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Preface

This eleventh Report reviews papers relevant to the chemistry of amino-acids, peptides, and proteins published in the main journals during 1978. Subject coverage is similar to that of previous volumes except that the usual chapter on peptides with structural features not typical of proteins has unfortunately had to be omitted.

Chapter 4 dealing with chemical structure and biological activity is selective rather than comprehensive. The present volume includes the biennial survey of metal derivatives of amino-acids, peptides, and proteins.

It is a pleasure once more to thank the many contributors to this volume whose names are listed opposite.

R. C. SHEPPARD

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Abbreviations

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

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

Ac	acetyl
Acm	acetamidomethyl
Ad	adamantyl
Adoc	adamantyloxycarbonyl
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-cyanobenzl
Bzl(O Me)	4-methoxybenzyl
$Bzl(NO_2)$	4-nitrobenzyl
$Bzl(2\text{-}NO_2)$	2-nitrobenzyl
c.d.	circular dichroism
Cha	cyclohexylamine
Cm	carboxymethyl
Cmc	*S*-carboxymethylcysteine
Cox	4,5-di-(4-chlorophenyl)-4-oxazolin-2-one amino-acid derivative

CPh_2Py	diphenyl-4-pyridylmethyl
Dcha	dicyclohexylamine
Ddz	3,5-dimthoxy($\alpha\alpha$-dimethyl)benzyloxycarbonyl
DMF	*NN*-dimethylformamide
DMSO	dimethyl sulphoxide
Dnp	2,4-dinitrophenyl
Dns	1-dimethylaminonaphthalene-5-sulphonyl (dansyl)
2,4-Dnps	2,4-dinitrosulphenyl
Dopa	3,4-dihydroxyphenylalanine
DP	degree of polymerization
Dpp	diphenylphosphinoyl
DPtd	4,6-diphenylthieno[3,4-*d*][1,3]dioxal-2-one 5,5-dioxide
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
Ec	ethylcarbamoyl
edta	ethylenediaminetetra-acetate
En	ethylene diamine
e.p.r.	electron paramagnetic resonance
e.s.r.	electron spin resonance
Et	ethyl
Gal	galactose
g.c.–m.s.	gas chromatograph–mass spectrometer combination
g.l.c.	gas–liquid chromatography
Glc	glucose
Glp	pyrrolid-2-one-5-carboxylic acid
Glx	glutamic acid or glutamine (not yet determined)
GTP	guanosine 5′-triphosphate
H.p.l.c.	high performance liquid chromatography
Iboc	isobornyloxycarbonyl
i.r.	infrared
Mal=	maleoyl
Man	mannose
Mbh	4,4-dimethoxybenzhydryl
Mbs	4-methoxybenzene sulphonyl
Me	methyl
Mea	mercaptoethylamine
Mhoc	1-methylcyclohexylcarbonyl
$MePh_2Peoc$	2-methyldiphenylphosphinioethyloxycarbonyl
Msc	2-(methylsulphonyl)ethoxycarbonyl
Mtc	2-methylthioethyloxycarbonyl
NAD	nicotinamide-adenine dinucleotide (NAD^+ oxidized: NADH, reduced)
NCA	*N*-carboxyanhydride
Nma	maleimido
Nmps	4-methyl-2-nitrosulphenyl
N.m.r.	nuclear magnetic resonance
Nsu	succinimido
Nps	*o*-nitrophenylsulphenyl

Np	4-nitrophenyl
ONp	*p*-nitrophenoxy
ONp(o)	*o*-nitrophenoxy
ONSu	succinimido-oxy
OPcp	pentachlorophenoxy
OPfp	pentafluorophenoxy
OPic	4-picolyloxy
Opop	phenacyl-2-hydroxyphenyl ester
o.r.d.	optical rotatory dispersion
OTcp	2,4,5-trichlorophenoxy
Pac	phenacyl
Pcp	pentachlorophenyl
Peoc	2-triphenylphosphinioethyloxycarbonyl
Ph(SMe)	*p*-methylthiophenyl
Pic	4-picolyl
Picoc	4-picolyloxycarbonyl
Pipoc	piperidino-oxycarbonyl
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'*-tetramethylethylene diamine
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
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

Chemical studies of amino-acids including structure determination, synthesis, analysis, and physical and chemical behaviour are dealt with in this Chapter. Although the objectives of most of these studies are in support of biological investigations, behaviour, metabolic studies, and papers covering the natural distribution of amino-acids are excluded, as in previous Volumes in this series.

Textbooks, Reviews, and General Articles.—Encyclopaedic[1] and other comprehensive reviews[2] have appeared. Specific topics dealt with in other reviews are: cross-linking amino-acids in proteins,[3] role and distribution of *N*-acetylaspartic acid[4] and L-canaline,[5] and biochemistry[6] and distribution in higher plants[7] of D-amino-acids. The view is developed further,[8] that the non-protein amino-acids present in unusually large amounts in many seeds may confer an evolutionary advantage by inhibiting the germination and growth of seeds of competing species. A fascinating account has been provided[9] of the activation of amino-acids by esterification to tRNA's carrying the appropriate anticodons.

Reviews dealing with topics in amino-acid chemistry are cited in the relevant sections of this Chapter.

2 Naturally Occurring Amino-acids

Occurrence of Known Amino-acids.—There is no opportunity in the space available in this Chapter to cover aspects of the distribution of the common amino-acids in the geosphere[10] and in the biosphere. The coverage concen-

[1] A. Yamamoto, 'Kirk-Othmer Encyclopaedia of Chemistry and Technology', Third Edition, ed. M. Grayson and D. Eckroth, Vol. 2, 1978, p. 376; T. Yoshida, *ibid.*, p. 410; H. Reisman, Encyclopaedia of Chemical Process Design, 1977, Vol. 3, p. 197.

[2] J.C. Johnson, 'Amino-acids Technology; Recent Developments', Noyes Data Corp., Park Ridge, New Jersey, 1978; Y. Izumi, I. Chibata, and T. Hoh, *Angew. Chem.*, 1978, **90**, 187; *Angew. Chem. Int. Ed.*, 1978, **17**, 176; J.H. Jones, in *'Comprehensive Organic Chemistry'*, ed. I.O. Sutherland, Vol. 2, 1979, p. 815; P.M. Hardy, *ibid.*, Vol. 5, ed. E. Haslam, p. 187.

[3] 'Protein Crosslinking: Nutritional and Medical Consequences', ed. M. Friedman, Plenum, New York, 1977.

[4] R. Bakhash and E. Westman, *Bios (Madison, N.J.)*, 1978, **49**, 13.

[5] G.A. Rosenthal, *Life Sci.*, 1978, **23**, 93.

[6] K. Soda, *Kagaku*, 1977, **32**, 517 (*Chem. Abs.*, 1978, **88**, 100 374).

[7] T. Ogawa, *Nippon Nogei Kagaku Kaishi*, 1978, **52**, R83 (*Chem. Abs.*, 1978, **89**, 176 255).

[8] M.F. Wilson and E.A. Bell, *Phytochemistry*, 1978, **17**, 403; E.A. Bell, *Pontif. Acad. Sci., Scr. Varia*, 1976, **41**, 571 (*Chem. Abs.*, 1979, **89**, 56 387).

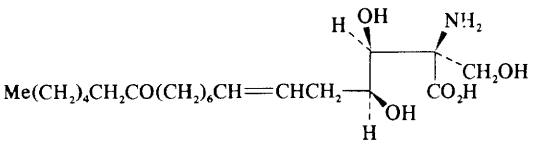
[9] E. Holler, *Angew. Chem. Int. Ed.*, 1978, **17**, 648.

[10] J.L. Bada, E.H. Man, and A.C. Walker, 'Initial Reports of The Deep Sea Drilling Project', ed. P.T. Robinson, GPO, Washington, D.C., Vol. 37, 1977, p. 633; L. Chichereau, G. Monteil, and J. Trichet, *Adv. Org. Geochem., (Proc. 7th Int. Meeting)*, ed. R. Campos and J. Goni, Empressa Nac. Adaro Invest. Minerassa, Madrid, 1977, p. 879; J.K. Whelan, *Geochim. Cosmochim. Acta*, 1977, **41**, 803.

trates on the first findings in Nature of known amino-acids, almost invariably concerning the 'uncommon' amino-acids, and on papers describing these compounds in unusual situations.

The non-protein amino-acids found in *Fagaceae* have been listed;[11] other plant sources reported are *Iris,* containing 3-(3-hydroxymethylphenyl)-L-alanine[12] (previously found in *Caesalpinia tinctoria* but of undetermined configuration) and *Iris pseudacorus,* shown to excrete 3-(3-carboxyphenyl)-alanine in large amounts into culture substrate;[13] *Afzelia bella* seeds contain (2*S*,4*R*)-dicarboxypyrrolidine and its 4-hydroxy-analogue (hydroxy-L-proline), the latter being one of the protein amino-acids but found now for the first time in the free amino-acid pool of a plant;[14] *Tephrosieae* contain 3-[2-amino-(2-imidazolin-4-yl)]alanine, enduracididine, homoarginine, γ-hydroxyhomoarginine, canavanine, and other guanidines,[15] while the report that soybean (*Glycine max.*) contains L-canavanine has been corrected[16] with the admonition that conclusions must not be drawn on the coincidence of ion-exchange retention time alone. *Lyngbya majuscula* contains (*S*)-α-butyramido-γ-butyrolactone.[17]

Microbial sources of amino-acids (excluding coverage of fermentative production[2,18a] of amino-acids, except for a representative citation of the formation of L-lysine and L-threonine by mutants of *Brevibacterium lactofermentum*[18b]) include *Streptomyces flocculus,* which synthesizes β-methyltryptophan as an intermediate *en route* to streptonigrin.[19] Configurational assignments have been made through chemical correlation and physical studies to (*S,S*)-3,5-diaminohexanoic acid (from *Clostridium sticklandii*),[20] (*S,S*)-2-amino-4-methylhex-5-enoic acid (from a New Guinea *Boletus*),[21] (*S,S*)-homoisoleucine (from *Aesculus californica*),[21] and to the antiobiotic thermozymocydin (*alias* myriocin), whose stereochemistry has been revized to the all-*cis* structure (1).[22]



(1)

[11] T. Kasai, P.O. Larsen, and H. Sorensen, *Phytochemistry,* 1978, **17,** 1911.
[12] P.O. Larsen, F.T. Sorensen, and E. Wieczorkowska, *Phytochemistry,* 1978, **17,** 549.
[13] M.C. Cardenas and R. Kickuth, *Angew. Bot.,* 1978, **52,** 203.
[14] A. Welter, M. Marlier, and G. Dardenne, *Phytochemistry,* 1978, **17,** 131.
[15] L.E. Fellows, R.M. Polhill, and E.A. Bell, *Biochem. Syst. Ecol.,* 1978, **6,** 213.
[16] G.A. Rosenthal, *Experientia,* 1978, **34,** 1539.
[17] F.J. Marner and R.E. Moore, *Phytochemistry,* 1978, **17,** 553.
[18] (*a*) P. Pilat, *Kvasny Prum.,* 1978, **24,** 67 (*Chem. Abs.,* 1979, **90,** 74 172); (*b*) O. Tosaka, K. Takinami, and Y. Hirose, *Agric. Biol. Chem.,* 1978, **42,** 745.
[19] S.J. Gould and D.S. Darling, *Tetrahedron Letters,* 1978, 3207.
[20] F. Kunz, J. Retey, D. Arigoni, L. Tsai, and T.C. Stadtman, *Helv. Chim. Acta,* 1978, **61,** 1139.
[21] E. Gellert, B. Halpern, and R. Rudzats, *Phytochemistry,* 1978, **17,** 802.
[22] C.H. Kuo and N.L. Wendler, *Tetrahedron Letters,* 1978, 211.

β-Alanine betaine ('β-homobetaine') occurs in the white muscles of New Zealand whiptail and southern blue whiting,[23] and dopa has been found in the shell protein of *Mytilus edulis* to the extent of not less than 3.6 %.[24] Two related sponges, *Haliclona* and *Chalinopsilla,* contain L-azetidine-2-carboxylic acid;[25] this functions as an antidermatophyte towards *Trichophtyon mentagrophytes.* A dioxopiperazine incorporating *N*-methyl-δδδ-trichloromethylleucine has been located in the sponge *Dysidea herbacea.*[26] Extensive studies continue[27] on the formation of *N*-(1-carboxyethyl) amino-acids in sunflower crown gall tissues incited by *Agrobacterium tumefaciens* (see also Vol. 10, p. 2), and *meso*-α-iminodipropionic acid found in squid muscle (*Todarodes pacificas*)[28a] and in the adductor muscle of the scallop *Patinopecten yessoensis*[28b] similarly incorporates a D-alanine moiety. Hypotaurine is present in the bivalve mollusc *Noetia ponderosa.*[29]

N-Terminal *N*-methylamino-acid residues in proteins described recently (Vol. 10, p. 3) have also been detected in pilin from *Pseudomonas aeruginosa*[30] and other gonoccocal and meningococcal proteins;[31] in all these examples, the residue is *N*-methylphenylalanine. *Halobacterium halobium* ferredoxin contains a single N^{ε}-acetyl-lysine residue.[32] γ-Carboxyglutamic acid has been found in mammalian ribosomal proteins,[33] and the occurrence of this amino-acid in vertebrates has been extended further to shark tooth,[34] fish otolith,[34] and fossil bones.[35]

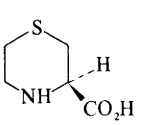
Rat-liver mitochondria are capable of the β-hydroxylation of N^{ε}-trimethyllysine, providing an intermediate in carnitine biosynthesis.[36]

Red feathers of the Rhode Island cock contain dopa and *S*-cysteinyldopa.[37]

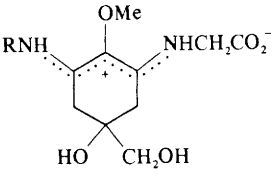
New Natural Free Amino-acids.—Plant sources of new amino-acids reported in 1978 are few in number, compared with sources at other biological levels. Accumulations of 'uncommon' amino-acids have been listed for 64 species of *Caesalpineae,* showing notable species variations.[38] One of these, *Peltophorum,* contains a previously undescribed imino-acid tentatively identified as a phosphate ester of 4-hydroxypipecolic acid.[38] Lathyrine {β-(2-aminopyrimidin-4-yl)alanine} and its γ-L-glutamyl derivative have been found in *Lathyrus*

[23] S. Konosu, M. Murakami, T. Hayashi, and S. Fuke, *Nippon Suisan Gakkaishi,* 1978, **44,** 1165.
[24] J.H. Waite, and S.O. Andersen, *Biochim. Biophys. Acta,* 1978, **541,** 107.
[25] B. Bach, R.P. Gregson, G.S. Holland, R.J. Quinn, and J.L. Reichelt, *Experientia,* 1978, **34,** 608.
[26] R. Kazlauskas, P.T. Murphy, and R.J. Wells, *Tetrahedron Letters,* 1978, 4945.
[27] J.D. Kemp, *Plant Physiol.,* 1978, **62,** 26; D.W. Sutton, J.D. Kemp, and E. Hack, *ibid.,* p. 363.
[28] (*a*) M. Sato, Y. Sato, and Y. Tsuchiya, *Nippon Suisan Gakkaishi,* 1977, **43,** 1441 (*Chem. Abs.,* 1978, **88,** 84 761); (*b*) *ibid.,* 1978 **44,** 247.
[29] L.M. Amende and S.K. Pierce, *Comp. Biochem. Physiol. B,* 1978, **59B,** 257.
[30] L.S. Frost, M. Carpenter, and W. Paranchych, *Nature,* 1978, **271,** 87; W. Paranchych, L.S. Frost, and M. Carpenter, *J. Bacteriol.,* 1978, **134,** 1179.
[31] M.A. Hermodson, K.C.S. Chen, and T.M. Buchanan, *Biochemistry,* 1978, **17,** 442.
[32] T. Hase, S. Wakabayashi, H. Matsubara, L. Kerscher, D. Oesterhelt, K.K. Rao, and D.O. Hall, *J. Biochem. (Tokyo),* 1978, **83,** 1657.
[33] J.J. van Buskirk and W.M. Kirsch, *Biochem. Biophys. Res. Comm.,* 1978, **80,** 1033; 1978, **82,** 1329.
[34] K. King, *Biochim. Biophys. Acta,* 1978, **542,** 542.
[35] K. King, *Nature,* 1978, **273,** 41.
[36] J.D. Hulse, S.R. Ellis, and L.M. Henderson, *J. Biol. Chem.,* 1978, **253,** 1654.
[37] G. Agrup, C. Hansson, H. Rorsman, A.M. Rosengren, and E. Rosengren, *Acta Derm.-Venereol.,* 1978, **58,** 269.
[38] C.S. Evans and E.A. Bell, *Phytochemistry,* 1978, **17,** 1127.

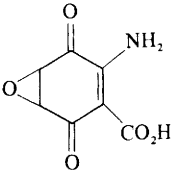
japonicus.[39] L-1,4-Thiazane-3-carboxylic acid (2) is a constituent of the alga *Heterochordaria abietina*,[40] while tetra-acetylclionamide (an N^{α}-acetyl-6-bromotryptophanamide from the sponge *Cliona celata*)[41] and four families of *N*-acyl β-methylene-β-alanine esters from an unidentified black sponge[42] are closer relatives of well-known natural free amino-acids. Particularly interesting structures have been established for palythine (3; R = H)[43] and derivatives palythene (3; R = *trans*-MeCH=CH–) and palythinol (3; R = $HOCH_2CHMe$) from the zoanthid *Palythoa tuberculosa*.[44] Palythene is optically-inactive.[43] While undeniably an amino-acid, antibiotic I-851 (4) is a structural type normally outside the scope of this Chapter; this chloramphenicol antagonist



(2)



(3)



(4)

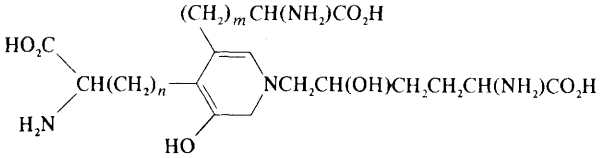
was isolated from *Streptococcus fulvoviolaceus*.[45] Related culture studies have led to the isolation of 4-[(*E*)-4′-hydroxy-3′-methylbut-2′-enyl]-L-tryptophan, in addition to the previously reported 4-dimethylallyltryptophan, from cell-free extracts of *Claviceps paspali* with isopentenyl pyrophosphate and L-tryptophan.[46] *Flavobacterium rigense* accumulates *O*-2-hydroxypropyl homoserine in a medium containing propane-1,2-diol as sole carbon source.[47]

New amino-acids from normal human urine are α-amino-γ,δ-dihydroxyadipic acid[48] and α-hydroxy-β-keto-γ-aminobutyric acid.[49]

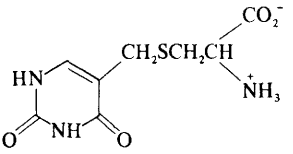
New Amino-acids from Hydrolysates.—The main interest in this section continues to be found in novel protein cross-linking residues and in peptide antibiotics.

