 95.
[234] C. M. Vos, G. Westera, and B. van Zanten, *J. Inorg. Biochem.*, 1980, **12**, 45.
[235] C. M. Vos, G. Westera, and D. Schipper, *J. Inorg. Biochem.*, 1980, **13**, 165.
[236] D. Solaiman, E. A. Rao, W. Antholine, and D. H. Petering, *J. Inorg. Biochem.*, 1980, **12**, 201.
[237] Y. Sugiura and K. Ishizu, *J. Inorg. Biochem.*, 1979, **11**, 171.
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[242] R. W. Hay and D. P. Piplani, *Kemiai Kozlemenyek*, 1977, **48**, 47.
[243] M.-J. Rhee and C. B. Storm, *J. Inorg. Biochem.*, 1979, **11**, 17.

albumin.[244] The reaction of the nickel(II)–glycylglycyl-L-histidine complex with molecular oxygen and the formation of a decarboxylated species have been studied by ^{1}H n.m.r. spectra.[245] The interaction of copper(II) and nickel(II) with L-histidine and glycylglycyl-L-histidine as an albumin model has been studied by the same group using c.d. and e.s.r.,[246] and the effect of non-co-ordinative axial blocking on the stability and kinetic behaviour of ternary 2,6-lutidine–nickel(II)–oligopeptide complexes based upon glycine investigated spectrophotometrically.[247]

A series of papers from the Margerum group have discussed copper(III) and nickel(III) peptides. Tripeptide complexes were, in general, found to be less stable to photochemical decomposition in acid solution than those of tetrapeptides or pentapeptides.[248] The pK_a values have been determined for the copper(III) complexes of eight peptides and peptide amides[249] and the electron-transfer reactions between copper(III)–peptide complexes and tris(1,10-phenanthroline)cobalt(II) studied in detail.[250] Electron-transfer reactions between copper(III)–peptide complexes and hexachloroiridate have also been studied.[251] The oxidative decarboxylation of glyoxalate in the presence of a deprotonated amine copper(III)–peptide complex is faster than with pyruvate, which in turn is faster than with phenylglyoxylate.[252] The reactions of copper(III) tetraglycine in acid and base have been studied and rate constants obtained.[253]

The reactivity of a variety of less common metal peptides has attracted attention. There has been a discussion of the functional design of haem proteins based upon an octapeptide model,[254] and the irreversible redox rearrangement of dioxygen complexes of cobalt(II) dipeptides has been studied by gas chromatography and mass spectroanalysis.[255]

Two papers on molybdenum peptides include the mononuclear molybdenum(V) complexes of a cysteinyl peptide[256] and the reactivity of polymer-anchored molybdenum(V and VI) tripeptide complexes.[257] The interaction of the throtropin releasing factor L-pyroglutamyl-L-histidyl-L-prolinamide with nickel(II) has been studied,[258] and the stereoselective reaction between formaldehyde and *N*-salicylideneglycyl-L-valinatocopper(II) monitored.[259] Stereoselective catalysis of the oxidation of ascorbic acid by iron(III) complexes supported on asymmetric polymers based upon poly(L-glutamate) and poly(D-glutamate) has been

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studied,[260] and the kinetics of disulphide bond cleavage by methylmercury have been investigated by n.m.r. techniques.[261]

4 Proteins

Work in this area continues apace. A new volume concerning iron in biochemistry and medicine[262] and a short review of metal–ligand complexing in biological systems, at the interface between protein and lower molecular weight ligands. have appeared.[263] Work on the biological functions of proteinases has been described in a recent book.[264] A range of new approaches to metalloprotein investigations has appeared in the literature. These include the use of diplatinum(II) octaphosphite as a potential probe for basic proteins,[265] the introduction of [cobalt{tris(3,5-dimethyl-1-parazolylmethyl)amine}H_2O]$^{2+}$, as a model for metalloenzymes containing zinc,[266] the binding of *N*-methyl isatin β-thiosemicarbazone–copper complexes to proteins in general,[267] and the use of metal chelating drugs to induce the synthesis of proteins normally found inside cells.[268]

In a previous review we have referred to the dangers involved in using models and metal-substitution reactions in order to obtain information about metalloproteins. This is particularly so when the metal is zinc, which is spectroscopically silent. Nevertheless, considerable progress is now being made in the area of zinc-containing metalloproteins. There is a recent report of the active site-specific reconstituted zinc(II) horse liver alcohol dehydrogenase being modelled, and conformational transitions being elucidated.[269]

Our last report listed a dozen papers concerning carbonic anhydrase. This zinc-containing enzyme continues to attract a great deal of attention. Bertini *et al.*[270] have studied the acid–base equilibria of the cobalt(II) and zinc(II) carbonic anhydrases in buffered solutions by 80 MHz n.m.r. and have also investigated the interaction of the cobalt(II) enzyme with trichloroacetaldehyde.[271] The work has recently been extended to electronic spectral studies of the cobalt(II) enzyme and the interaction of cyanometallates with this derivative.[272, 273] It is interesting that heavy metal cyanides do not show appreciable affinity for the cobalt(II) enzyme. Copper(II) binding to carbonic anhydrase B has recently been studied by the same group.[274] The reaction of carbon dioxide with water to produce bicarbonate is, of course, water dependent and a recent study has established the rate of water

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exchange at the active-site zinc of human carbonic anhydrase. The influence of various external buffers is also reported.[275] ^{17}O n.m.r. relaxation rates have been measured for carbonic anhydrase and several of its metal derivatives.[276] One of the newer physical chemical approaches to enzymology is that of photochemically induced dynamic nuclear polarization (CIDNP) n.m.r. and this has now been applied to the interaction between carbonic anhydrase and B-sulphanilamide.[277] Meanwhile, the more traditional method of optical spectroscopy has been used for cobalt(III) substituted enzymes.[278] The mechanism of carbonic anhydrase activity is still open to question and letters are still appearing discussing new aspects; for example, a model has been proposed for one of the active sites in carbonic anhydrase[279] and a discussion of the evidence for a two-site mechanism has recently been given.[280]

The zinc-containing metalloenzyme carboxypeptidase still attracts the interests of co-ordination chemists. Boyer has used the zinc complexes of naturally occurring 2-hydroxyacids as models for the zinc activated hydrolysis of esters using carboxypeptidase as a catalyst.[281] Polydentate ligands such as EDTA and NTA do not accelerate the rate at which zinc can be extracted from a metalloprotein, whereas simpler bidentate or tridentate ligands are able to attack and remove the zinc at the active site.[282] Apparently, the former ligands completely saturate the co-ordination sites on the zinc ion and preclude the formation of the enzyme–zinc–ligand ternary complex, which is an essential feature of the mechanism. By substituting cobalt(II) for zinc, and concentrating on the reaction between 1,10-phenanthroline and the substituted enzyme, a mechanism has been proposed to explain the pH profile of metalloenzyme–substrate interactions.[283] These mechanisms are not simple; for example, it has been possible to quantify the catalytic effect of a third ligand such as amino-acid upon the mechanism of transfer of zinc from enzyme to EDTA.[284] Apparently, the amino-acid anion can act as an intermediary and helps to remove the zinc whereas, as mentioned in reference 282, the EDTA is unable to attack the zinc in the metalloenzyme. Zinc may be substituted by cobalt(II) or cobalt(III). The latter metal centre has the advantage of being both substitution inert and spectroscopically active. Cobalt(III) derivatives of carbonic anhydrase and carboxypeptidase have been studied spectroscopically[285] in an effort to define the ligating groups at the active site of the enzymes. In similar vein, visible and magnetic c.d. spectra of cobalt(II)-substituted zinc metalloenzymes have been used to reveal the distorted tetrahedral stoicheiometry prevailing around the metal ion.[286] A newer approach

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involves labelling specifically not the zinc in the active site but rather the tyrosyl-248 residue by chemical modification with diazotized *p*-arsanilic acid as a label.[287] This produces a sensitive dynamic probe of the local environment of the active centre and permits resonance Raman spectroscopy to be used as a tool. Two other enzymes requiring zinc for activation are β-lactamase II and insulin.[288, 289] β-Lactamase II has been treated with cobalt(II) and zinc(II) by equilibrium dialysis, whereas the method of choice for studying insulin–metal interactions is that of copper(II) substitution and electron paramagnetic resonance of the freeze dried hexamer.