Bovine bone collagen contains pyridinoline (5; $n + m = 3$); further studies are in hand to resolve the structural ambiguities.[50] U.v. irradiation (253 nm) induces cross-links, tentatively identified as α-*S*-cysteinylthymine (6), between

[39] S. Hatanaka and S. Kaneko, *Phytochemistry*, 1978, **17**, 2027.
[40] K. Kawauchi, T. Kosuru, S. Ota, and T. Suzuki, *Nippon Suisan Gakkaishi*, 1977, **43**, 1293.
[41] R.J. Andersen, *Tetrahedron Letters*, 1978, 2541.
[42] M.B. Yunker and P.J. Scheuer, *Tetrahedron Letters*, 1978, 4651.
[43] S. Takano, D. Uemura, and Y. Hirata, *Tetrahedron Letters*, 1978, 2299.
[44] S. Takano, D. Uemura, and Y. Hirata, *Tetrahedron Letters*, 1978, 4909.
[45] Y. Imagawa, S. Shima, A. Hirota, and H. Sakai, *Agric. Biol. Chem.*, 1978, **42**, 681.
[46] R.J. Petroski and W.J. Kelleher, *Lloydia*, 1978, **41**, 332.
[47] S. Yamada, K. Nabe, T. Ujimaru, N. Izuo, and M. Chibata, *Appl. Environ. Microbiol.*, 1978, **35**, 1046.
[48] S. Yuasa, *Biochim. Biophys. Acta*, 1978, **540**, 93.
[49] M. Kinuta, *Biochim. Biophys. Acta*, 1978, **542**, 56.
[50] D. Fujimoto, T. Moriguchi, T. Ishida, and H. Hayshi, *Biochem. Biophys. Res. Comm.*, 1978, **84**, 52.



(5)



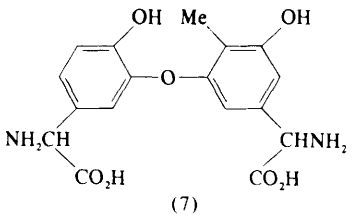
(6)

protein and polynucleotide in phage fd gene 5 protein complexes with fd DNA or poly(dT).[51]

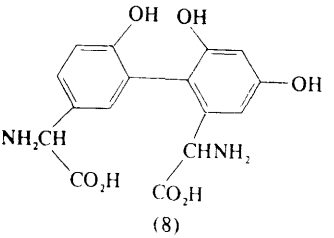
Further progress has been made in structural assignments to amino-acids released from ristomycins[52,53] and related antibiotics (vancomycin, *alias* vancosamine[54]) on hydrolysis. The oxygen-bridged phenylglycine derivative (7; ristomycinic acid[52]) and the biphenyl (8; actinoidinoic acid [52]) have been identified as components of ristomycins, and the evidence for the presence of two β-hydroxychlorotyrosine, three oxygenated phenylglycines, on *N*-methylleucine, and an aspartic acid residue in vancomycin, has been reviewed.[54] N^{β}-Methyl-L-β-arginine $H_2NC(=NH)NHCH_2CH_2CH(NHMe)CH_2CO_2H$ is a constituent of the newly discovered antibiotic LL-BM547β.[55]

3 Chemical Synthesis and Resolution of Amino-acids

General Methods of Synthesis of α-Amino-acids.—Alkylation of glycine derivatives is formally a general synthetic route to α-amino-acids but is fre-



(7)



(8)

[51] P. Paradiso, Y. Nakashima, and W. Konigsberg, 'Biomolecules: Structure and Function', ed. P.F. Agris, Academic Press, New York, 1977, p. 581.
[52] G.S. Katrukha, P.B. Terentev, B. Diarra, and E.S. Gershtein, *Khim. prirod. Soedinenii*, 1978, 141.
[53] C.M. Harris, J.J. Kibby, and T.M. Harris, *Tetrahedron Letters*, 1978, 705.
[54] G.M. Sheldrick, P.G. Jones, O. Kennard, D.H. Williams, and G.A. Smith, *Nature*, 1978, **271**, 223.
[55] S. Nomoto and T. Shiba, *Chem. Letters*, 1978, 589.

quently inefficient, owing to low reactivity. When activated halides are used as alkylating agents to overcome this source of low yields, appreciable amounts of unwanted $\alpha\alpha$-dialkylated products may be formed. Phase transfer alkylation of the stable Schiff base $Ph_2C{=}NCH_2CO_2Et$ is something of an improvement over earlier methods based on this process[56] (though some α-benzylphenylalanine is formed using benzyl halides), and the corresponding Schiff base $Ph_2C{=}NCH_2CN$ can be employed analogously with non-activated halides.[57] Examples of the related azlactone synthesis[58,59] and other examples of the use of an aldehyde to create an amino-acid side-chain $Me_2CHCHO \longrightarrow Me_2CHCH(OH)CN \longrightarrow Me_2CHCH(OSOCl)CN \longrightarrow$ DL-valine;[60] methyl isocyanoacetate with R^1COR^2 giving cycloalk-1-enylglycines after hydrolysis,[61] or Schiff bases of $\alpha\beta$-dehydro-amino-acids $R_2NCH{=}NC(=CR^1R^2)CO_2Me$ *via* Michael addition of the amine to the isocyanoacrylate, followed by synchronous elimination – insertion of the amine into the isocyano-group[62]). Related applications of methyl isocyanoacetate [$\longrightarrow RCOCH(NH_2)CO_2Me$ with RCOCl or $(RCO)_2O$, thence to the substituted oxazole-4-carboxylate and to the erythro-β-hydroxyamino-acid after hydrolysis and hydrogenation over Pt, or $NaBH_4$ reduction;[63] $CNCH_2CONR^1R^2 + MeCHO \longrightarrow$ *trans*-5-methyloxazolin-2-yl-4-carboxamide $\longrightarrow$ N^α-formyl-DL-threoninamides with formic acid[64]] illustrate novel routes to β-hydroxy-α-amino-acids. Condensation of a methyl 2-isocyanoalkanoate with 1-phenylethylamine, followed by cyclization (BuLi) gives the corresponding 4-substituted *N*-(l-phenylethylimidazolidin-5-one), which can be alkylated further, giving $\alpha\alpha$-dialkyl-α-amino-acids.[65] Further examples of Ugi four-component condensation reactions, leading to N^α-benzyl-amino-acid amides,[66,67] have been described; one of these studies[67] involves CO_2 as a reactant.

Carbon–carbon bond formation methods described in the preceding paragraph include several processes in which bis-alkylation is avoided, as is also the case with the acetamidomalonate route.[21,68–73] 2-Amino-4-methylhexanoic acid,[21] 2-amino-4-(2-aminoethoxy)-*trans*-but-3-enoic acid[68] and its 4-methoxy analogue,[73] and acetyltyrosine[69] have been prepared by this method, and 3,5-

[56] M.J. O'Donnell, J.M. Boniece, and S.E. Earp, *Tetrahedron Letters*, 1978, 2641.
[57] M.J. O'Donnell and T.M. Eckrich, *Tetrahedron Letters*, 1978, 4625.
[58] E.I. Karpeiskaya, E.S. Neupokoeva, L.F. Godunova, I.P. Murina, A.P. Kharchevnikov, and E.I. Klabunovskii, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1978, 1368; L.F. Godunova, E.S. Neupokoeva, E.I. Karpeiskaya, and E.I. Klabunovskii, *ibid.*, p. 1363.
[59] M.A. Rakshinda and N. H. Khan, *Indian J. Chem.*, 1978, **16B**, 634.
[60] J.W. Davis, *J. Org. Chem.*, 1978, **43**, 3980.
[61] M. Suzuki, K. Nunami, and N. Yoneda, *J.C.S. Chem. Comm.*, 1978, 270.
[62] M. Suzuki, K. Nunami, T. Moriya, K. Matsumoto, and N. Yoneda, *J. Org. Chem.*, 1978, **43**, 4933.
[63] M. Kirihata, H. Tokumori, I. Ichimoto, and H. Ueda, *Nippon Nogei Kagaku Kaishi*, 1978, **52**, 271.
[64] Y. Ozaki, K, Matsumoto, and M, Miyoshi, *Agric. Biol. Chem.*, 1978, **42**, 1565.
[65] U. Schöllkopf, H.H. Hansberg, I. Hoppe, M. Segal, and U. Reiter, *Angew. Chem.*, 1978, **90**, 136.
[66] H.R. Divanfard, Z. Lysenko, P.-C. Wang, and M.M. Joullie, *Synthesis Comm.*, 1978, **8**, 269.
[67] E. Haslinger, *Monatsh. Chem.*, 1978, **109**, 749.
[68] D.D. Keith, R. Yang, J.A. Tortora, and M. Weigele, *J. Org. Chem.*, 1978, **43**, 3713.
[69] L. Florval, S.B. Ross, S.O. Ogren, and N.E. Stjernstrom, *Acta Pharm. Suec.*, 1978, **15**, 13.
[70] H.J. Teuber, H. Krause, and V. Berariu, *Annalen*, 1978, 757.
[71] W. Oppolzer and H. Andres, *Tetrahedron Letters*, 1978, 3397.
[72] G.A. Dilbeck, L. Field, A.A. Gallo, and R.J. Gargiulo, *J. Org. Chem.*, 1978, **43**, 4593.
[73] D.D. Keith, J. A. Tortora, and R. Yang, *J. Org. Chem.*, 1978, **43**, 3711.

di-t-butyltyrosine and (3,5-di-t-butyl-4-hydroxyphenyl)glycine have been prepared *via* the rarely-used formamidomalonate.[70]

An interesting synthesis of arylglycines from the corresponding aldehyde, bromoform, and NH_3–KOH–$LiNH_2$ involves a bromo-oxirane intermediate.[74] Further examples of arylglycine synthesis using β-hydroxyglycine derivatives have been described.[75]

Electroreductive amination of α-keto-acids in aqueous NH_4OH–NH_4Cl at mercury electrodes proceeds with low yields in some cases owing to competing condensation reactions.[76] Radical amination of corresponding hydroxy acids in aqueous NH_4OH under γ-irradiation has been shown to produce alanine, β-alanine, glycine, aspartic acid, and α- and γ-aminobutyric acids.[77]

Synthesis of β-Amino-acids and Higher Homologues.—Mention is made in the preceding section, and elsewhere in this Chapter,[78,79] of the synthesis of homologues of α-amino-acids. Little scope exists for generalizations covering synthetic methods for these compounds, but the use of the Arndt–Eistert reaction for conversion of N^{α}-toluene-*p*-sulphonyl-N^{δ}-Boc-L-ornithine into the β-lysine derivative, followed by treatment with *O*-methyl-*N*-nitroisourea, in the synthesis of N^{α}-methyl-L-β-arginine,[55] and electroreduction of cyanoacetic acid to β-alanine,[80] illustrate two standard methods.

Asymmetric Synthesis.—Increased interest in this topic, though using well-established principles, is noticeable. Asymmetric hydrogenation of α-acylaminoacrylic acids catalysed by chiral Rh^{I}–phosphine complexes[81] now interests several research groups. Optical purity 73—93 %[81] and 'reaching 100 %'[82] have been recorded in the synthesis of enantiomers of alanine[81,83] and phenylalanine.[82,85–87] Optically-active β-amino-acids are available through the same process applied to β-acylamino-acrylates.[88] L-Dopa and L-phenylalanine can be obtained from the corresponding azlactones by hydrogenation over Pd in the presence of (*S*)-phenylethylamine.[58]

Hydrogenolysis is a key step in asymmetric transamination procedures employing chiral benzylamines. Asymmetric synthesis of alanine from ethyl pyruvate *via* the Schiff base formed with (*R*)-$H_2NCHPhCO_2R$, on treatment with H_2–Pd to give the secondary amine (highest optical purity with a bulky ester grouping R), acid hydrolysis, and hydrogenolysis, leads to the L-isomer

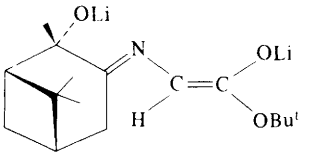
[74] E.L. Compere and D.A. Weinstein, *Synthesis*, 1977, 852.
[75] D. Ben-Ishai, J. Altman, Z. Bernstein, and N. Peled, *Tetrahedron*, 1978, **34**, 467; A. Schouteeten, Y. Christidis, and G. Mattioda, *Bull. Soc. chim. France*, 1978, 248.
[76] E.A. Jeffrey and A. Meisters, *Austral. J. Chem.*, 1978, **31**, 73; E.A. Jeffrey, O. Johansen, and A. Meisters, *ibid.*, p. 79.
[77] K. Ema, T. Kato, and M. Shinagawa, *Radioisotopes*, 1978, **27**, 445.
[78] D.H. Rich, E.T. Sun, and A.S. Boparai, *J. Org. Chem.*, 1978, **43**, 3624.
[79] J.A. Yankeelov, K.F. Fok, and D.J. Carothers, *J. Org. Chem.*, 1978, **43**, 1623.
[80] V. Krishnan, K. Ragapathy, and H.V.K. Udupa, *J. Appl. Electrochem.*, 1978, **8**, 169.
[81] K. Hanaki, K. Kashiwabara, and J. Fujita, *Chem. Letters*, 1978, 489.
[82] J. Vilim and J. Hetflejs, *Chem. prumysl*, 1978, **28**, 135 (*Chem. Abs.*, 1978, **89**, 180 317).
[83] K. Herrmann, *Nachr. Chem., Tech. Lab.*, 1978, **26**, 651 (*Chem. Abs.*, 1978, **89**, 215 729).
[84] N. Takaishi, H. Imai, C.A. Bertelo, and J.K. Stille, *J. Amer. Chem. Soc.*, 1978, **100**, 264.
[85] G. Descotes, D. Lafont, and D. Sinou, *J. Organometallic Chem.*, 1978, **150**, C14.
[86] R. Jackson and D.J. Thompson, *J. Organometallic Chem.*, 1978, **159**, C29.
[87] T. Masuda and J.K. Stille, *J. Amer. Chem. Soc.*, 1978, **100**, 268.
[88] K. Achiwa and T. Soga, *Tetrahedron Letters*, 1978, 1119.

predominantly when hydrogenation is carried out at −10 ° to +20 °C, but to the D-enantiomer at higher temperatures.[89] Chiral serines are obtained in optical purity 28—39 % by successive hydrolysis and hydrogenolysis of the chiral aziridinecarboxylates formed between αβ-dibromopropionates and (*R*)-phenylethylamine;[90] aspartic acids have been obtained analogously from dibromosuccinates in rather better optical yields.[91] Only low optical yields (2—18 %) have so far been achieved by alkylation of *N*-[(*S*)-phenylethyl]-formamidoacetonitrile followed by hydrolysis and hydrogenolysis, indicating minimal stereoselectivity in the deprotonation step (NaH) preceding alkylation.[92] Alkylation of the di-anion (9) from (+)-pinanone and glycine t-butyl ester followed by hydrolysis gives the corresponding D-amino-acid t-butyl ester with regeneration of the chiral reagent,[93] which is an improvement relative to corresponding steps in the transamination of α-keto acids with the hydrazide analogue of (−)-ephedrine[94] and with a chiral pyridoxamine analogue.[95] Asymmetric alkylation of (+)-menthyl α-isocyanopropionate has been described.[96]

Asymmetric synthesis of β-amino-acids *via* Reformatsky reactions using (−)-menthyl bromoacetate and chiral Schiff bases $R^1CH{=}NR^2$[R^2 = (*R*)-phenylethyl] favours the (*S*)-enantiomers to the extent of 2—28 %.[97]

Prebiotic Synthesis; Model Reactions.—As in the preceding section, there are more papers eligible for citation here, compared with previous Volumes, but most of the work described involves development of familiar approaches.

The current work of virtually all the active research groups in this field has been collected in a '*Proceedings*' volume.[98] The temptation to select material from this volume for discussion has been resisted, since any selection would have been unrepresentative, but the material has been published already in the primary sources.



(9)

[89] K. Harada and Y. Kataoka, *Chem. Letters*, 1978, 791; *Tetrahedron Letters*, 1978, 2103.
[90] K. Harada and I. Nakamura, *J.C.S. Chem. Comm.*, 1978, 522.
[91] K. Harada and I. Nakamura, *Chem. Letters*, 1978, 9.
[92] K. Harada, M. Tamura, and S. Suzuki, *Bull. Chem. Soc. Japan*, 1978, **51**, 2171.
[93] T. Oguri, N. Kawai, T. Shioiri, and S. Yamada, *Chem. Pharm. Bull.*, 1978, **26**, 803.
[94] H. Takahashi, H. Neguchi, K. Tomita, and H. Otomasu, *Yakugaku Zasshi*, 1978, **98**, 618.
[95] H. Kuzuhara, T. Komatsu, and S. Emoto, *Tetrahedron Letters*, 1978, 3563.
[96] M. Kirihata, S. Mihara, I. Ichimoto, and H. Ueda, *Agric. Biol. Chem.*, 1978, **42**, 185.
[97] M. Furukawa, T. Okawara, Y. Noguchi, and Y. Terewaki, *Chem. Pharm. Bull.*, 1978, **26**, 260.
[98] 'Origins of Life,' Proceedings of the 2nd ISSOL Meeting 1977, ed. H. Noda, Bus. Cent. Acad. Soc., Tokyo, 1978; *inter alia* see I. Draganic, Z. Draganic, A. Shimoyama, and C. Ponnamperuma, p. 53 (*Chem. Abs.*, 1979, **90**, 49 864); A. Shimoyama, N. Blair, and C. Ponnamperuma, p. 95; C.S. Matthews, p. 123.

Perhaps the most interesting work, from a chemist's viewpoint, is the oligomerization of HCN in 0.1M aqueous solutions at pH 9.2, subsequent hydrolysis giving purines, pyrimidines, and amino-acids.[99] This topic has been discussed in earlier volumes in this series, as have studies of contact glow-discharge electrolysis, extended to the amination of aliphatic cyanides in aqueous NH_4OH[100] and the cyanation of aliphatic amines,[100] hydroxyamino-acid formation from alkenoic acids in aqueous NH_4OH,[101] β-hydroxyaspartic acid formation from maleic and fumaric acids,[101] and conversion of β-amino-acids formed in this way into α-amino-acids (through excision of the α-methylene or -methine grouping[102]). Electric discharge through CH_4–NH_3–H_2O mixtures gives amino-nitriles in very low yields[103] and analogous mixtures subjected to high-frequency plasmolysis[104] give products in proportions influenced by catalysts, pH, and the characteristics of the energy source. Aqueous solutions of metal cyanides exposed to multi-krad doses from a ^{60}Co source give uncharacterized material which releases five protein amino-acids and five non-protein amino-acids on hydrolysis.[105a] Similar treatment of aqueous MeCN yields glycine, lysine, aspartic acid, serine, and glutamic acid,[106,105b] while EtCN gives nine amino-acids.[105b] γ-Irradiation of cyanamide, H_2NCN, in aqueous solutions gives arginine and other compounds.[107]

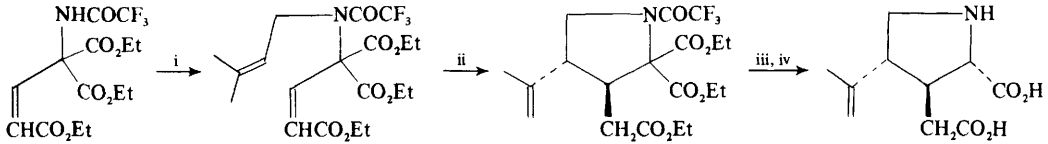
Glycine is formed in 2.5 % aqueous formaldehyde, saturated with N_2 and subjected to ultrasonic sound.[108]

Protein Amino-acids and Other Naturally-occurring Amino-acids.—The preceding sections have described synthetic methods which have been used, more often than not, for the synthesis of naturally-occurring amino-acids. This section deals with specific rather than general synthetic methods.

The synthesis of lysine from ε-caprolactam,[109] and the routes available to canaline [$H_2NOCH_2CH_2CH(NH_2)CO_2H$][110] have been reviewed. Full details of the synthesis of DL-capreomycidine (Vol. 10, p. 9) from *threo*-β-hydroxy-ornithine have been published.[111]

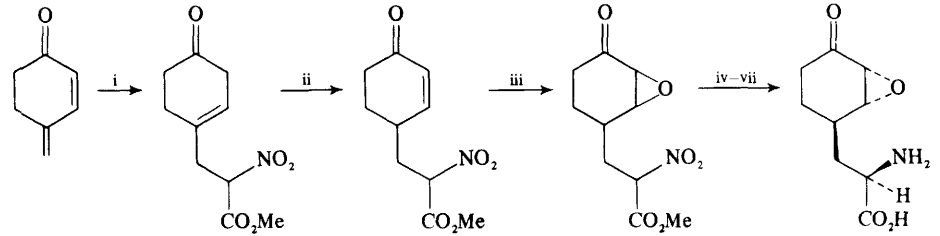
Trichloroethene and amino-acetonitrile are intermediates in the synthesis of glycine from tetrachloroethanes in aqueous NH_4OH at 160—170 °C.[112] Mechanistically interesting syntheses have also been reported for α-allokainic

[99] J.P. Ferris, P.C. Joshi, E.H. Edelson, and J.G. Lawless, *J. Mol. Evol.*, 1978, **11**, 293.
[100] K. Harada, S. Suzuki, and H. Ishida, *BioSystems*, 1978, **10**, 247.
[101] K. Harada, S. Suzuki, and H. Ishida, *Experientia*, 1978, **34**, 17.
[102] S. Suzuki, M. Tamura, J. Teresawa, and K. Harada, *Bio-org. Chem.*, 1978, **7**, 111.
[103] H. Ebisawa, S. Mankino, E. Miyoshi, T. Shirai, and S. Yanagisawa, *Nippon Kagaku Kaishi*, 1978, 351 (*Chem. Abs.*, 1978, **88**, 169 292).
[104] C.I. Simionescu, S. Dumitriu, V.I. Popa, V. Bulacovschi, and B. Simionescu, *Rev. Roumaine Chim.*, 1978, **23**, 89.
[105] (*a*) Z. Draganic, I. Draganic, A. Shimoyama, and C. Ponnamperuma, *Origins of Life*, 1977, **8**, 371; (*b*) *ibid.*, p. 377.
[106] M.J. Shushtarian, *Q. Bull. Fac. Sci., Tehran Univ.*, 1976, **8**, 19 (*Chem. Abs.*, 1978, **88**, 90 006).
[107] Z.D. Draganic, I.G. Draganic, and S.V. Jovanovic, *Radiation Res.*, 1978, **75**, 508.
[108] A.V. Sokolskaya, *Zhur. obshchei Khim.*, 1978, **48**, 1407.
[109] M. Ciha, E. Stefanikova, and A. Zvakova, *Chem. listy*, 1978, **72**, 1066.
[110] T. Korpela, J. Lundell, and P. Pasanen, *Org. Prep. Proced. Int.*, 1977, **9**, 57.
[111] T. Wakamiya, K. Mizuno, T. Ukita, T. Teshima, and T. Shiba, *Bull. Chem. Soc. Japan*, 1978, **51**, 850.
[112] M. Inoue and S. Enomoto, *Chem. Letters*, 1978, 1231.



Reagents: i, NaH–1-bromo-3-methylbut-1-ene; ii, 170 °C, 10 min; iii, 0.5M NaOH, reflux; iv, H_3O^+

Scheme 1



(10)

Reagents: i, $NO_2CH_2CO_2Me$; $PhCH_2\overset{+}{N}Me_3\bar{O}H$; ii, 1% aq.HCl–DMSO (50% conversion; separate on silica gel); iii, alkaline H_2O_2–MeOH– –20 °C; iv, H_2–Raney Ni–Ac_2O; v, silica gel chromatography; vi, alkaline hydrolysis (1.25M NaOH, 5 °C, 15 min); vii, hog kidney acylase

Scheme 2

acid (Scheme 1; in which the ingredients for an ene reaction are set up, starting from diethyl *N*-trifluoroacetylaminomalonate),[71] and for the antibiotic anticapsin (10; for which the obvious starting point, a reduced tyrosine, was precluded by the ease of intramolecular addition of the amino-function to the conjugated ketone).[113]

Stereospecific synthesis of the chiral lactone ester analogue establishes the all-*cis* stereochemistry (1) for the antibiotic thermozymocydin (myriocin).[22] The synthesis of (3*S*,5*S*)-5-hydroxyhexahydropyrazine-3-carboxylic acid, a constituent of the monamycin antibiotics, has been established.[114] The starting point, pyridazino[1,2-*b*]phthalazine, proved to be a suitable substrate for stereospecific introduction of the hydroxy- and carboxy-groups, the phthaloyl residue being cleaved at the end of the synthesis with hydrazine.

The pepstatin constituent, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid ('statine'), has been synthesized from Boc-L-leucine methyl ester *via* Bu^t_2AlH reduction to the leucinal, followed by addition of $LiCH_2CO_2Et$ and conventional subsequent steps.[78]

Aliphatic Amino-acids.—Hydrogenation of (*R*)-phenylglycine over $Pd(OH)_2$ around neutral pH gives (*R*)-cyclohexylglycine.[115]

α-Alkyl Analogues of Protein Amino-acids.—A synthesis of α-methylarginine, produced by Streptomyces X-11837,[116] involves the cleavage of 2-methyl-2-acetamidovalerolactone with 30% HBr, and elaborating the resulting $R(CH_2)_3CMe(NHAc)CO_2H$ (R = Br) successively into intermediates [R = PhCH=N(O)] and (R = HONH).[117] An asymmetric synthesis of α-methylornithine depends on Michael addition of (+)-menthyl α-isocyanopropionate carbanion to acrylonitrile.[96]

α-Hetero-atom Substituted α-Amino-acids.—Full details are reported for the conversion of *N*-acetylamino-acid methyl esters into α-methoxy-analogues using Bu^tOCl and MeOH.[118] α-Chloro-α-acylamido-acids formed[119] with AcCl from α-hydroxy-α-acylamidoacids (*i.e.* amide–glyoxylic acid adducts)[75] can be converted into alkoxy-, alkythio-, or alkanesulphonyl-analogues through nucleophilic substitution.[119]

α-Halogenoalkyl-α-amino-acids.—Alkylation of the carbanion derived from benzylidenealanine methyl ester with halogenomethanes gives α-halogenomethyl α-amino-acids.[120]

Aliphatic Amino-acids Carrying Hydroxy Groups in Side-chains.—Hydroxyalkylation of *N*-salicylideneglycine ethyl ester by acetaldehyde in the presence of base gives the threonine derivative, exemplifying further this well-studied

[113] R.W. Rickards, J.C. Rodwell, and K.J. Schmalzl, *J.C.S. Chem. Comm.*, 1977, 849.
[114] C.H. Hassall and K.L. Ramachandran, *Heterocycles*, 1977, **7**, 119.
[115] M. Tamura and K. Harada, *Synthesis Comm.*, 1978, **8**, 345.
[116] T.C. Demny and H. Maehr, U.S.P. 4 061 542 (*Chem. Abs.*, 1978, **88**, 72 986).
[117] H. Maehr and M. Leach, *J. Antibiotics*, 1978, **31**, 165.
[118] H. Poisel, *Chem. Ber.*, 1978, **111**, 3136.
[119] D. Matthies, *Synthesis*, 1978, 53.
[120] P. Bey and J.P. Vevert, *Tetrahedron Letters*, 1978, 1215.

addition reaction (see also Vol. 10, p. 10).[121] A different approach to converting one amino-acid into another is the chiral synthesis of Boc-D-homoserine lactone through treatment of *N*-Boc-*S*-methyl-D-methionone trimethylsilyl ester with $KOBu^t$ in refluxing THF.[122]

A new synthesis of γ-hydroxyglutamic acid involves hydrogenolysis of 3-ethoxycarbonyl-5-methoxycarbonylisoxazoline (the 1,3-dipolar adduct of methyl acrylate and ethyl cyanoformate-*N*-oxide).[123]

To examples elsewhere in this Chapter of higher homologous hydroxyalkyl-amino-acids can be added the synthesis of 4-amino-3-hydroxybutanoic acid.[124]

α-Amino-acids with Unsaturated Side-chains.—The two main structural types covered by this section, 'αβ-dehydro-amino-acids' $R^1R^2C{=}C(NH_2)CO_2H$, and alkenyl or alkynyl amino-acids, are represented increasingly regularly in the literature. Dehydration of *N*-protected serine and threonine esters can be performed with Ph_3P and diethyl azodicarboxylate, giving the protected dehydro-amino-acids ($R^1 = R^2 = H$) and ($R^1 = H$, $R^2 = Me$), respectively.[125] Amino-substituted analogues ($R^1 = H$, $R^2 = NH_2$) can be prepared from ethyl pyruvate, after conversion into the protected dehydro-alanine $CH_2{=}CH(NHZ)CO_2Et$, by successive *N*-bromination (NBS), *N*-azidation (NaN_3) and spontaneous N→C shift, and Raney nickel reduction.[126]

A novel approach to the synthesis of α-vinyl-α-amino-acids depends on the exclusive α-alkylation of the carbanion formed with $LiN(SiMe_3)_2$ from the Schiff base of a dehydro-amino-acid ($R^1 = H$, $R^2 = Me$): $\overset{\frown}{CH_2{\cdots}CH{\cdots}C}(N{=}CHPh)CO_2Me \rightarrow CH_2{=}CHCR(N{=}CHPh)CO_2Me$.[127] In a related approach,[128] the di-anion formed from *N*-ethoxycarbonyl trimethyl-silyglycine methyl ester by treatment with $LiNPr^i_2$ in HMPA undergoes mono-alkylation to give α-ethynyl-α-amino-acids. This synthon rearranges into the allenic isocyanate $Me_3SiCH{=}C{=}C(N{=}C{=}O)CO_2Me$ when used in an attempt to prepare the first member of the series, 2-aminobutynoic acid.[128]

Aromatic and Heterocyclic Amino-acids.—Aromatic and heteroaromatic side-chains may be introduced into glycine derivatives through general synthetic routes described in an earlier section, and this section deals with subsequent modifications to side-chains, and the construction of heteroaromatic rings from acyclic precursors.

Benzylideneazlactone on treatment with hydroxylamine gives the ring-opened adduct PhCH(NHOH)CH(NHCOPh)CONHOH, from which β-

[121] Y.N. Belokon, V.M. Belikov, V.A. Maksakov, N.G. Faleev, and E.A. Paskonova, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1978, 1026.
[122] R.D.G. Cooper, F. Jose, L. McShane, and G.A. Koppel, *Tetrahedron Letters*, 1978, 2243.
[123] T. Kusumi, H. Kakisawa, S. Suzuki, K. Harada, and C. Kashima, *Bull. Chem. Soc. Japan*, 1978, **51**, 1261.
[124] M. Pinzi and G. Pifferi, *J. Pharm. Sci.*, 1978, **67**, 120.
[125] H. Wojciechowska, R. Pawlowicz, R. Andruszkiewicz, and J. Grzybowska, *Tetrahedron Letters*, 1978, 4063.
[126] C. Shin, K. Watanabe, H. Ohmatsu, and J. Yoshimura, *Tetrahedron Letters*, 1978, 4535.
[127] W.J. Greenlee, D. Taub, and A.A. Patchett, *Tetrahedron Letters*, 1978, 3999.
[128] P. Casara and B.W. Metcalf, *Tetrahedron Letters*, 1978, 1581.

aminophenylalanine can be obtained by hydrogenation (over Pd–C) followed by acid hydrolysis.[129]

L-Histidine carrying a trifluoromethyl group at C-2 of the imidazole ring can be obtained by Bamberger cleavage of the amino-acid followed by recyclization in refluxing TFAA.[130] Fischer indolization of *N*-acetyl-L-glutamic *γ*-semi-aldehyde with *p*-methoxy- or -phenoxy-phenylhydrazine gives the corresponding 5-methoxy- or -phenoxy-L-tryptophans.[131] A synthesis of racemic *α*-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid, an analogue of ibotenic acid, has been accomplished, the key step being the cyclization of the corresponding oxime.[132]

***N*-Substituted Amino-acids.**—Continuing efforts aimed at the synthesis of *N*-methylamino-acids are represented by a paper from one of the main innovators in this field, demonstrating reductive *NN*-dimethylation of Boc-amino-acids in a MeOH–O_2–Pd/C brew, equivalent to methanolic formaldehyde.[133] *N*-Hydroxytyrosine has been synthesized from the keto-acid oxime by $Na(CN)BH_3$ reduction.[134] *N*-(Alkylthiomethyl)amino-acids are readily obtained using formaldehyde and a thiol.[135]

Excluding citations of *N*-protected amino-acids prepared for use in peptide synthesis, two topics remain, *viz.* the preparation of a wide variety of *N*-alkylated DL-phenylalanines by reduction of the corresponding Schiff bases,[136] and continuing Australian work (preparation of *N*-substituted analogues)[137] on L-mimosine (3-hydroxy-4-oxo-1,4-dihydropyrid-1-yl)-L-alanine, an amino-acid known to cause defleecing of sheep.[138]

***α*-Amino-acids Containing Sulphur.**—Carefully worked out procedures, some amounting to improvements of known methods, have been described for the synthesis of heterocyclic analogues of cysteine, norcysteine, and homocysteine (*e.g.* 1,3-thiazane-6-carboxylic acid).[139] 5-Mercapto-4,4-dimethyl-2-amino-pentanoic acid, a bis homologue of penicillamine has been synthesized from acetamidomalonate and $Me_2C{=}CHCH_2CL$, followed by BF_3-catalysed addition of $PhCH_2SH$ (the first example of BF_3-catalysed Markownikoff addition of a thiol to an alkene).[72] 3-Alkyl analogues of the same homologue have been prepared through the azlactone route, using $R^1SCR^2R^3CH_2COR^4$ and hippuric acid.[59]

[129] R.M. Ali and N.H. Khan, *Synthesis Comm.*, 1978, **8**, 497.
[130] H. Kimoto, K.L. Kirk, and L.A. Cohen, *J. Org. Chem.*, 1978, **43**, 3403.
[131] S. Iriuchijima and G. Tsuchihashi, *Agric. Biol. Chem.*, 1978, **42**, 843.
[132] S.B. Christensen and P. Krogsgaard-Larsen, *Acta Chem. Scand.*, 1978, **B32**, 27.
[133] F.M.F. Chen and N.L. Benoiton, *Canad. J. Chem.*, 1978, **56**, 150.
[134] B.K. Malik and I.J. McFarlane, *Indian J. Chem. B*, 1978, **16B**, 76.
[135] K. Ito, R. Komaki, and M. Sekiya, *Chem. Pharm. Bull.*, 1977, **25**, 3385.
[136] S. Takemura, H. Terauchi, K. Kowata, K. Nakano, Y. Okumura, K. Li, and Y. Inamori, *Yakagaku Zasshi*, 1978, **98**, 869.
[137] F.H.C. Stewart, *Austral. J. Chem.*, 1978, **31**, 1861.
[138] P.J. Reis, *Austral. J. Agric. Res.*, 1978, 29, 1043; P.J. Reis and D.A. Tunks, *ibid.*, p. 1057; P.J. Reis, D.A. Tunks, and A.M. Downes, *ibid.*, p. 1065.
[139] N.J. Lewis, R.L. Inloes, J. Hes, R.H. Matthews, and G. Milo, *J. Med. Chem.*, 1978, **21**, 1070.

A List of Amino-acids which have been Synthesized for the First Time.—New amino-acids not mentioned elsewhere in this Chapter are collected here.