Plastocyanin is a low molecular weight protein, consisting of just 99 amino-acid residues. An *X*-ray structural determination down to 2.7 Å resolution has been published.[290] The copper in spinach plastocyanin is ligated by two histidines, one methionine, and one cysteine in a somewhat distorted tetrahedral stereochemistry. This small protein has focused attention on the chemistry of copper in a mixed nitrogen–sulphur donor environment. The kinetics and pK_a values for ligands binding to the active site of plastocyanin have been noted [291] and the thermodynamics of electron transfer reactions [292] correlated with the hydrophobicity of the redox sites in stellacyanin, plastocyanin, and azurin. The reactivity and spectroscopic properties of these last three proteins have now been studied [293, 294] and correlated with c.d. and magnetic c.d. spectra. Bands, which correspond to those derived from ligand-field calculations based on distorted tetrahedral stereochemistry on the metal, are observed. Three important papers concerning the n.m.r., *X*-ray diffraction , and *X*-ray photoelectron spectrometry of plastocyanins and their model compounds have helped to build up our knowledge of this intriguing protein.[295–297]

Electron nuclear double resonance spectra of stellacyanin have been measured. This is claimed to be the first published observation of copper ENDOR in a protein. The results confirm the presence of two nitrogen donors and the flattened tetrahedral geometry observed with plastocyanin.[298] Activation parameters for the oxidation of copper(I) stellacyanin have been determined.[299] The 270 MHz ^{1}H n.m.r. spectra of both copper(II) and copper(I) apostellacyanin indicate that two histidines are bound to the metal ion and a structure for the copper binding site

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has been proposed.[300] Models which approximate to the active site of these small copper proteins have produced useful data. It is particularly noteworthy that the copper(I) proteins are exceptionally stable.[301] Azurin also gives a stable copper(I) derivative and two histidine residues ligate the metal ion.[302] This work has been substantiated by metal substitution studies of azurin and 270 MHz n.m.r. spectroscopy of the protein.[303, 304] Ligand field and Jahn–Teller calculations of the bonding in blue copper proteins are helping to contribute towards our knowledge of the spectroscopic properties of these flattened tetrahedral chromophores.[305]

Other copper–protein interactions recently reported include those of the catecholase–copper–phthalazine complex studied by electronic spectra,[306] the spectroscopic and catalytic properties of laccase in the copper depleted state,[307] thermodynamic studies of the copper(II)–human serum albumin equilibrium,[308] and the effects of anions and ferricyanide on the copper(II) site of the histidine and tryptophan modified versions of galactose oxidase.[309]

Copper binding in haemocyanin has been studied by *X*-ray absorption fine-structure analysis[310] and resonance Raman spectroscopy.[311] These techniques suggest that the two copper atoms are both bound to the proteins by three histidine ligands and one other donor group, possibly oxygen from a tyrosine residue. Purple haemocyanin is formed when ethyleneglycol is added to oxyhaemocyanin[312] with a concomitant distortion of the active site. This site was further examined using absorption spectra c.d., e.s.r., and Raman spectroscopy.[313] An equilibrium between the faintly denatured species, which produces the purple colour, and the intensively denatured species, which contains e.s.r. detectable copper(II), is suggested.[313] These haemocyanins have now been compared with the copper active sites of tyrosinase proteins, another protein that contains a strongly coupled binuclear copper active site but which, of course, has completely different biological functions.[314] E.s.r. spectra and n.m.r. analysis have been applied to the interaction of manganese(II) with oxyhaemocyanin.[315] The bridging between the two coppers in the active sites has been studied by examining a series of haemocyanin derivatives such as half-met-L haemocyanin where L stands for a range of ligands from cyanide through nitrogen donors through

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halides to acetate and water.[316] A comparison of the ligand substitution of these various forms suggested that there may well be an endogenous protein bridge between the two coppers in haemocyanin.[316] Small fragments of β_c-haemocyanin have been subjected to resonance Raman spectroscopy[317] and chemical and spectroscopic comparisons of mollusc and arthropod haemocyanins have found that the strain in the active site is greater for arthropods than for molluscs.[318]

Cytochrome *c* and related myoglobin and haemoglobin proteins have been studied in the presence of copper complexes. The ensuing redox kinetics are consistent with the classical outer-sphere electron transfer mechanism involving the haem edge of the haem proteins.[319,320] Iron(II) cytochrome P-450 models (high spin) have been complexed with alkyl mercaptides and studied using ^{1}H n.m.r. spectroscopy. During the normal catalytic cycle of bacterial cytochrome P-450, the iron porphyrin active site is known to exhibit rich co-ordination and redox chemistry; low-spin iron(III), high-spin iron(III), high-spin iron(II), low-spin iron(II), and conceivably iron(IV) oxidation states all play a role. Proton n.m.r. spectroscopy of the mercaptide complexes of naturally occurring and synthetic porphyrins has been used to elucidate the different spin and oxidation states and the preferred complexes.[321] Similarly, cobalt(III), copper(II), iron(II), manganese(III), nickel(II), and zinc(II) derivatives of horse cytochrome *c* have been subjected to n.m.r. studies.[322] The phenylalanine residues of eukaryotic cytochrome *c* have been characterized.[323] Electron transfer between stellacyanin and cytochrome *c* has been studied by stopped-flow and temperature-jump techniques. The equilibrium constants obtained have values that correspond to the expected redox potentials for these respective proteins.[324]

The iron-containing protein haemoglobin continues to attract a great deal of attention. Spectrophotometric and stopped-flow techniques have established that copper(II), when mixed with either the oxy- or deoxy-forms of haemoglobin, rapidly promotes the oxidation of all four iron atoms to give low-spin iron(III) forms (called hemichromes).[325] A variety of experimental methods have been used to investigate haemoglobin and myoglobin: the low temperature Soret-excited resonance Raman spectroscopy of myoglobin–hydrogen peroxide complexes has been reported;[326] magnetic c.d. spectra for a range of five co-ordinated haem [iron(III)] protoporphyrin dimethylester complexes were observed from the near u.v. to the i.r. regions and the results correlated with molecular orbital calculations;[327] nitrosyl transfer from cobalt nitrosyls to haemoglobin and myo-

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globin was studied by u.v. and visible spectra;[328] and a fascinating range of studies whereby the thermodynamics of oxygen binding to native myoglobins and their cobalt analogues were investigated for sperm whale, grey whale, and sea lion myoglobins, *etc.*[329] It is suggested that the significant difference in the oxygen binding properties arises from, or is reflected in, differing enthalpy and entropy compensation effects.

Other haem protein research work reported includes the synthesis and n.m.r. characterization of chelated haem models of haemoproteins,[330] electron nuclear double resonance studies of both the high- and low-spin iron(III) haemoglobins and myoglobins,[331] investigations of porphyrin core expansion and the so-called 'doming effect' in haem proteins studied by resonance Raman spectroscopy,[332] similar Raman studies on the nitrosyl haem proteins and their porphyrin analogues,[333] *X*-ray diffraction studies of a range of models related to the active site of oxygen binding in haemoproteins,[334] a comparative study of carbon monoxide and isocyanide binding to cyclophane models of haems wherein the magnitude of the distal side steric effects are established,[335] Griffith binding *versus* Pauling binding for a range of dioxygen complexes of manganese porphyrins,[336] and the enthalpies and binding constants for the oxygenation of iron(II) and cobalt(II) 'capped' porphyrins.[337]

Kinetic studies involving reactions of iron–sulphur proteins naturally centre around ferredoxin. A recent paper has identified the specific binding sites using redox inactive $Cr(NH_3)_6^{3+}$ and $Cr(en)_3^{3+}$.[338] A model for unconstrained structural changes in ferredoxin type proteins has been used to characterize the elongated core structure and structural changes therein when this protein reacts.[339, 340] Finally, the exchange reactions of cysteine–glycine peptides with the iron–sulphur cluster compound bis(tetramethylammonium)tetrakis(μ_3-sulphido-t-butylthioiron) have used equilibrium constants as a model of ferredoxin activity.[341]