Compound	*Ref.*
$N^{\beta}N^{\beta}$-Dialkylasparagines	140
$N^{\gamma}N^{\gamma}$-Dialkylglutamines	140
L-Cysteic acid sulphonamides, H-Cys(SO$_2$NHR)-OH (R = *O*-alkyl; CH_2CH_2Cl; $CH_2C_6H_4$-*p*-SO_2F; $NHCOC_6H_4$-*p*-SO_2F; *trans*-NHCOCH=CHCO$_2$Et)	141
2′-Deoxyadenosyl-L-homocysteine	142
(2,3-Epoxycyclohexyl)glycine	143
(3,5-Di-t-butyl-4-hydroxyphenyl)glycine	144
N-Bromoacetyl-*p*-azido-L-phenylalanine methyl ester	145
3-(OR)-4(OR′)-Dopas (R = Me, Et, Pr, or Bu^s; R′ = H, Me, or Bu)	146
5-Fluorodopa{(3,4-dihydroxy-5-fluorophenyl)alanine}	147
2-Arylazo-L-histidines	148
4-Arylazo-L-histidines	148
2,4-Bis-arylazo-L-histidines	148

Labelled Amino-acids.—The synthesis and metabolism of chirally labelled α-amino-acids have been reviewed.[149]

Further studies of ^{3}H-labelling of amino-acids by ^{3}H atoms formed by microwave discharge in $^3H_2$ (see also Vol. 10, p. 16) have been described;[150,151] ^{2}H atoms at 77 K degrade *ca.* 40 % of a sample of D-alanine, giving also α- and β-exchanged products in the ratio 88 : 12.[151] [2-^{2}H]-3-Fluoro-DL-alanine is formed by reductive amination in aqueous NH_4OH containing NaB_2H_4 with highly specific incorporation of the label.[152] (1*R*)-[1-^{3}H,^{2}H]-3-phenylpropanol was formed from (1*S*)-[1-^{2}H]-3-phenylpropanol, [1-^{3}H]ethanol, and yeast alcohol dehydrogenase, and transformed into (4*R*)-[4-^{3}H,^{2}H]-DL-homoserine

[140] T. Caplaneris, P. Cordopatis, J. Matsoukas, and D. Theodoropoulos, *Tetrahedron*, 1978, **34**, 969.
[141] S. Brynes, G.J. Burckhart, and M. Mokotoff, *J. Med. Chem.*, 1978, **21**, 45.
[142] Y. Wang and H.P.C. Hogenkamp, *J. Org. Chem.*, 1978, **43**, 998.
[143] M. Dzieduszycka, M. Smelkowski, T. Czarnowska, and E. Borowski, *Pol. J. Chem.*, 1978, **52**, 933.
[144] F.R. Hewgill and R.J. Webb, *Austral. J. Chem.*, 1977, **30**, 2565.
[145] Y.S. Klausner, A.M. Feigenbaum, N. De Groot, and A.A. Hochberg, *Arch. Biochem. Biophys.*, 1978, **185**, 151.
[146] J. Barth and C.G. Wermuth, *Bull. Soc. chim. France*, 1977, 956.
[147] E.S. Garnett, G. Firnau, P.K.H. Chan, S. Sood, and L.W. Belbeck, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 464.
[148] G. Montagnoli, O. Pieroni, L. Nannicini, and A. Muttini, *Gazzetta*, 1977, **107**, 409.
[149] R.J. Parry, *Bio-org. Chem.*, 1978, **2**, 247.
[150] B.W. Wessels, D.J. McKean, N.C. Lien, C. Shinnick, P.M. De Luca, and O. Smithies, *Radiat. Res.*, 1978, **74**, 35.
[151] E.F. Siminov, M.S. Unukovich, E.S. Filatov, and A.V. Shishkov, *Khim. vysok. Energii*, 1978, **12**, 8.
[152] U.H. Dolling, A.W. Douglas, E.J.J. Grabowski, E.F. Schoenewaldt, P. Sohar, and M. Sletzinger, *J. Org. Chem.*, 1978, **43**, 1634.

[the (4*S*)-diastereoisomer was formed analogously], but 2-phenylethanol underwent negligible H-exchange and a corresponding synthesis of asymmetrically [^{3}H,^{2}H]-labelled serine was not feasible.[153]

Nine carboxyl-^{11}C-labelled amino-acids have been prepared by an appropriately rapid synthesis (high temperature, high pressure) from K^{11}CN, NH_4Cl, and $(NH_4)_2CO_3$ (a modified Bucherer–Strecker synthesis),[154] also used[155] under more normal laboratory conditions for the synthesis of DL-[1-^{13}C]methionine. *S*-Adenosyl-L-methionines, labelled in the *S*-methyl group by either ^{2}H or ^{13}C have been prepared from the methionines and *Saccharomyces cerevisiae*.[156] ^{13}C-or ^{14}C-Enriched CO_2 and HCHO have been used, with the reduced form of cobaloxime, to synthesize *N*-bromomethylphthalimide, from which labelled glycines have been prepared.[157] While ^{14}C-β-bromo-α-aminobutyric acid, can be prepared from ^{14}C-L-threonine by conventional substitution reactions,[158] the ^{125}I-γ-iodo-analogues and the corresponding γ-iodo-α-aminopentanoic acid are unstable and are rapidly de-iodinated.[158] Unusual synthetic methodology is involved in the route to [1-^{14}C]-*N*-benzyloxycarbonylphenylalanine methyl ortho-ester: $Ph(CH_2)_2Cl \rightarrow Ph(CH_2)_2{}^{14}C(OMe)=NCl \rightarrow PhCH_2CH(NHZ)$-$^{14}C(OMe)_3$[159] while more conventional steps (and eight of them) are involved in the synthesis of the ^{14}C-labelled nitrogen mustard *p*-$[Cl(^{14}CH_2)_2]_2N$-$C_6H_4CH_2CH(NH_2)CO_2H$ from L-phenylalanine.[160]

Small quantities of labelled N^6-methyl-lysine can be prepared from [*U*-^{14}C]lysine with a *Neurospora crassa* or *Salmonella typhimurium* culture, or in an alternative labelling pattern from reductive methylation of a protein with $^{14}CH_2O$ at pH 9.0, followed by hydrolysis and ion-exchange separation.[161] A new synthesis of ^{14}C-labelled azaserine from *N*-trifluoroacetyl-L-serine benzyl ester and ^{14}C-labelled glycine proceeds *via* esterification of the *Z*-glycine, generation by HNO_2 of the diazoacetate after de-protection of the glycyl moiety, and use of acylase I for enantiospecific deacylation.[162]

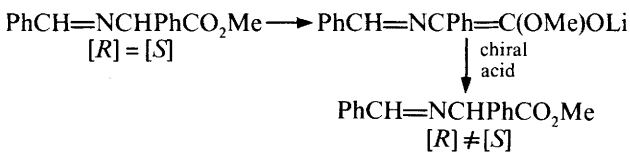
To citations in the preceding paragraphs for halogen isotope-labelled amino-acids can be added the synthesis of ^{18}F-5-fluorodopa, employing known methodology.[147]

Resolution of Amino-acids.—Judging by the scope of the 1978 literature, many hours were spent in many laboratories in the resolution of amino-acids. As usual, most of the procedures are based on well-known physical separations of diastereoisomeric salts. Space is found here for mention of only one paper in this area, because of its special interest; preferential separation from a solution of an *N*-benzyloxycarbonyl-DL-amino-acid and (±)-ephedrine seeded with the

[153] C. Fuganti, O. Ghiringhelli, and P. Grasselli, *Experientia*, 1978, **34**, 297.
[154] R.L. Hayes, L.C. Washburn, B.W. Wieland, T.T. Sun, J.B. Anon, T.A. Butler, and A.P. Callahan, *Internat. J. Appl. Radiat. Isotopes*, 1978, **29**, 186.
[155] B.D. Andersen, *J. Labelled Comp. Radiopharm.*, 1978, **14**, 589.
[156] M.F. Hegazi, R.T. Borchardt, S. Osaki, and R.L. Schowen, *Nucleic Acid Chem.*, 1978, **2**, 889.
[157] G.L. Blackmer and C.-W. Tsai, *J. Organometallic Chem.*, 1978, **155**, C17.
[158] H.F. Kung, S. Gilani, and M. Blau, *J. Nuclear Med.*, 1978, **19**, 393.
[159] C.R. Partington and M.P. Mertes, *J. Labelled Comp. Radiopharm.*, 1978, **14**, 223.
[160] C. Nicolas and D. Godeneche, *J. Labelled Comp. Radiopharm.*, 1978, **14**, 205.
[161] W.K. Paik, P. Dimaria, E. Pearson, and S. Kim, *Analyt. Biochem.*, 1978, **90**, 262.
[162] T.J. Curphey and D.S. Daniel, *J. Org. Chem.*, 1978, **43**, 4666.

desired chiral salt is a process which amounts to the simultaneous resolution of two pairs of racemates.[163]

Enantioselective protonation of the lithium enolate of the Schiff base of a DL-amino-acid methyl ester can be brought about to the extent of 62 % enantiomeric enhancement, when using a di-acyltartaric acid with bulky acyl groups:[164]



Column techniques involving a chiral stationary phase continue to receive attention, particularly the use of polystyrene beads to which an amino-acid residue is covalently attached, capable of complexing copper(II) ions.[165] This technique (co-ordination exchange chromatography), applied to amino-acid resolution, offers much scope for experimental appraisal of factors involved, a process already largely completed by Cram and co-workers in their distantly related work on the resolution of amino-acid ester salts with macrocyclic chiral polyethers.[166]

Enantiomeric discrimination, the principle underlying enzymic 'resolution' of DL-amino-acid derivatives, is also the basis of attempts to account for the ascendance of L-amino-acids after 'chance' synthesis in prebiotic times. Two different approaches using hydantoins as substrates produce interesting contrasts, with the hydrolysis of racemic hydroxybutylhydantoin in aqueous $Ba(OH)_2$ at 121 °C followed by incubation with D-amino-acid oxidase giving a mixture of L-amino-acid and the corresponding α-keto-acid,[167] while incubation of DL-5-(2-methylthioethyl)hydantoin with *Pseudomonas striata* gives *N*-carbamyl-D-methionine.[168] Examples of conventional methods are the use of hog renal acylase in the last stage of the total synthesis of L-2-amino-4-methoxy-*trans*-but-3-enoic acid (from *Pseudomonas aeruginosa*),[73] discriminatory metabolism by *Escherischia coli* of *N*-phenylacetyl-DL-methionine and N^{α}-phenylacetyl-N^{ϵ}-benzoyl-DL-lysine giving the L-amino-acids through cleavage of the N^{α}-acyl group[169] (this hydrolytic process is catalysed by benzylpenicillinacylase,[170] and may be useful in the assignment of absolute configur-

[163] C.-H. Wong and K.-T. Wang, *Tetrahedron Letters*, 1978, 3813.
[164] L. Duhamel and J.C. Plaquevent, *J. Amer. Chem. Soc.*, 1978, **100**, 7415.
[165] D. Muller, M.A. Petit, A. Szubarga, and J. Jozefonovicz, *Compt. rend. Acad. Sci., Ser. C*, 1977, **285**, 531; J. Jozefonovicz, M.A. Petit, and A. Szubarga, *J. Chromatog.*, 1978, **147**, 177; V.A. Davankov, Y.A. Zolotarev, and A.B. Tevlin, *Biorg. Khim.*, 1978, **4**, 1164; I.A. Yamskov, B.B. Berezin, V.E. Tikhonov, L.A. Belchich, and V.A. Davankov, *ibid.*, p. 1170; V.A. Davankov and Y.A. Zolotarev, *J. Chromatog.*, 1978, **155**, 285, 295, 303; I.A. Yamskov, B. Berezin, and V.A. Davankov, *Makromol. Chem.*, 1978, **179**, 2121.
[166] J.M. Timko, R.C. Helgeson, and D.J. Cram, *J. Amer. Chem. Soc.*, 1978, **100**, 2828.
[167] B.A. Kern and R.H. Reitz, *Agric. Biol. Chem.*, 1978, **42**, 1275.
[168] H. Yamada, S. Takahashi, Y. Kii, and H. Kumagai, *J. Ferment. Technol.*, 1978, **56**, 484.
[169] Y. Kameda and Y. Kameda, *Chem. Pharm. Bull.*, 1978, **26**, 2236.
[170] D. Rossi, A. Romeo, and G. Lucente, *J. Org. Chem.*, 1978, **43**, 2576.

ation to α-amino-acids), and the use of immobilized trypsin for the preparation of L-lysine from DL-lysine methyl ester.[171] This enzyme, in the free form, was shown earlier[172] to release D-lysine from DL-lysine ethyl ester; it seems unlikely that the change of ester grouping, or a modified specificity brought about by immobilizing the enzyme, can reverse the enantiospecificity of trypsin, as these results imply, and the lack of evidence for stereochemical purity of the product from the recent study[171] suggests that a re-appraisal is required.

Some theoretical considerations, that enantioselection favouring the survival of L-amino-acids in prebiotic times was due to intrinsic energy differences between enantiomeric configurations, or that elliptically polarized radiation was responsible for preferential destruction of the D-enantiomer,[173] can be linked with recent experimental results (see Volume 10, p. 17 and earlier Volumes). β-Irradiation in itself has been deduced to be insufficiently energetic to discriminate between amino-acid enantiomers[174] and there is experimental support for this[175] in the finding that crystalline ^{14}C-labelled DL-amino-acids do not undergo asymmetric degradation on β-radiolysis, in contrast with accelerated electrons reported earlier. ^{60}Co-γ-Irradiation of enantiomers of leucine, and aqueous solutions of their Na salts, causes degradation, and *ca.* 5 % of the residual amino-acid was racemized when this process was continued to the extent of 68 % degradation.[176] Little or no racemization accompanied γ-radiolysis of other amino-acids, as their hydrochlorides, in aqueous solution.[177] Perhaps the experiment with more promise, along these lines, will be the irradiation of a DL-amino-acid obtained in a crystalline modification involving an asymmetric distribution of paired enantiomers.

4 Physical and Stereochemical Studies of Amino-acids

Crystal Structures of Amino-acids and Their Derivatives.—*X*-Ray crystal analysis of both naturally occurring [L-methionine hydrochloride,[178] L-cystathionine,[179] (2*RS*, 4RS)-2-hydroxy-4-aminopentanoic acid,[180] and lycoperdic acid[181]] and closely related amino-acids [LL- and *meso*-di-aminopimelic acid hydrochlorides,[182] *p*-nitrophenyl-DL-alanine hydrochloride,[183] and 4′-methoxy-3,5,3′-trimethyl-L-thyronine (a halogen-free thyroxine analogue[184])] has been reported. The conformation of the latter was

[171] P. Monsan and G. Durand, *Biochim. Biophys. Acta,* 1978, **523,** 477.
[172] J.E. Purdie, R.E. Demayo, J.H. Seely, and N.L. Benoiton, *Biochim. Biophys. Acta.,* 1972, **268,** 523.
[173] B. Norden, *J. Mol. Evol.,* 1978, **11,** 313.
[174] C. Fajszi and J. Csege, *Origins of Life,* 1977, **8,** 277.
[175] W.A. Bonner, R.M. Lemmon, and H.P. Noyes, *J. Org. Chem.,* 1978, **43,** 522.
[176] W.A. Bonner and R.M. Lemmon, *J. Mol. Evol.,* 1978, **11,** 95.
[177] W.A. Bonner and R.M. Lemmon, *Bio-org. Chem.,* 1978, **7,** 175.
[178] B. De Blasio, V. Pavone, and C. Pedone, *Cryst. Struct. Comm.,* 1977, **6,** 845.
[179] C.S. Chen, T. Srikrishnan, and R. Parthasarathy, *Biochim. Biophys. Acta,* 1978, **538,** 534.
[180] L. Brehm and T. Honore, *Acta Cryst.,* 1978, **B34,** 2359.
[181] J. Lamotte, B. Oleksyn, L. Dupont, O. Dideberg, H. Campsteyn, M. Vermeire, and N. Rmugenda-Banga, *Acta Cryst.,* 1978, **B34,** 3635.
[182] S.E. Hull, O. Kennard, H.J. Rogers, and M.V. Kelemen, *Acta Cryst.,* 1977, **B33,** 3832.
[183] K.K. Chacko, S. Swaminathan, S. Parthasarathy, and S. Natarajan, *Acta Cryst. B,* 1978, **B34,** 1208.
[184] V. Cody, *Science,* 1978, **201,** 1131.

very similar to that of the 3,5,3′-tri-iodo analogue, suggesting that no special role is played by the iodine atoms in sustaining a physiologically significant conformation.

Amino-acid derivatives *N*-acetyl-α-amino-isobutyric acid methylamide,[185] *N*-acetyl-*S*-nitroso-DL-penicillamine,[186] and alaproclate $H_2NCHMeCO_2CMe_2$-CH_2-*p*-Cl-C_6H_4, a potent inhibitor of 5-hydroxytryptamine accumulation in brain,[187] have also been subjected to *X*-ray analysis. Crystal data for 27 *N*-acylamino-acid derivatives reveal the existence of intermolecular hydrogen bonds between the carboxyl proton and the acyl oxygen atom in the free acids, but a variety of hydrogen bonding patterns for corresponding amides, in which intermolecular bonding between the N–H proton of the acylamido-group and the oxygen atom of the amide group is preferred.[188]

N.M.R. Spectrometry.—Much of the work in this area continues along well-established lines of enquiry, with solution conformational studies predominating.

The influence of pH on the solution conformations of leucine, isoleucine, and valine[189] and of arginine and lysine[190] has been assessed through variable temperature ^{1}H-n.m.r. studies. Lysine adopts an intramolecularly hydrogen bonded structure in aqueous solutions;[190] four distinct zwitterionic forms of arginine can be discerned, each at maximum concentration at pH values $\leqslant$ 1, 8, 11—12, and $\geqslant$ 13.[190] Related studies of the protonation of the imidazole moiety of N^{α}-acetylhistidine methylamide have been described[191] (see also Vol. 10, p. 19). Increasing amounts of adenosine-5′-monophosphate cause upfield shifts of imidazole proton resonances of L-histidine, interpreted[192] to indicate interaction of the solutes through stacking involving the heteroaromatic groupings.