Adrenodoxin, which plays a role in the oxidation–reduction process in adrenal mitochondrial steroid hydroxylation, is a single polypeptide chain containing Fe_2S_2 redox centres and 114 amino-acid residues, which include five cysteine residues per molecule. The kinetics of this compound reacting with a range of

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methylmercury reagents have been reported and a mechanism has been suggested.[342] Similar work has been reported for the ferrodoxins reacting with a range of cobalt and platinum low-molecular-weight ligand complexes.[343] Low-molecular-weight forms of iron are required for transmission through membranes and so the mobilization of iron from ferritin by chelating agents is an important topic. Crichton *et al.* have studied a range of chelating agents such as desferrioxamine, rhodotorulic acid, and derivatives and their effectiveness in iron mobilization.[344] An interesting article concerning the ability of transferrin and ferritin to exchange vanadium suggests that, although the exchange cannot be produced *in vitro* at physiological pHs, it does occur *in vivo*.[345] This suggests that a special biological factor is required which is only found within cells. Water may be decomposed to generate hydrogen using visible light in the presence of a hydrogenase–ruthenium(II) carbonyltetraphenylporphyrin–methyl viologen reducing agent.[346] The phenolate oxygen of tyrosine amino-acid residues is claimed to be involved in the metal-ion binding in transferrins. These reports are now supported by studies whereby copper and iron(III) transferrins have been subjected to spectrophotometric, n.m.r, and fluorescence analysis.[347]

Metallothioneins are well known as forms in which excess of metal ions, essential or polluting, are accumulated and stored in the liver and the kidneys. The metabolic interaction between cadmium and zinc in metallothioneins has been reported,[348] also the binding of gold(I) to metallothionein.[349] In the intestine there is mutual antagonism between copper and zinc metallothioneins,[350] and animals caused acute stress by chloroform inhalation produced increased amounts of zinc metallothionein in their livers.[351] The binding of cadmium to metallothioneins has been investigated by spin coupling in the n.m.r. spectrum of cadmium(II) metallothionein from rabbit liver and the results are the first direct evidence for the existence of a polynuclear cluster arrangement in this protein.[352] The binary zinc thiolates, $Zn(SR)_2$, and their derivatives, have been investigated in detail as models for the unknown structures of metallothionein proteins.[353]

Mammalian cells contain two types of superoxide dismutase. The cytosolic enzyme contains both copper and zinc and the manganese-containing enzyme is present in the matrix of the mitochondria. All normal mammalian cells contain these two types of enzyme, with the exception of erythrocytes, which lack the manganese form. Interestingly, the quantities of both forms of superoxide dismutase are diminished in tumour cells. These observations have attracted a variety of chemical investigations. For example, the adrenalin autoxidation of the copper and zinc form has been investigated by 360 MHz ^{1}H n.m.r. measure-

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ments;[354] a mathematical model has been proposed for a whole range of metal complexes of superoxide dismutase and their rates of reaction.[355] The reversible removal of copper and zinc from the enzyme at low pH [356] has been investigated and the possibility of imidazolate bridging between adjacent metal ions in their active sites has been tested. The binding site of the copper in bovine superoxide dismutase thiocyanate has been investigated using e.s.r. electronic absorption spectra, and ^{1}H, ^{13}C, and $N^{13}CS$ n.m.r. spectroscopies.[357]

Molybdoenzymes are a relatively new field entering into these reports and in previous times apparently played a detailed role in chemical evolution, whereas more recently they have become involved in nitrogen fixation reactions. The chemical evolution of molybdoenzymes has been discussed from the point of view of their molybdenum–sulphur bonding,[358] and the structure of the active site in such enzymes has been modelled and discussed at length using *X*-ray absorption spectroscopy.[359] The molybdenum is apparently bound to a mixture of oxo- and sulphur-donor ligands. The configurations and bond lengths of these species are listed in the reference. An intriguing parallel has been drawn between the ability of C_2H_2 to reduce peptide chains from bovine insulin that have been complexed with MoO_4^{2-} with the similar reducing power of $NaBH_4$ for iron–molybdenum cofactor nitrogenase systems.[360] These molybdoinsulin systems of nitrogenase simulate many of the steric hindrance effects seen in reductions involving acetylene and substituted acetylenes. Similarly, the reduction of molecular nitrogen with molybdoinsulin catalysts has been found to have parallels which bear upon theories of chemical evolution.[361] These molybdoenzymes can only be prepared within cells and a recent paper [362] reports that molybdenum(VI) did not react with human erythrocyte membranes, whereas molybdenum(V) did. The molybdenum-containing centre of xanthine oxidase has been studied by EXAFS approaches and it was found that the cyanolysable sulphur atom of the functional xanthine oxidase is probably tightly bound to the molybdenum at a distance of $\leqslant 2.3$ Å.[363] The spectrum of the rapid molybdenum(V) e.p.r. signal from xanthine oxidase dissolved in ^{17}O-enriched water has been recorded,[364] and finally the molybdenum binding site in xanthine oxidase has been studied using *X*-ray absorption spectroscopy. This is said to be the first definitive structural characterization of this binding site.[365]

The binding of manganese to deoxyribonuclease has been studied by conductimetric methods. It was found that the site used for binding manganese 2+ ions is

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different to that used for magnesium and calcium ions.[366] Only a few enzymes require manganese and to date these have been limited to those involving manganese(II). However, recent magnetic susceptibility and e.s.r. experiments have demonstrated that the manganese bound to superoxide dismutase is trivalent. The manganese(II)-containing acid phosphatase has now been isolated and this chromophore has been characterized.[367] The chromium, manganese, cobalt, and copper complexes of human lactoferrin have been assessed using electronic spectra and metal binding sites assigned to tyrosyl residues and possibly a histidine nitrogen.[368] A model for iron(II)- and manganese(II)-binding to aconitase and citrate ions has been applied to a consideration of the gastrointestinal absorption of essential metal ions.[369] Similarly, a detailed study of the respective binding sites for iron(III) and vanadyl(IV) to conalbumin has been effected and details of the respective binding sites for these two cations have been itemized.[370,371]

Turning to enzymes which are predominantly dependent upon non-transition metals, thermolysin, a protease that contains one zinc and four calcium ions, has been studied by *X*-ray diffraction methods.[372] The metal ion co-ordination sites are found to be pseudo-tetrahedral, a result that parallels the carboxypeptidase observations reported earlier in this report. Bovine prothrombin is involved in the calcium ion mediated binding of vitamin K in blood. The reactions between this agent and lanthanide(III) ions have been studied using luminescence and n.m.r. spectroscopy.[373] Details of the different distinct types of binding sites are tabulated. A different type of substitution reaction has been attempted with apo-yeast enolase. The parent enzyme occurs as a magnesium complex but in order to gain e.p.r. data the apo-enzyme has now been complexed with copper(II).[374] Additional information concerning the geometry and location of the divalent cation binding sites and in particular the question of whether imidazole is involved has all been produced by this study. A range of divalent metal ions that inhibit yeast enolase activity through binding at sites remote to those metal binding sites normally associated with catalytic activity have been studied; the inhibition by these metal ions may be alleviated by the addition of ligands.[375] This work has been broadened to consider the conformation of the metal ions producing the enzymatic activity. Criteria for catalysis and its prevention have been established from spectrophotometric titrations.[376]

The kinetic analysis of calcium binding to concanavalin A produces a K_a value for the binding in reasonable agreement with those values obtained by equilibrium methods.[377] Protein–protein interaction sites in the calcium modulated skeletal

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muscle troponin complex and its peptide fragments have suggested means for site recognition.[378] A model for coenzyme–metal ion–apoenzyme interactions involving thiamine pyrophosphate (the cofactor form of vitamin B_1) centres around the divalent metal ions with magnesium(II) and manganese(II). The presence of conformational equilibrium is suggested from these studies.[379]

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