Full details (see Vol. 10, p. 19) of ^{1}H-n.m.r. studies of phenylalanine derivatives show their remarkable conformational dependence on solvent polarity.[193]

Structural information with either more detail or greater reliability can usually be obtained by subjecting a sample to scrutiny by more than one physical technique. This may involve the co-operative use of ^{1}H- and ^{13}C-n.m.r., as employed in a study of the *cis–trans* isomerism of acylsarcosine esters,[194] ^{1}H-, ^{13}C-, and ^{15}N-n.m.r. in continuing studies (see Vol. 10, p. 19) of the assessment of the populations of the three staggered C-1–C-2 rotamers in solutions of [4,5-$^2H_2$]-, [1-^{13}C,2,4,5-$^2H_3$]- and [^{15}N, 2,3,4-$^2H_3$*]-labelled leucine,[195] and ^{1}H-n.m.r. and Raman spectroscopic studies, showing that

[185] A. Aubry, J. Protas, G. Boussard, M. Marraud, and J. Neel, *Biopolymers*, 1978, **17**, 1693.
[186] L. Field, R.V. Dilts, R. Ravichandran, P.G. Lenhert, and G.E. Carnahan, *J.C.S. Chem. Comm.*, 1978, 249; G.E. Carnahan, P.G. Lenhert, and R. Ravichandran, *Acta Cryst.*, 1978, **B34**, 2645.
[187] U.H. Lindberg, S.B. Ross, S.-O. Thorberg, and S.-O. Ögren, *Tetrahedron Letters*, 1978, 1779.
[188] C.-S. Chen and R. Parthasarathy, *Internat. J. Peptide Protein Res.*, 1978, **11**, 9.
[189] V.P. Tikhonov and N.A. Kostromina, *Teor. i eksp. Khim.*, 1977, **13**, 496.
[190] V.P. Tikhonov and N.A. Kostromina, *Ukrain khim. Zhur.*, 1978, **44**, 451.
[191] M. Tanokura, M. Tasumi, and T. Miyazawa, *Chem. Letters*, 1978, 739.
[192] H.H. Mautsch and K. Neurohr, *F.E.B.S. Letters*, 1978, **86**, 57.
[193] J. Kobayashi and U. Nagai, *Biopolymers*, 1978, **17**, 2265.
[194] H.R. Kricheldorf and G. Schilling, *Makromol. Chem.*, 1977, **178**, 3115.
[195] A.J. Fischman, H.R. Wyssbrod, W.C. Agosta, and D. Cowburn, *J. Amer. Chem. Soc.*, 1978, **100**, 54.

4-aminobutanoic acid is flexible in aqueous solution, with the three main staggered rotamers equally populated, over a wide range of pH, while 4-amino-3-hydroxybutanoic acid exists in the *trans–trans* and *gauche–trans* rotameric forms predominantly at neutral pH, and in the *trans–trans* conformation at other pH values.[196]

The effects of pH on ^{13}C-n.m.r. chemical shifts and spin–spin coupling constants of [*U*-^{13}C]aspartic and glutamic acids have been assessed.[197]

Cyclic α-imino-acids have provided interesting results to reward the efforts expended in their study by n.m.r. methods. Papers from some leading investigators describe calculations of *exo–endo* ratios and their pH dependence for proline,[198] and similar studies of L-thiazolidine-4-carboxylic acid[199] which, with its enlarged ring size compared with proline, adopts an average planar conformation at pH 6.1 but with *exo* and *endo* puckered conformations predominating at lower pH values. Concentrated aqueous solutions of proline show some of the characteristics of hydrophilic colloids,[200] and ^{1}H-n.m.r. studies show that solute ordering, through a stacking structure sustained by strong hydrogen bonds with solvent, offers a plausible explanation.[200]

^{15}N-N.m.r. studies can provide information concerning interactions involving the α-amino-group, and of nitrogen-containing functional groups in side-chains, through the sensitivity of chemical shift to electronic and conformational changes, as well as through interpretations of ^{15}N–^{13}C and ^{15}N–^{1}H coupling constants.[201] Effects of pH on the proton distribution in arginine[202,203] and complex formation involving this amino-acid with Cl^-, PO_4^{3-}, and ATP,[203] are described in recent papers; Roberts and co-workers used samples containing ^{15}N at natural abundance.[202,203]

Continuing studies of solid amino-acids (proton-selective spin-lattice relaxation rates[204]) and metal-binding properties, of methylmercury compounds to tryptophan,[205] of lanthanide ions to γ-carboxyglutamic acid (binding involves both γ-carboxy groups),[206] and of manganese(II) ions to amino-acids (resulting in selective broadening of proton resonances in order $\alpha > \beta > \gamma$).[207]

Assignment of absolute configuration and enantiomeric purity can be accomplished through n.m.r. study of Eu(fod)$_3$ complexes of (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl derivatives of amino-acids.[208]

[196] K. Tanaka, H. Akutsu, Y. Ozaki, Y. Kyogoku, and K. Tomita, *Bull. Chem. Soc. Japan*, 1978, **51**, 2654.
[197] R.E. London, T.E. Walker, V.H. Kollmann, and N.A. Matwiyoff, *J. Amer. Chem. Soc.*, 1978, **100**, 3723.
[198] K. Jankowski, F. Soler, and M. Ellenberger, *J. Mol. Struct.*, 1978, **48**, 63.
[199] F. Piriou, K. Lintner, T.H. Lam, F. Toma, and S. Fermandjian, *Tetrahedron*, 1978, **34**, 553.
[200] B. Schobert and H. Tschesche, *Biochim. Biophys. Acta*, 1978, **541**, 270.
[201] F. Blomberg and H. Rueterjans, *Jerusalem Symp. Quantum Chem. Biochem.*, 1978, **11**, 231.
[202] R.E. London, T.E. Walker, T.W. Whaley, and N.A. Matwiyoff, *Org. Magn. Resonance*, 1977, **9**, 598; I. Yavari and J.D. Roberts, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 635.
[203] K. Kanamori, A.H. Cain, and J.D. Roberts, *J. Amer. Chem. Soc.*, 1978, **100**, 4979.
[204] E.R. Andrew, W.S. Hinshaw, M.G. Hutchins, and R.O.I. Sjöblom, *Mol. Phys.*, 1977, **34**, 1695; N. Niccolai, M.P. Leon de Miles, S.P. Hehir, and W.A. Gibbons, *J. Amer. Chem. Soc.*, 1978, **100**, 6528; U. Pande and R.C. Gupta, *Indian J. Pure Appl. Phys.*, 1977, 15, 777.
[205] P. Svejda, A.H. Maki, and R.R. Anderson, *J. Amer. Chem. Soc.*, 1978, **100**, 7138.
[206] R. Sperling, B.C. Furie, M. Blumenstein, B. Keyt, and B. Furie, *J. Biol. Chem.*, 1978, **253**, 3898.
[207] E. Tiezzi and G. Valensin, *Biofizika*, 1976, **21**, 401.
[208] F. Yasuhara, K. Kabuto, and S. Yamaguchi, *Tetrahedron Letters*, 1978, 4289.

Optical Rotatory Dispersion and Circular Dichroism.—Conventional approaches are reflected in interpretations of sign of longest-wavelength Cotton effect of a chromophoric derivative of an amino-acid with absolute configuration. *N*-Dithioethoxycarbonyl-L-*β*-amino-acids show negative $n \rightarrow \pi^*$ Cotton effects in $CHCl_3$ or benzene solutions,[209] and Schiff bases $Me_2CHCR^1{=}NCHR^2CO_2R^3$ show a c.d. maximum at 240 nm.[210]

Increasing interest in vibrational c.d. studies, involving absorption and Raman scattering, can be discerned through the general literature, and although complexities of peak assignments are fully displayed in studies of L-alanine,[211] some assignments to C–H stretching modes have been possible in this case.

Mass Spectrometry.—An increasing variety of ionization techniques is being assessed through fundamental studies of many compound types, including amino-acids. Chemical ionization (c.i.m.s.) studies of free amino-acids involve additional problems in interpretation since solvated protonated species are formed when the samples are volatilized by rapid heating in NH_3, leading to (4M + 1) ions in some cases.[212] The most recent paper culled from the literature[213] covers c.i.m.s. of arginine hydrochloride using isobutane. Field desorption (f.d.m.s.) studies of free amino-acids (methionine[214] and a representative group of amino-acids[215]) cover continuing scrutiny of origins of fragment ions[214] and effects of emitter temperature on fragment ion intensities.[215] Negative-ion mass spectra using 2—4 eV electrons of 20 free amino-acids have been compared with positive-ion spectra obtained using 6—16 eV electrons.[216] Absolute secondary-ion emission yields have been reported[217] for parent-like secondary ions [$(M + H)^+$ or $(M-H)^-$] and large characteristic fragments produced through this variation of the ion–molecule ionization procedure.[218]

Amino-acid derivatives chosen for their higher volatility are featured in the majority of recent papers. Per-trimethylsilylated amino-acids,[219] triazines,[220] and phenylthiohydantoins derived from amino-acids[221] have not displaced *N*-acyl amino-acid esters as the most commonly used derivatives (*N*-acetylamino-acidmethyl[222] and n-propyl esters,[223] *N*-trifluoroacetyl-amino-acid n-butyl esters,[223] *N*-heptafluorobutyryl-amino-acid n-butyl esters,[223] *N*-acetyl-amino-

[209] T. Yamada, S. Kuwata, and H. Watanabe, *Tetrahedron Letters*, 1978, 1813.
[210] H. Takahashi and H. Otomasu, *Chem. Pharm. Bull.*, 1978, **26**, 466.
[211] M. Diem, P.J. Gotkin, J.M. Kupfer, and L.A. Nafie, *J. Amer. Chem. Soc.*, 1978, **100**, 5644.
[212] J.S. Gaffney, R.C. Pierce, and L. Friedman, *Internat. J. Mass Spectrom. Ion Phys.*, 1977, **25**, 439.
[213] R.J. Cotter, *Analyt. Chem.*, 1979, **51**, 317.
[214] J. van der Greef, N.M.M. Nibbering, H.R. Schulten, and W.D. Lehmann, *Z. Naturforsch. Anorg. Chem. Org. Chem.*, 1978, **33b**, 770.
[215] H.U. Winkler, H.B. Linden, and H.D. Beckley, *Adv. Mass Spectrom.*, 1978, **7A**, 104.
[216] D. Voigt and J. Schmidt, *Biomed. Mass Spectrom.*, 1978, **5**, 44.
[217] A. Benninghoven, D. Jaspers, and W. Sichtermann, *Adv. Mass Spectrom.*, 1978, **7B**, 1433.
[218] A. Benninghoven and W.K. Sichtermann, *Analyt. Chem.*, 1978, **50**, 1180.
[219] K.R. Leimer, R.H. Rice, and C.W. Gehrke, *J. Chromatog.*, 1977, **141**, 355.
[220] K. Maekawa, E. Taniguchi, and E. Kuwano, *Org. Mass Spectrom.*, 1978, **13**, 4.
[221] J.B. Laudenslager and L.P. Theard, *Adv. Mass Spectrom.*, 1978, **7B**, 1388.
[222] J.M.L. Mee and B. Halpern, '4th Int. Symposium on Recent Developments in Mass Spectrometry in Biochemistry and Medicine', ed. A. Frigerio, Plenum, New York, Vol. 1, 1978, p. 291.
[223] P. Padieu, J. Desgres, B.F. Maume, G. Vander Velde, and R.S. Skinner, *Adv. Mass Spectrom.*, 1978, **7B**, 1604.

acid *p*-nitrobenzyl esters,[224] and *N*-benzoyl-amino-acid methyl esters,[225] have been featured), and further examples of amino-acid derivatives employed in analysis are described in the later section dealing with gas – liquid chromatography. Some of these studies involve c.i.m.s.[222,223,225] techniques, and dissociation resonance capture[224] (avoiding extensive fragmentation) and Penning ionization variations.[221] Application of the derivatization approach to the analysis of γ-aminobutyric acid has been demonstrated.[226]

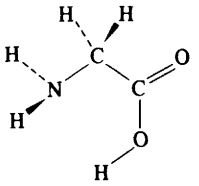
Other Physical and Theoretical Studies.—The major collective headings for papers described in this section, as in previous Volumes, are: spectroscopic studies not covered in preceding sections; classical studies of fundamental physical properties, including those with possible relevance to abiotic synthesis of peptides; and molecular orbital calculations.

Raman spectroscopic studies of tryptophan in neutral aqueous solutions,[227] of solid β-alanine, taurine, and ciliatine,[228] and of L-histidine in aqueous solutions[229] have been reported. The tautomer ratio N^{τ} : N^{π} = *ca.* 4 : 1 for imidazole-protonated L-histidine, from which a difference in stability of *ca.* 1 kcal mol^{-1} has been calculated for these tautomers.[229] Among microwave spectroscopic studies[230—233] are results for glycine interpreted to show that a compact conformation (11) is adopted in the vapour phase.[231—233] This is in disagreement with the conformation (12) deduced from *ab initio* calculations[231,233] (see Volume 10, p. 22) but it has been reasoned[231] that both conformations should exist in the vapour phase, but that (12) should not contribute significantly to the observed spectra. Ordered layers formed through adsorption of amino-acids on copper (100) and copper (101) have been revealed by electron-diffraction.[234] Photoelectron spectroscopy of amino-acids and their methyl esters yields the generalization that the gas-phase electronic structure of the amino-acid can be deduced from results obtained for its ester.[235] Ultrasonic absorption spectra have been reported for aqueous solutions of L-proline and of L-serine.[236]

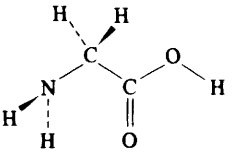
A large number of papers[237] have been published covering solutions (solubility of common amino-acids in water;[237] influence of electrolytes on solubility;[238] free energy of interaction of glycine with LiCl, NaCl, and CsCl in

[224] C.L. Brown and C.L. Chan, *Adv. Mass Spectrom.*, 1978, **7A**, 371.
[225] G. Höfle, G. Höhne, J. Respondek, and H. Schwarz, *Org. Mass Spectrom.*, 1977, **12**, 477.
[226] J.D. Huizinga, A.W. Teelken, F.A.J. Muskiet, H.J. Jeuring, and B.G. Wolthers, *J. Neurochem.*, 1978, **30**, 911.
[227] A.Y. Hirakawa, Y. Nishimura, T. Matsumoto, M. Nakanishi, and M. Tsuboi, *J. Raman Spectroscopy*, 1978, **7**, 282.
[228] C. Garrigou-Lagrange, *Canad. J. Chem.*, 1978, **56**, 663.
[229] I. Ashikawa and K. Itoh, *Chem. Letters*, 1978, 681.
[230] E.J. Croom, R. Shack, J.C.W. Sheppard, and R.J. Sheppard, *J. Microwave Power*, 1977, **12**, 111 (*Chem. Abs.*, 1978, **88**, 70 693).
[231] H.L. Sellers and L. Schafer, *J. Amer. Chem. Soc.*, 1978, **100**, 7728.
[232] R.D. Suenram and F.J. Lovas, *J. Mol. Spectroscopy*, 1978, **72**, 372.
[233] R.D. Brown, P.D. Godfrey, J.W.V. Storey, and M.P. Bassez, *J.C.S. Chem. Comm.*, 1978, 547.
[234] L.L.A. Fanasoska, J.C. Buchholz, and G.A. Somorjai, *Surface Sci.*, 1978, **72**, 189.
[235] L. Klasinc, *Internat. J. Quantum Chem., Quantum Biol.*, 1978, **5**, 373.
[236] N. Inoue, *Japanese J. Appl. Phys.*, 1978, **17**, 1699; *Acustica*, 1978, **40**, 91 (*Chem. Abs.*, 1978, **89**, 163 929).
[237] H.B. Bull, K. Breese, and C.A. Swenson, *Biopolymers*, 1978, **17**, 1091.
[238] A.J.H. Hernandez, *Acta Cient. Venez.*, 1978, **29**, 158.

aqueous solution;[239] dissociation constants for $H_3\overset{+}{N}(CH_2)_nCO_2H$, $n = 1—10$, interpreted in terms of a conformation intermediate between the random-coil and fully-extended states;[240] dissociation constants of α-amino-acids in various media[241]) and thermodynamic properties (heats of sublimation of sarcosine and L-proline;[242] heats of dissociation of proline in different electrolytes;[243] heat capacities of amino-acids in aqueous solutions;[244] heats of solution of optically-active amino-acids in water at 298. 15 K, several kJ mol^{-1} lower than those for corresponding DL-forms, except for threonine;[245] apparent molal heat capacities of amino-acids in aqueous mannitol or glycerol;[246] and apparent molal volumes and adiabatic compressibilities of amino-acids in water at 25 °C[247]). Reverse osmosis separation of amino-acids has been demonstrated, using a cellulose acetate membrane.[248]



(11)



(12)

Adsorption studies range from fundamental properties relevant to laboratory techniques (adsorption of aliphatic amino-acids at a mercury–water interface;[249] adsorption of amino-acids at the electrode inhibits the anodic oxidation of glucose[250]) to uptake on solids [251—253] with possible relevance to abiotic condensation. Glycine is adsorbed weakly by apatite,[251] and aspartic acid (but not tryptophan) is adsorbed on monodispersed chromium hydroxide sol.[252] Aqueous micelles (hexadecyltrimethylammonium hydroxide) are more effective concentrating media at pH 8 for amino-acids than clays.[253] Preferential

[239] B.P. Kelley and T.H. Lilley, *J.C.S. Faraday I*, 1978, **74**, 2771, 2779.
[240] J.T. Edward, P.G. Farrell, J.L. Job, and B.-L. Poh, *Canad. J. Chem.*, 1978, **56**, 1122.
[241] H. Matsui, *Denki Kagaku Oyobi Kogyo Butsuri Kagaku, 1977*, **45**, 734 (*Chem. Abs.*, 1978, **88**, 116 547); A.P. Kreshkov, N.S. Aldarova, A.N. Yarovenko, B.B. Tanganov, K.N. Shulunova, T.K. Batorova, and T.E. Batlaeva, *Zhur. fiz. Khim.*, 1978, **52**, 456; O.I. Zinovev and L.F. Pulatova, *ibid.*, p. 631.
[242] R. Sabbah and M. Lafitte, *Bull. Soc. Chim.*, 1978, 50.
[243] V.P. Vasilev, L.A. Kochergina, and T.B. Sokolova, *Zhur. obshchei Khim.*, 1978, **48**, 650.
[244] J.C. Ahluwalia, C. Ostiguy, G. Perron, and J.E. Desnoyers, *Canad. J. Chem.*, 1977, **55**, 3364.
[245] M. Matsumoto and K. Amaya, *Chem. Letters*, 1978, 87.
[246] G. Di Paola and B. Belleau, *Canad. J. Chem.*, 1978, **56**, 1827.
[247] F.J. Millero, A. Lo Surdo, and C. Shin, *J. Phys. Chem.*, 1978, **82**, 704.
[248] O. Tozawa and D. Nomura, *Nippon Kagaku Kaishi*, 1978, 1291.
[249] T. Kakinchi and M. Senda, *J. Electroanal. Chem. Interfacial Electrochem.*, 1978, **88**, 219.
[250] J.R. Rao, G.J. Richter, G. Luft, and F. von Sturm, *Biomaterials, Medical Devices, and Artificial Organs*, 1978, **6**, 127.
[251] C. Rey, J.C. Trombe, and G. Montel, *J. Chem. Research (S)*, 1978, 188.
[252] H. Kumanomido, R.C. Patel, and E. Matijevic, *J. Colloid Interface Sci.*, 1978, **66**, 183.
[253] D.W. Armstrong, R. Seguin, and J.H. Fendler, *J. Mol. Evol.*, 1977, **10**, 241.

adsorption of the L-enantiomer has been established for the DL-α-amino-propionitrile-*d*-quartz system.[254] Direct correlations exist between the hydrophobicity or hydrophilicity of the homocodonic amino-acids and their anticodon nucleotides.[255]

Conformational energy calculations for cystine,[256] glycine,[257] and for other amino-acids[258] continue using established methods[256,257] together with attempts to break new ground.[258] The equilibrium conformation of the glycine zwitterion is planar, stabilized by an intramolecular hydrogen bond, and is 29 kcal mol^{-1} above the energy of the corresponding conformation of the non-zwitterion form. A hydration energy of *ca.* 50 kcal mol^{-1} can be deduced for the gas phase zwitterion.[257]

5 Chemical Studies of Amino-acids

Racemization.—The main areas of interest developed in recent years provide the basis of all papers published in 1978 on this topic. Analytical procedures for assessing accurate enantiomer ratios have been developed to meet the quantitative demands of these studies, and the reader is referred to Section 6 and to the coverage earlier in this Chapter (on preferential degradation of one enantiomer in a racemic mixture) for a complete review.

Interpretation of D–L-ratios for amino-acids present in fossil materials[259] in terms of the age of the samples has been criticized because the ratios may have been influenced by differential leaching of enantiomers over the intervening period. A study[35] of the relationship between γ-carboxyglutamic acid content of fossil bones and their leaching histories suggests a basis for checking this aspect. High D–L-ratios are found for aspartic acid present in water-insoluble protein from cataracts, suggesting a reason for the precipitation.[260] A review has appeared[261] on the more general topic of racemization of amino-acid residues in proteins in relationship to signs of ageing. Variations in D–L-aspartic acid ratios for samples of plants uniformly preserved for *ca.* 11 000 years in an ancient packrat midden have been observed,[262] implying either that complex relationships exist between bound aspartic acid and the lignin–cellulose matrix, or that the neighbouring sequence to the protein-bound aspartic acid residue in these samples controls the racemization rate of that residue.[262] A similar species-dependent racemization rate for isoleucine has been demonstrated for fossil planktonic foraminifera.[263]

Mechanistic studies relevant to metabolic processes have been described for copper(II) catalysed racemization of Schiff bases derived from L-amino-acids

[254] S. Morimoto, K. Kawashiro, and H. Yoshida, *Origins of Life*, 1977, **8**, 355.
[255] A.L. Weber and J.C. Lacey, *J. Mol. Evol.*, 1978, **11**, 199; J.R. Jungck, *ibid.*, p. 211.
[256] L.A. Eslava, J.B. Putnam, and L. Pedersen, *Internat. J. Peptide Protein Res.*, 1978, **11**, 149.
[257] Y.C. Tse, M.D. Newton, S. Vishveshwara, and J.A. Pople, *J. Amer. Chem. Soc.*, 1978, **100**, 4329.
[258] J. Snir, R.A. Nemenoff, and H.A. Scheraga, *J. Phys. Chem.*, 1978, **82**, 2527.
[259] P.M. Masters and J.L. Bada, *Advances in Chemistry Series*, No. 171 (1978), p. 117.
[260] P.M. Masters, J.L. Bada, and J.S. Zigler, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 1204.
[261] L. Poplin and R. De Long, *Gerontology*, 1978, **24**, 365.
[262] M.H. Engel, J.E. Zumberge, B. Nagy, and T.R. Van Devender, *Phytochemistry*, 1978, **17**, 1559.
[263] K. King and C. Neville, *Science*, 1977, **195**, 1333.

and salicylaldehyde,[264] pyruvic acid,[265] or pyridoxal.[266] Oxazolone intermediates are plausible sources of the racemization observed for L-proline in trifluoroacetic anhydride[267] or aroyl-L-leucine *p*-chlorophenyl esters in basic media.[268]

The racemization of amino-acid derivatives has been reviewed.[269]

General Reactions.—De-aminative bromination of α-amino-acids using $NaNO_2$–NaBr with retention of configuration in the cases of 3,5-dichloro-L-tyrosine[270a] and L-aspartic acid[270b] is accounted for by neighbouring group participation of the phenoxide and α-carboxy groups, respectively. Inversion of configuration is observed in the corresponding reaction with α-methyl- and αβ-dimethylaspartates.[270b] This stereospecific process is exploited in the conversion of D-leucine into (*R*)-2-bromo-4-methylpentanoic acid for the synthesis of (*S*)-2-(2-aminoethylthio)-4-methylpentanoic acid.[79] There are relatively few unexplored reactions, apparently, of free amino-acids, and routine work covering the decomposition kinetics of crystalline amino-acids,[271] the Maillard reaction (fusion of an amino-acid with glucose[272,273] or a triglyceride[273] at 100—120 °C), and conversion into aldehydes through heating with $KMnO_4$,$K_2Cr_2O_7$ in acid media, or $KBrO_3$ or $KClO_4$ in alkaline media[274] has been described.

Results with amino-acid derivatives include oxidative decarboxylation of *N*-acylamino-acid *p*-nitrophenyl esters with *m*-chloroperbenzoic acid,[275] preparation of *N*-acylamino-acid t-butylthioesters *via* $LiSBu^t$,[276] esterification of *N*-acylamino-acids with MeOH or EtOH with BF_3–Et_2O (amino-acids give methyl esters in 14–82 % yield by this procedure),[277] formation of difluoroboron heterocycles from *N*-alkylamino-acids and BF_3–Et_2O,[278] conversion of *N*-(*o*-nitrophenylsulphenyl)amino-acid amides into corresponding nitriles without racemization using $POCl_3$ and pyridine,[279] and conversion of amino-acid esters into hydroxamic acids.[280] *N*-Acylamino-acids, after conversion into

[264] M. Ando and S. Emoto, *Bull. Chem. Soc. Japan*, 1978, **51**, 2366; Y.N. Belokon, M.M. Dolgaya, M.B. Saporovskaya, V.I. Tararov, and V.M. Belikov, *J. Mol. Catal.*, 1978, **4**, 289; V.M. Velikov, Y.N. Belokon, V.A. Karginov, N.S. Martinkova, and M.B. Saporovskaya, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1976, 1276.

[265] R.D. Gillard and P. O'Brien, *J.C.S. Dalton*, 1978, 1444.

[266] M.-D. Tsai, H.J.R. Weintraub, S.R. Byrn, C.-J. Chang, and H.G. Floss, *Biochemistry*, 1978, **17**, 3183.

[267] I. Tomida and T. Kuwahara, *Agric. Biol. Chem.*, 1978, **42**, 1059.

[268] J.P. Morawiec and I.Z. Siemion, *Roczniki Chem.*, 1977, **51**, 1867.

[269] M. Lebl and K. Jost, *Chem. listy*, 1978, **72**, 252.

[270] (*a*) K. Koga, M.J. Tzuoh, and S. Yamada, *Chem. Pharm. Bull.*, 1978, **26**, 178; (*b*) Y. Murakami, K. Koga, and S. Yamada, *ibid.*, p. 307.

[271] G.W.C. Hung, *Thermochim. Acta*, 1978, **23**, 233.

[272] F. Orsi and E. Dworschak, *Acta Ailment. Acad. Sci. Hung.*, 1978, **7**, 41.

[273] S. Saito and K. Shirai, *Nippon Daigaku Nojuigakubu Gakujutsu Kenkyu Hokoku*, 1978, **35**, 186 (*Chem. Abs.*, 1978, **89**, 24 752).

[274] L. Lipparini and M.R. Cavana, *Rass. Chim.*, 1978, **30**, 19 (*Chem. Abs.*, 1978, **89**, 161 856).

[275] G. Lucente, F. Pinnen, and G. Zanotti, *Tetrahedron Letters*, 1978, 3155.

[276] U. Schmidt and F. Steindl, *Synthesis*, 1978, 544.

[277] T. Yamada, N. Isono, A. Inui, T. Miyazawa, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1978, **51**, 1897.

[278] J. Halstrom, E. Nebelin, and E.J. Pedersen, *J. Chem. Research (S)*, 1978, 80.

[279] K. Kawashiro, H. Yoshida, and S. Morimoto, *Bull. Chem. Soc. Japan*, 1977, **50**, 2956.

[280] H. Hjeds and T. Honore, *Acta Chem. Scand.*, 1978, **B32** 187.

oxazol-5-ones, can be alkylated to give α-alkyl analogues $R^1CONHCR^2R^3$-CO_2H, after hydrolysis: $Pb(OAc)_4$ oxidation causes loss of the acylamido and carboxy groups, yielding ketones R^2COR^3.[281] This new ketone synthesis portrays amino-acids as carbonyl synthons. *N*-Thiobenzoylamino-acids give 5-(2′-benzamidoacyloxy)-2-phenylthiazoles, and corresponding amides give 5-trifluoroacetamido analogues, with trifluoroacetic anhydride;[282] these reactions involve *S*-trifluoroacetyl intermediates, since corresponding esters are converted into benzamido-analogues.

1,3-Dipolar reactivity of amino-acid Schiff bases (see Vol. 10, p. 27) has been illustrated further, in reactions with acrylonitriles to give pyrrolidines,[283] and in reactions of pyridoxal derivatives of possible metabolic significance.[284]

The chemical basis of colorimetric analysis procedures for amino-acids has been studied. The formation of 1-alkyl-and aryl-thio-2-alkylisoindoles from *o*-phthalaldehyde, a thiol, and an amino-acid[285,286] proceeds at maximum rate at pH 9.7—10,[287] but for amino-acid esters the maximum rate is at pH 5—6. This permits the quantitative analysis of mixtures containing amino-acids and their esters.[288] The *o*-phthalaldehyde procedure is ten times more sensitive than the ninhydrin reaction,[287] but the latter can be rendered 2—3 times more sensitive by the addition of Fe^{II}, Mn^{II}, or Mo^{II} salts, bringing the lower detection limit down to 3 nmol.[289] The basis of the colour reaction between amino-acids and *o*-acetylbenzophenone,[290] and that of hydroxyproline, chloramine T, and 4-dimethylaminbenzaldehyde,[291] has been assessed.

Specific Reactions of Natural Amino-acids.—A group of papers which have the common feature of reporting reactions of side-chain functional groups in natural amino-acids is collected in this section. Conversion of *N*-trityl-*O*-toluene-*p*-sulphonyl-L-serine benzyl ester into the L-aziridine-2-carboxylate,[292] decarboxylation of kainic acid (the C-4 epimer of the product shown in Scheme 1) with sodium periodate,[293] the relative stability of 4-hydroxyproline towards HNO_2 or chloramine-T, compared with the 3-hydroxy-isomer,[294] formation of homolanthionine, homocysteic acid, and homolanthionine sulphone from DL-homocystine and H_2O_2 in aqueous HCl,[295] EDTA-stimulation of Cu^{2+}-catalysed autoxidation of cysteine,[296] oxidation of methionine derivatives to sulphoxides by H_2O_2, $NaIO_4$, $NaBrO_3$, *N*-chlorosuccinimide, or chloramine T,

[281] R. Lohmar and W. Steglich, *Angew. Chem.*, 1978, **90**, 493; *Angew. Chem. Internat. Edn.*, 1978, **177**, 450; W. Steglich, *Chimia*, 1978, **32**, 394.
[282] G.C. Barrett, *Tetrahedron*, 1978, **34**, 611.
[283] M. Joucla and J. Hamelin, *Tetrahedron Letters*, 1978, 2885.
[284] R. Grigg and J. Kemp, *Tetrahedron Letters*, 1978, 2823.
[285] S.S. Simons and D.F. Johnson, *J. Org. Chem.*, 1978, **43**, 2886.
[286] S.S. Simons, *Analyt. Biochem.*, 1978, **90**, 705.
[287] V. Svedas, I.Y. Galaev, and I.V. Berezin, *Biorg. Khim.*, 1978, **4**, 19.
[288] V. Svedas, I.Y. Galaev, and I.V. Berezin, *Biorg. Khim.*, 1978, **4**, 130.
[289] J.V. Singh, S.K. Khanna, and G.B. Singh, *Analyt. Biochem.*, 1978, **85**, 581.
[290] S. Nanya and E. Maekawa, *Nippon Kaguku Kaishi*, 1977, 1750 (*Chem Abs.*, 1979, **90**, 105 587).
[291] U. Pindur, *Z. Lebensm.-Untersuch.*, 1978, **166**, 148.
[292] K. Nakajima, F. Takai, T. Tanaka, and K. Okawa, *Bull. Chem. Soc. Japan*, 1978, **51**, 1577.
[293] R.D. Allan, *Tetrahedron Letters*, 1978, 2199.
[294] G. Szymanovicz, O. Mercier, A. Randoux, and J.P. Borel, *Biochimie*, 1978, **60**, 499.
[295] S.H. Lipton, *J. Agric. Food Chem.*, 1978, **26**, 1406.
[296] A. Hanaki and H. Kamida, *Chem. Pharm. Bull.*, 1978, **26**, 325.

and removal of unwanted sulphone by crystallization,[297] oxidation of methionine with iodine to yield products derived from an intermediate cyclic sulphimine,[298] formation of 3-chlorotyrosine through the hydrolysis in air of glycoproteins by hot hydrochloric acid,[299] formation of 3-phenyl- and 2-amino-5-phenylpyridines by pyrolysis of phenylalanine,[300] conversion of histidine into imidazoleacetic acid and imidazole-lactic acid by peroxides and hydroperoxides derived from methyl linoleate,[301] oxidative processes with tryptophan (Cu_2Cl_2–py–O_2 causes indole cleavage;[302] photo-oxygenation yields the intramolecular addition product involving the amino and indole groups when sensitized by Rose Bengal,[303] then N^{ind}-formylkynurenine;[304] ozonolysis in methanol also yields the latter product *via* the N^{ind}-methoxyhydroperoxide[305]); formation of tryptathionine from tryptophan and cystine under conditions normally used to hydrolyse proteins (6M-HCl) and its subsequent conversion into cysteine and β-(3-oxoindolyl)alanine by hydrolysis;[306] similar involvement of C-2 of the indole moiety of tryptophan in its condensation with 4-dimethylaminobenzaldehyde to give 1 : 1- and 2 : 1-adducts;[307] in the substitution of *N*-acetyltryptophan methyl ester by 2′,3′-*O*-isopropylidene-5-bromouridine through acetone-sensitized irradiation,[308] and in the reduction of L-tryptophan derivatives with BH_3-py to 2,3-dihydro-derivatives;[309] and conversion of tryptophan into indoleacetamide *via* an electronically-excited intermediate, judging by the accompanying luminescence and formation of pyridoxoic acid, through reaction with pyridoxal phosphate, and horseradish peroxidase, in the presence of manganese(II) ions.[310]

Specific Reactions of Amino-acids Related to Biochemical Processes.—While some of the papers in preceding sections would be equally appropriate here, two papers have been selected to represent the continuing interest in model reactions and studies of properties. Duplex formation involving tryptophan methyl ester and oligodeoxyribonucleotides, studied by c.d. and fluorescence methods, does not follow an intercalation mechanism.[311] In Zn^{2+}-mediated

[297] N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, 1978, **26**, 650.
[298] P.R. Young and L.-S. Hsieh, *J. Amer. Chem. Soc.*, 1978, **100**, 7121.
[299] M. Havlikova, P. Smolek, G. Entlicher, and J. Kocourek, *J. Chromatog.*, 1978, **154**, 336.
[300] K. Tsuji, T. Yamamoto, H. Zenda, and T. Kosuge, *Yakugaku Zasshi*, 1978, **98**, 910 (*Chem. Abs.*, 1978, **89**, 163 925).
[301] S.H. Yong and M. Karel, *J. Amer. Oil Chemists' Soc.*, 1978, **55**, 352.
[302] E. Balogh-Hergovich and G. Speier, *Magyar Kém. Folyóirat*, 1977, **83**, 188.
[303] M. Nakagawa and T. Hino, *Heterocycles*, 1977, **6**, 1675.
[304] M. Nakagawa, H. Watanabe, S. Kodato, H. Okajima, T. Hino, J.L. Flippen, and B. Witkop, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4730; J.P. McCormick and T. Thomason, *J. Amer. Chem. Soc.*, 1978, **100**, 312.
[305] F. Sakiyama, N. Masuda, T. Nakazawa, and Y. Katsuragi, *Chem. Letters*, 1978, 893.
[306] T. Ohta and T. Nakai, *Biochim. Biophys. Acta*, 1978, **533**, 440.
[307] U. Pindur, *Arch. Pharm.*, 1978, **311**, 615.
[308] I. Saito, S. Ito, and T. Matsuura, *J. Amer. Chem. Soc.*, 1978, **100**, 2901.
[309] Y. Kikugawa, *J. Chem. Research (S)*, 1978, 184.
[310] O. Augusto and G. Cilento, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 1238.
[311] N.G. Dolinnaya, E.S. Gromova, A.P. Razzhirin, Z.A. Shabarova, and M.A. Prokofev, *Doklady Akad. Nauk S.S.S.R.*, 1978, **239**, 603.

ternary complex formation involving ATP and aromatic amino-acids, an order Trp > Tyr > Phe has been established[312] for complex-forming efficiency.

Effects of Electromagnetic Radiation on Amino-acids.—An increasing number of papers deserves discussion, but again as in previous volumes, little more than a brief summary is possible in the space available.

The two major topic groupings are degradation of amino-acids and their derivatives under short wavelength irradiation, and electronic excitation at longer wavelengths. In the former category, γ-radiolysis of crystalline amino-acids,[313] *N*-acetyl-L-valine,[314] and solutions of sulphur-containing amino-acids[315] and e.s.r.-monitoring to assess radical-pair formation,[313] corresponding *X*-irradiation studies of crystalline hydrochlorides of L-phenylalanine and L-tryptophan,[316] and irradiation of L-tryptophan hydrochloride[317] and other amino-acids and peptides[318] with high-energy electrons, have been described. A common feature of the *X*-ray and electron-irradiation studies[316—318] is the formation of a C-2 radical by deamination, but additional radicals formed by H-abstraction and addition processes are also observed. There is a continuing interest in enantiomeric discrimination possibilities under irradiation[173—177] and further discussion of the consequences of positron-irradiation of chiral molecules has been published.[319] The category in which this type of study belongs also embraces an interesting project,[320] in which the selective photo-destruction of amino-acids has been assessed. Thirty five amino-acids have been found, so far, in extracts from the Murchison meteorite, and many more are believed to be present;[320] of the various types present, α-amino-acids are selectively destroyed as their copper complexes at 254 nm (order of stability $\alpha > \beta > \gamma = \delta = \varepsilon = 0$), while for dicarboxylic amino-acids, $\alpha > \beta \approx \gamma$. Scope for speculation that the meteorite may have originally contained a much higher α-amino-acid content arises from the finding[320] that when all the α-amino-acids in a mixture have been photodegraded, *ca.* 80% of the initial concentrations of β- and γ-amino-acids remains.

One paper,[321] describing the formation of tyrosine by the hydroxylation of phenylalanine by hydroxide radicals generated by photoreduction of anthraquinone-sulphonate, is located in this section, while others on photochemistry of amino-acids can be found in the preceding section. Amino-acid fluorescence studies described in the 1978 literature almost exclusively concern tryptophan and its derivatives,[322—328] dealing with the pH-dependence of the fluorescence intensity of the tryptophan–histidine charge-transfer complex,[322] fluorescence

[312] J.J. Toulme, *Bio-inorg. Chem.*, 1978, **8**, 319.
[313] T. Matsuyama and H. Yamaoka, *J. Phys. Soc. Japan*, 1978, **45**, 717.
[314] G.H. Schnepel, *Radiat. Res.*, 1978, **74**, 240.
[315] T. Oku, *Nippon Daigaku Nojnigakubu Gakujutsu Kenkyu Hokoku*, 1978, **35**, 163 (*Chem. Abs.*, 1978, **89**, 24 748); p. 181 (*Chem. Abs.*, 1978, **89**, 24 749).
[316] W. Flossmann and E. Westhof, *Radiat. Res.*, 1978, **73**, 75; *Internat. J. Radiat. Biol., and Related Studies in Phys., Chem., Med.*, 1978, **33**, 139.
[317] E. Sagstuen, H.G. Byrkjeland, and T. Henriksen, *Radiat. Res.*, 1978, **74**, 10.
[318] M.D. Sevilla, J.B. D'Arcy, and D. Suryanarayana, *J. Phys. Chem.*, 1978, **82**, 2589.
[319] Y.-C. Jean and H.J. Ache, *J. Phys. Chem.*, 1977, **81**, 1157.
[320] N. Levi and J.G. Lawless, *Analyt. Biochem.* 1978, **90**, 796.
[321] S.I. Skuratova and G.V. Fomin, *Zhur. fiz. Khim.*, 1978, **52**, 2971.
[322] S.V. Koner, V.P. Khrapovitskii, and S.N. Cherenkevich, *Zhur. priklad. Spektrosk.*, 1978, **29**, 37.

enhancement accompanying rapid transfer of tryptophan from ^1H_2O to ^2H_2O,[323] and more routine studies[324—326] of the amino-acid itself, including a detailed study of emission from the excited triplet state.[326] N^{α}-Acetyltryptophanamide fluorescence[327] is reduced by the addition of Eu-$(NO_3)_2$.[328] Fluorescence attributable to the tyrosinate anion, previously observed only at pH values above 10.3, can be discerned at neutral pH in concentrated buffer solutions.[329]

6 Analytical Methods

Reviews of sub-nanomole amino-acid analysis[330] and appropriate internal standards for ion-exchange analysis[331] form parts of a recent serial issue. Separations of 'uncommon' amino-acids have been reviewed.[332] The second edition of a standard orienting text has appeared.[333]

Gas–Liquid Chromatography.—There is some overlap with the material discussed in an earlier section dealing with the mass spectrometry of amino-acids. The largest body of literature dedicated to this section concerns the quantitative analysis of amino-acid mixtures, but the use of g.l.c. to identify derivatives of amino-acid residues cleaved from peptides through sequence analysis is also covered.

The conversion of amino-acids into volatile derivatives continues to offer problems of choice of method rather than inherent practical difficulties. Recent papers describe the development of established derivatization methods and routine extensions of them; *N*-trifluoroacetylamino-acid n-butyl esters have featured in several studies,[334—338] some of which deal with particular amino-acids (glutamic acid and glutamine;[335] 4-nitrohistidine[336]), *N*-heptafluorobutyroylamino-acid iso-amyl esters[338] and their isobutyl,[339,340] butyl,[341] or propyl[340] analogues providing alternative types of volatile derivatives. Bis(pentafluoropropionyl) derivatives of histidine and 1,4-dimethylhistidine

[323] M. Nakanishi and M. Tsuboi, *Chem. Phys. Letters*, 1978, **57**, 262.
[324] L.C. Pereira, I.C. Ferreira, M.P.F. Thomaz, and M.I.B.M. Jorge, *J. Photochem.*, 1978, **9**, 425.
[325] D.M. Rayner and A.G. Szabo, *Canad. J. Chem.*, 1978, **56**, 743.
[326] A.L. Kwiram, J.B.A. Ross, and D.A. Deranleau, *Chem. Phys. Letters*, 1978, **54**, 506.
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[337] N. Yoshida, T. Yoneda, and Y. Ishihara, *Hokkaido Daigaku Suisan Gakubu Kenkyu Iho*, 1977, **28**, 165.
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[340] D.J. de Lange, C.M. Potgieter, and Z.J. Bosch, *S. Afr. J. Dairy Technol.*, 1977, **9**, 81.
[341] C.F. Poole and M. Verzele, *J. Chromatog.*, 1978, **150**, 439.

have proved suitable for g.l.c.–m.s. purposes.[342] γ-Aminobutyric acid can be determined at picomole levels in tissue samples by g.l.c.–m.s. of its *N*-dinitrophenyl ethyl ester.[343] The use of 2,2-bis(chlorodifluoromethyl) 1,3-oxazolidinones, formed from amino-acids by reaction with 1,3-dichlorotetrafluoroacetone and pyridine followed by cyclization with trifluoroacetic anhydride, has been advocated further (see Vol. 10, p. 20), this time for use with glutamic and aspartic acids and their side-chain amides.[344] Homologous *N*-carboxyalkanoyl amino-acid methyl esters,[345] and *NN*-dimethylamino-acid methyl or butyl esters (prepared from the esters by treatment with HCHO and sodium cyanoborohydride),[346] are new variations of conventional structural types. Trimethylsilyl derivatives[347—349] continue to be used for g.l.c.–m.s. identification and quantitative analysis of amino-acids.

Automation of g.l.c. procedures for amino-acid analysis has been explored further.[350]

Acylation with *N*-trifluoroacetyl-L-prolyl chloride (see also Vol. 10, p. 28) yields a diastereoisomer mixture with partly-racemized amino-acids, and this general approach forms the basis of enantiomer analysis of amino-acids in fossils.[351]

Analysis at picomole levels can be achieved by g.l.c. (electron capture detection) for pentafluorophenylthiohydantoins produced by modified Edman degradation of peptides.[352]

Ion-exchange Chromatography.—The substantial literature covering the instrumental aspect of amino-acid analysis cannot be covered fully here, but topic areas are represented by citations for automated analysers[353—356] (inexpensive[353] or otherwise), mathematical modelling aimed at optimized operating conditions,[357] factors determining the effectiveness of ion-exchange resins used in amino-acid chromatography,[358] assessment of a short programme analysis procedure[359] and a single column procedure using stepwise elution[360] and a study of the use of titanous chloride as a reducing agent for ninhydrin, aimed at improving the precision of the colorimetric stage in amino-acid analysis.[361]

[342] M. Nahy and E. Gelpi, *Chromatographia*, 1978, **11**, 573.
[343] B.N. Colby and M.W. McCaman, *Biomed.Mass Spectrom.*, 1978, **5**, 215.
[344] P. Husek and V. Felt, *J. Chromatog.*, 1978, **152**, 363, 546.
[345] N. Gregersen, I. Gron, K. Rasmussen, and S. Kolvraa, *Biomed. Mass Spectrom.*, 1978, **5**, 80.
[346] T. Severin and H. Popp-Ginsbach, *J. Chromatog.*, 1978, **147**, 473.
[347] M. Takimoto, T. Takeda, S. Takahashi, and T. Murata, *Shimadzu Hyoron*, 1977, **34**, 159 (*Chem. Abs.*, 1978, **89**, 163 926).
[348] N. Heki, *Nippon Naibumpi Gakkai Zasshi*, 1978, **54**, 1157.
[349] S. Yamada, *GC–MS News*, 1978, **6**, 76 (*Chem. Abs.*, 1979, **90**, 18 417).
[350] B.M. Nair, *J. Chromatog.*, 1978, **155**, 249.
[351] E.A. Hoopes, E.T. Peltzer, and J.L. Bada, *J. Chromatog. Sci.*, 1978, **16**, 556.
[352] B.H. Robertson, R.M. Wolcutt, and J.C. Bennett, *Proc. Soc. Exp. Biol. Med.*, 1977, **155**, 287.
[353] G.R. Beecher, *Adv. Exp. Med. Biol.*, 1978, **105**, 827.
[354] V.R. Villanneva and R.C. Adlakha, *Analyt. Biochem.*, 1978, **91**, 264.
[355] R.W. Welch, *J. Chromatog.*, 1978, **152**, 528.
[356] C.St. Pierre and R. Paquin, *J. Chromatog.*, 1978, **156**, 79.
[357] V.G. Maltsev, B.G. Belenkii, and M.L. Aleksandrov, *Zhur. analit. Khim.*, 1978, **33**, 798.
[358] J. Rahm, *Chem. listy*, 1978, **72**, 381.
[359] K. Olek, S. Uhlhaas, and P. Wardenbach, *J. Clin. Chem. Clin. Biochem.*, 1978, **16**, 283.
[360] S. Thoenes, B. Furch, and R. Tutschek, *J. Chromatog.*, 1978, **153**, 536.
[361] L.B. James, *J. Chromatog.*, 1978, **152**, 298.

Precautions to be taken to obtain reliable analysis of arginine, using the amino-acid analyser, have been described.[362] The behaviour in the amino-acid analyser of side-chain *N*-methyl arginines,[363] γ-carboxyglutamic acid (present to the extent of 9.6 % in prothrombin),[364] cystine (lower detection limit 1 nmol),[365] common aromatic amino-acids, together with 3-iodo-, 3,5-di-iodo-, 3,5-dibromo-3-nitro, 3,5-dinitro-, and 3-amino-tyrosines and 4-nitrophenylalanine,[366] and αε-di-aminopimelic acid,[367] has been reported. The separation of the last-named amino-acid from a mixture by anion-exchange chromatography presents no difficulties.[368] β-Alanine and β-aminoisobutyric acid assays based on ion-exchange separation have been developed with special relevance to the analysis of physiological fluids.[369]

The amino-acid analyser equivalent of the enantiomeric analysis of a partly-racemic amino-acid by g.l.c.[351] is similarly based on diastereoisomeric peptide formation; coupling with Boc-L-leucine *N*-hydroxysuccinimide ester (a procedure involving no detectable racemization, *i.e.* < 0.1 %) followed by de-protection gives a DL–LL peptide pair, which through quantitative analysis provides an accurate D–L-assay (less than one part of D-amino-acid in the presence of 1000 parts of the L-enantiomer can be detected).[370]

Further development of the chromatofuge principle, in which centrifugal force replaces hydraulic pressure, has been reported for preparative ion-exchange separation of amino-acids.[371]

Thin-layer and Paper Chromatography.—Papers of a routine nature have been excluded, which amounts to the majority of published work on this well-established technique. Analytical separations of amino-acids by t.l.c. (leucine from isoleucine,[372] ^{125}I-labelled thyroxine, tri-iodothyronine, and iodide ion[373]), by concurrent two-dimensional electrophoresis and paper- or t.l.c.,[374] and by ascending ion-exchange paper chromatography on carboxymethylcellulose (for N^{τ}-methylhistidine)[375] have been reported. Colour reactions of particular importance are *o*-phthalaldehyde and ninhydrin, of which the former is preferable for the visualization of ^{14}C-labelled amino-acids on t.l.c. since it does not cause loss of carbon.[376] Tryptophan can be detected on t.l.c. at 40–850 pmol levels through the yellow-orange fluorescence caused by spraying with 70 % $HClO_4$ before [377] or after[378] a fluorescamine spray.

[362] J.B. Lewis, *J. Chromatog.*, 1978, **166**, 333.
[363] C.G. Zarkadas, *Canad. J. Biochem.*, 1978, **56**, 952.
[364] F. Yamagata, H. Kasai, and T. Okuyama, *Bunseki Kagaku*, 1977, **26**, 819 (*Chem. Abs.*, 1978, **88**, 85 398).
[365] D. Allard, P. Parvy, J.C. Castets, Y. Huang, and P. Kamoun, *Ann. Clin. Biochem.*, 1978, **15**, 161.
[366] K. Ohtsuki and H. Hatano, *J. Chromatog.*, 1978, **148**, 536.
[367] J. Mzik, J.P. Hogan, J.R. Lindsay, and P. Davis, *J. Chromatog.*, 1978, **152**, 269.
[368] U. Chetal, U.R. Mehra, B.P. Singh, and Y.R. Saxena, *Sep. Sci. Technol.*, 1978, **13**, 831.
[369] K.C. Kuo, T.F. Cole, C.W. Gehrke, T.P. Waalkes, and E. Borek, *Clin. Chem.*, 1978, **24**, 1373.
[370] A.R. Mitchell, S.B.H. Kent, I.C. Chu, and R.B. Merrifield, *Analyt. Chem.*, 1978, **50**, 637.
[371] J.W. Finley, J.M. Krochta, and E. Heftmann, *J. Chromatog.*, 1978, **157**, 435.
[372] E.V. Degterev and G.P. Podgornova, *Khim.-Farm. Zhur.*, 1978, **12**, 133.
[373] M. Dobias, J. Mucha, and P. Talan, *Radiochem. Radioanayt. Letters*, 1978, **33**, 179.
[374] R.L. Munier and S. Meunier, *Chromatographia*, 1978, **11**, 508.
[375] N. Nishizawa, T. Noguchi, S. Hareyama, and R. Funabiki, *J. Chromatog.*, 1978, **151**, 424.
[376] H.M. Davies and B.J. Miflin, *J. Chromatog.*, 1978, **153**, 284.
[377] H. Nakamura and J.J. Pisano, *J. Chromatog.*, 1978, **152**, 167.
[378] H. Nakamura and J.J. Pisano, *J. Chromatog.*, 1978, **152**, 153.

Dansylamino-acids detected[379—381] at very low levels[379,390] (< 1 pmol in nanolitre samples of solutions using the ^{14}C variation[379]), and procedures for the analysis of PTH's[382] and diphenylindenonylthiohydantoins,[383] illustrate development of techniques for the identification of amino-acid derivatives.

High Performance Liquid Chromatography.—Although much more recent in its origins, h.p.l.c., like t.l.c., has spawned a routine literature, some of which is included in the present survey. Sophisticated procedures, such as the automated procedure employing *o*-phthalaldehyde colorimetry for the analysis of γ-aminobutyric acid in cerebrospinal fluid,[384] have been developed for the support of particularly active areas of research, but off-the-shelf equipment is used more generally, with *o*-phthalaldehyde detection for dopa (to 4.6 ng levels),[385] and 5-hydroxytryptophan,[386] and with electrochemical detection for cysteine and homocysteine[387] and for dopa and *S*-cysteinyl-dopa.[388] Cation exchange separation of tryptophan and its metabolites[389] and separation of thyroidal amino-acids[390] have been described, and h.p.l.c. determination of valine as its dansyl derivative (down to 100 fmol levels) has been reported in a new Journal devoted to this technique.[391]

H.p.l.c. analysis of PTH's is being studied in many laboratories,[392—395] some points of interest arising in the lower detection limit set at 10 pmol,[393] and in the possibility for a short programme (20 min).[394] Effects of pH and eluent composition have been determined for the liquid chromatographic separation of PTH's on a stationary phase carrying L-valyl-L-valyl-L-proline linked through its carboxy group.[395] H.p.l.c. effects the separation of dimethyl-

[379] M. Weise and D.E. Oken, *J. Chromatog.*, 1978, **152**, 175.
[380] D. Kocmierska-Grodzka, A. Mazanowska, and G.B. Gerber, *Pol. J. Pharmacol. Pharm.*, 1978, **30**, 17.
[381] R.M. Metrione, *J. Chromatog.*, 1978, **154**, 247.
[382] R.L. Munier and A.M. Drapier, *Compt. rend.*, 1978, **286**, 433. E. Soczewinski, J. Iskierbo, and B. Kanadys, *Chem. Anal.*, 1978, **23**, 533 (*Chem. Abs.*, 1979, **90**, 2531).
[383] I. Mancheva and Y. Vladovska-Yukhnovska, *J. Chromatog.*, 1978, **151**, 207.
[384] P. Boehlen, P.J. Schechter, W. Van Damme, G. Coquillat, J.C. Dorsch, and J. Koch-Weser, *Clin. Chem.*, 1978, **24**, 256.
[385] P.M. Froehlich and T.D. Cunningham, *Analyt. Chim. Acta*, 1978, **97**, 357.
[386] F. Engbaek and I. Magnussen, *Clin. Chem.*, 1978, **24**, 376.
[387] R. Saetre and D.L. Rabenstein, *Analyt. Biochem.*, 1978, **90**, 684.
[388] C. Hansson, L.E. Edholm, G. Agrup, H. Rorsman, A.M. Rosengren, and E. Rosengren, *Clin. Chim. Acta*, 1978, **88**, 419.
[389] A. Saito, K. Maeda, K. Kobayashi, F. Yazawa, and Y. Yamamoto, *Igaku No Ayumi*, 1978, **106**, 37 (*Chem. Abs.*, 1978, **89**, 142 736).
[390] M.T.W. Hearn, W.S. Hancock, and C.A. Bishop, *J. Chromatog.*, 1978, **157**, 337.
[391] R. Bongiovanni and W. Dutton, *J. Liq. Chromatog.*, 1978, **1**, 617.
[392] A. Devillers-Thiery and G. Blobel, *Analyt. Biochem.*, 1978, **87**, 66; C.L. Zimmermann and J.J. Pisano, *Methods in Enzymol.*, 1977, **47**, 45; A.S. Bhown, J.E. Mole, W.L. Hollaway, and J.C. Bennett, *J. Chromatog.*, 1978, **156**, 35; R. Zeeuws and A.D. Strosberg, *F.E.B.S. Letters*, 1978, **85**, 68; J. Elion, M. Downing, and K. Mann, *J. Chromatog.*, 1978, **155**, 436; J. Van Beeumen, J. Van Damme, and J. De Ley, *F.E.B.S. Letters*, 1978, **93**, 373; M. Abrahamsson, K. Groningsson, and S. Castensson, *J. Chromatog.*, 1978, **154**, 313; D.J.P. Raulais, P.C. Rivaille, B. Daguilhanes, G.J.G. Lefevre, and G. Milhaud, *J. Chem. Research (S)*, 1978, 11; G. Oestvold, E. Jensen, and T. Greibrokk, *Medd. Nor. Farm. Selsk.*, 1978, **40**, 173.
[393] M.N. Margolies and A. Brauer, *J. Chromatog.*, 1978, **148**, 429.
[394] A.S. Bhown, J.E. Mole, A. Weissinger, and J.C. Bennett, *J. Chromatog.*, 1978, **148**, 532.
[395] G.W.K. Fong and E. Grushka, *Analyt. Chem.*, 1978, **50**, 1154.

aminoazobenzenesulphonyl amino-acids,[396] and resolves (–)-2-methoxy-α-methyl-1-naphthaleneacetyl-DL-amino-acid ester pairs.[397]

Fluorimetry.—Detection of amino-acids and their derivatives after ion-exchange or t.l.c. separation can be achieved by their conversion into fluorescent derivatives, and reviews of the use of fluorescamine[398] or *o*-phthalaldehyde[399] as reagents for this purpose have appeared. There have been suggestions that the fluorescamine reaction does not go to completion, and that substantial differences exist between the different amino-acids in their respective yields in this reaction.[400] Greater conversions in the fluorescamine reaction were achieved by sequential addition of fluorescamine, indicating that a competitive reaction causing inactivation of the reagent was in operation.[400] Analysis of amino-acid mixtures at picomole levels has been advocated for the fluorescamine procedure, but care must be taken to exclude amine contaminants from buffers, since they have been shown to yield non-fluorescent products at low pH.[401]

The procedure involving the conversion of amino-acids into 1-alkylthio-2-alkylisoindoles with *o*-phthalaldehyde and an alkanethiol[285—288] continues to gain adherents, and picomole level analysis of amino-acids,[402,403] including a fluorimetric procedure for estimating transmitter amino-acids (taurine, aspartic acid, glutamic acid, glycine, and γ-aminobutyric acid) in rat-brain tissue,[403] has been described.

Alternative general fluorimetric procedures based on dansylation[404] and reaction with 7-chloro-4-nitrobenzofurazan[405] have been developed further, the latter providing a more sensitive assay for hydroxyproline in blood plasma than colorimetric procedures employing ninhydrin or *p*-dimethylaminobenzaldehyde.[405] Specific fluorimetric assays for cysteine [conversion into the N^{α}-acetyl-*S*-(*N*-acridinylmaleimidyl) derivative][406] and arginine (sub-μg assay after reaction with 9,10-phenanthrenequinone at high pH)[407] have been described.

Other Separation Methods.—Established principles are exploited in separations of amino-acid mixtures by ligand exchange chromatography on Cu^{2+}-loaded silylated glass,[408] and by gel-electrofocusing.[409] Electrophoretic separation of hydroxylysine from its glycosides has been described.[410]

[396] J. Lammens and M. Verzele, *Chromatographia*, 1978, **11**, 376.
[397] J. Goto, M. Hasegawa, S. Nakamura, K. Shimada, and T. Nambara, *J. Chromatog.*, 1978, **152**, 413.
[398] S. Udenfriend and S. Stein, *Proc. Fifth Amer. Peptide Symp.*, ed. M. Goodman and J. Meienhofer, Wiley, New York, 1977, p. 14.
[399] K.S. Lee and D.G. Drescher, *Internat. J. Biochem.*, 1978, **9**, 457.
[400] R.F. Chen, P.D. Smith, and M. Maly, *Arch. Biochem. Biophys.*, 1978, **189**, 241.
[401] N.S. Nadi and F.L. Margolis, *Analyt. Biochem.*, 1978, **91**, 180.
[402] D.J. Reeder, L.T. Sniegoski, and R. Schaffer, *Analyt. Biochem.*, 1978, **86**, 490.
[403] K. Aoki and Y. Kuroiwa, *Chem. Pharm. Bull.*, 1978, **26**, 2684.
[404] P.K. Macnicol, *Analyt. Biochem.*, 1978, **85**, 71.
[405] M. Roth, *Clin. Chim. Acta*, 1978, **83**, 273.
[406] M. Machida, T. Takahashi, K. Itoh, T. Sekine, and Y. Kanaoka, *Chem. Pharm. Bull.*, 1978, **26**, 596.
[407] R.E. Smith and R. MacQuarrie, *Analyt. Biochem.*, 1978, **90**, 246.
[408] R.G. Masters and D.E. Leyden, *Analyt. Chim. Acta*, 1978, **98**, 9.
[409] P.G. Righetti and A. Chrambach, *Analyt. Biochem.*, 1978, **90**, 633.
[410] C. Cintrou, B.D. Peczon, and C.L. Kublin, *Analyt. Biochem.*, 1978, **87**, 622.

Determination of Specific Amino-acids.—This section may give the impression that specific colorimetric assays are represented to a small extent, relative to specific enzyme-based procedures. Several papers in the former category, however, have been cited in preceding sections, and are not repeated here; but nevertheless, enzymatic analyses are being taken up more widely. Estimation of tyrosine and dopa (tyrosinase),[411] branched chain aliphatic amino-acids (leucine dehydrogenase),[412] lysine or ornithine (L-lysine: α-ketoglutarate ε-aminotransferase),[413] are representative. Electrochemical assays of L-amino-acids using the appropriate oxidase and an iodide-selective electrode to determine the concentration of H_2O_2 released in the enzymatic degradation,[414] and for lysine or arginine using membrane-immobilized decarboxylase and diamine oxidase against an oxygen electrode,[415] represent continuing development of well-established principles. Radioenzymic methods for dopa (conversion into [^{3}H]-3-*O*-methyldopamine)[416] and for *S*-adenosyl-L-methionine[417] have been described.

In addition to alternative methods cited elsewhere in this Chapter, γ-aminobutyric acid is amenable to assay by a sensitive radioreceptor method exploiting its natural transmitter function.[418]

Colorimetric assays of protein amino-acids have been developed for glycine (formation of the azlactone with *p*-dimethylaminobenzaldehyde after conversion into hippuric acid),[419] hydroxyproline (development of photometric[420] and colorimetric[421] methods), methionine[422,423] (decolourization of halide platinates[422]) and cystine,[423] tyrosine (coupling with 1-nitroso-2-naphthol),[424] and tryptophan (fluorimetry after conversion into norharman;[425] condensation with α-ketoglutaric acid[426]). Colorimetric procedures with non-protein amino-acids have been described for hydroxylysine and its glycosides,[427] and N^{τ}-methylhistidine.[428]

[411] Y. Menezo, C. Voulot, J.P. Ortonne, and C. Khatchadourian, *Arch. Dermatol. Res.*, 1978, **263**, 267.
[412] T. Ohshima, H. Misono, and K. Soda, *Agric. Biol. Chem.*, 1978, **42**, 1919.
[413] K. Soda, T. Hirasawa, and T. Fukumura, *Analyt. Biochem.*, 1978, **87**, 283.
[414] M. Mascini and G. Palleschi, *Anal. Chim. Acta*, 1978, **100**, 215.
[415] L. Macholan, *Coll. Czech. Chem. Comm.*, 1978, **43**, 1811.
[416] G.A. Johnson and J.M. Green, *Clin. Chem.*, 1978, **24**, 1927.
[417] P.H. Yu, *Analyt. Biochem.*, 1978, **86**, 498.
[418] J.W. Ferkany, L.A. Smith, W.E. Seifert, R.M. Caprioli, and S.J. Enna, *Life Sciences*, 1978, **22**, 2121.
[419] S. Ohmori, M. Ikeda, Y. Watanabe, and K. Hirota, *Analyt. Biochem.*, 1978, **90**, 662.
[420] K. Lindenhayn, H. Kretschmer, and R. Rahn, *Z. Med. Laboratoriumsdiagn.*, 1978, **19**, 246.
[421] J.O. Gordeladze, J. Halse, O. Djoeseland, and H.N. Haugen, *Biochem. Med.*, 1978, **20**, 23.
[422] D.J. Collins, R. Robinson, and A.J. Knell, *Clin. Chim. Acta*, 1978, **88**, 277.
[423] C.O. Moorhouse, A.R. Law, and C. Maddix, in *Technicon Int. Congress on Advances in Automated Analysis*, ed. E.C. Barton, Mediad Inc., Tarrytown, N.J., U.S.A., 1977.
[424] T. Forgach and S.A. Balakrishnan, *Proc. Hung. Ann. Meeting Biochem.*, 1978, **18**, 223.
[425] C. Escande, B. Bousquet, and C. Dreux, *Ann. Biol. Clin.*, 1977, **35**, 387.
[426] N.E. Anderson and F.M. Clydesdale, *J. Food Sci.*, 1978, **43**, 1595.
[427] W.C. Bisbee and P.C. Kelleher, *Clin. Chim. Acta*, 1978, **90**, 29.
[428] L.C. Ward, *Analyt. Biochem.*, 1978, **88**, 598.

2

Structural Investigations of Peptides and Proteins

BY R. HARRISON, M. RANGARAJAN, A. DELL, D. MERCER, R.H. PAIN, AND OTHERS

PART IA: Protein Isolation and Characterization *by R. Harrison*

1 Introduction

This year's Report follows closely the pattern used last year, and many of the more general comments made then are equally applicable now. With the ever increasing volume of publications it has become necessary to be selective in virtually all aspects of protein isolation and characterization. It is hoped that such selection has not diminished the depth of the field covered, but merely taken out some of the filling.

2 Protein Isolation Methodology

A number of review articles on the methodology of protein isolation have appeared during the year. In addition to a text dealing with affinity chromatography, useful both as a reference work (literature cited up to 1976) and as a practical guide,[1] a number of more specialized articles are gathered together in the publication of lectures delivered to the First Tiselius Symposium on Modern Biochemical Separation Methods.[2] Those dealing with specific isolation methodologies are covered in the appropriate section. Others include isolation of specific classes of proteins such as ribonucleases,[3] receptors,[4] membrane proteins,[5] and isoenzymes (hexokinases and myosin).[6] I.U.B. guidelines for the nomenclature of multiple forms of enzymes have been published in a number of journals.[7]

General Comments on Isolation Methodology.—A major consideration in any isolation procedure is whether or not biospecific affinity ligands can be used. While there are obvious attractions, a large number of proteins are still isolated using conventional procedures. Thus a 15 000-fold purification of the dnaB protein of *E. coli* (present at about 20 molecules/cell) was obtained without recourse to affinity adsorbents,[8] and other workers have achieved consistently high yields (85 %/step) over a large number of sequential ion-exchange and gel filtration chromatographic separations.[9] These procedures should be com-

[1] J. Turková, 'Affinity Chromatography,' Elsevier (Amsterdam, New York, Oxford), 1978.
[2] First Tiselius Symposium on Modern Biochemical Separation Methods, *J. Chromatog.*, 1978, **159**, 1.
[3] S. Moore, *J. Chromatog.*, 1978, **159**, 3.
[4] L. Hudson, *J. Chromatog.*, 1978, **159**, 123.
[5] S. Hjertén, *J. Chromatog.*, 1978, **159**, 85.
[6] I.P. Trayer, *J. Chromatog.*, 1978, **159**, 93.
[7] Nomenclature of Multiple Forms of Enzymes, *European J. Biochem.*, 1978, **82**, 1.
[8] K. Ueda, R. McMacken, and A. Kornberg, *J. Biol. Chem.*, 1978, **253**, 261.
[9] T. Sakai and H.-K. Jun, *F.E.B.S. Letters*, 1978, **86**, 174.

pared with the injudicious use of affinity adsorbents, such as the 1.8-fold purification of sheep liver pyridoxal kinase on ADP-Sepharose with a 23 % yield.[10] Also of interest is the rejection of biospecific procedures in favour of salt precipitation and chromatography in the isolation of *Dichtyostelium discoidum* actin, with a concomitant increase in yield from 3 % to 32 %.[11] The potential of affinity adsorbents is illustrated in the isolation of a cathepsin E-like acid protease from rat spleen with a 52-fold purification in a single affinity step, but only an average 2-fold purification in the six remaining non-affinity steps.[12] Other workers have found an affinity step necessary for the retention, on storage, of enzymic activity, although with the step a considerable loss of activity occurred, with no apparent increase in purification.[13] An intriguing graphical presentation of purification data which facilitates evaluation of specific steps has been used.[14] In several cases, specific induction of a particular protein's synthesis has aided its subsequent isolation. One example of this is the 300-fold increase in β-glucuronidase levels in the urine of androgen-treated female mice.[15]

Conflicting data on the resolving power of a new gel filtration matrix, Sephacryl S-200, have been presented. In one report it was found to compare unfavourably with Sephadex G-150, and to behave as a cation exchanger above pH 7.4[16] Others have found that while at pH's below 5.5 and above 8.0 the matrix binds proteins, this effect can be eliminated by the introduction of 0.2M-NaCl into the elution buffer (which is required, in many instances, to prevent protein–protein interactions), and that it gives better resolution and less zone-spreading than the corresponding Sephadex.[17] Sepharose is another gel filtration matrix on which protein adsorption (to sulphate and carboxyl groups, below pH 4) can occur. In addition, many proteins bind to Sepharose in the presence of high salt concentrations. Comparison of this adsorption with that to polytetrafluoroethylene, an experimental matrix, has suggested that it is not hydrophobic in nature, and the possibility of hydrogen-bond formation between the protein and the matrix has been raised.[18] Adsorption mediated by hydrogen-bond formation between γ-globulin and agarose has also been suggested.[19] Whatever the nature of the interaction, several proteins have been isolated by virtue of their adsorption to agarose at high salt concentrations and elution at low ionic strength,[20—24] although in one instance, this adsorption was found to be highly variable.[21] Protein-binding ligands have been used in successive gel filtrations of heparin (+ and – antithrombin),[25] a protein kinase (+ and –

[10] E. Karaurya and M.L. Fonda, *Analyt. Biochem.*, 1978, **90**, 525.
[11] D.G. Uyemura, S.S. Brown, and J.A. Spudich, *J. Biol. Chem.*, 1978, **253**, 9088.
[12] K. Yamamoto, N. Katsuda, and K. Kato, *European J. Biochem.*, 1978, **92**, 499.
[13] J.A. Sharp and M.R. Edwards, *Biochem. J.*, 1978, **173**, 759.
[14] O. Henriksen, E.A. Robinson, and E. Appella, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3322.
[15] N.C. Mills, C. Gupta, and C.W. Bardin, *Arch. Biochem. Biophys.*, 1978, **185**, 100.
[16] G. Morgan and D.B. Ramsden, *J. Chromatog.*, 1978, **161**, 319.
[17] M. Belew, J. Porath, J. Fohlman, and J.-C. Janson, *J. Chromatog.*, 1978, **147**, 205.
[18] S. Hjertén, *J. Chromatog.*, 1978, **159**, 47.
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[24] S.L. Petrović, M.B. Novaković, and R.M. Metlaš, *Analyt. Biochem.*, 1978, **90**, 365.
[25] R.D. Rosenberg, G. Armand, and L. Lam, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3065.

cAMP),[26] a dehydrogenase (+ and − Blue Dextran),[27] and prephrenate dehydratase of *B. subtilis* [+ Phe (monomer) and + Met (tetramer)][28] as a means of increasing the purification obtainable at that specific step. A combination of a ligand-induced solubility shift and adsorption onto Sepharose at high ionic strength has been used in the purification of some tRNA synthetases. General application of such a procedure requires that the ligand–protein complex (in this instance, cognate tRNA–protein) is stable at high ionic strength.[29]

Highly specific steps are used frequently to effect the removal of particular contaminating proteins. This is necessary for a number of reasons, among them being the removal of enzymes with a deleterious effect on subsequent affinity matrices (*e.g.* DNAse prior to Mg^{2+}-dependent binding of RNA polymerase to DNA-cellulose[30]) or elimination of those with similar activities to the protein being purified (*e.g.* a glucuronidase from rat-liver α-D-mannosidase[31] and aminopeptidase M from a porcine dipeptidyl peptidase[32]). In the latter case, removal was effected with an immunoadsorbent against aminopeptidase M. Specific immunoadsorbents have also been used to remove albumin[33] and IgG[34] from plasma proteins, and complex immunoadsorbents have been used to remove contaminating proteins from a human enterokinase[35] and the C5b6 complex of human complement.[36] Albumin, the bulk component of plasma, has also been removed from plasma proteins by virtue of its binding to Cibacron Blue-Sepharose[37—39] and its lack of binding to Concanavalin A-Sepharose.[40] Another plasma component, plasminogen, whose removal is essential for the recovery of many protease-activated proteins in their native form, has been specifically removed on lysine-Sepharose.[41] Other instances of procedures designed primarily for the removal of contaminating proteins are found in the isolation of the dnaB protein of *E. coli*[42] and a rabbit-liver mannan-binding protein.[43]

Ligand Affinity Chromatography.—*General Comments.* Perhaps one of the more salutary papers dealing with ligand affinity chromatography to be published during the past year showed that the binding of the C2 component of complement to Sepharose containing bound C4 (with which it has been claimed to form a complex) was in fact a property of cyanogen bromide-

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[29] F. von der Haar, *F.E.B.S. Letters*, 1978, **94**, 371.
[30] K.K. Cheung and A. Newton, *J. Biol. Chem.*, 1978, **253**, 2254.
[31] D.J. Opheim and O. Touster, *J. Biol. Chem.*, 1978, **253**, 1017.
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[35] D.A.W. Grant, A.I. Magee, and J. Hermon-Taylor, *European J. Biochem.*, 1978, **88**, 183.
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[38] A. Koj, M.W.C. Hatton, K.-L. Wong, and E. Regoeczi, *Biochem. J.*, 1978, **169**, 589.
[39] M.J. Iqbal, P. Ford, and M.W. Johnson, *F.E.B.S. Letters*, 1978, **87**, 235.
[40] D.C. Parmelee, M.A. Evenson, and H.F. Deutsch, *J. Biol. Chem.*, 1978, **253**, 2114.
[41] I.A.M. van Ruijven-Vermeer and W. Nieuwenhuizen, *Biochem. J.*, 1978, **169**, 653.
[42] L.J. Reha-Krantz and J. Hurwitz, *J. Biol. Chem.*, 1978, **253**, 4043.
[43] T. Kawaski, R. Etoh, and I. Yamashina, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 1018.

activated and aged Sepharose, and was totally ligand independent.[44] Biospecific retardation and elution from h.p.l.c. columns containing immobilized AMP or anti-albumin, together with reaction schemes for ligand immobilization, have been described. As the authors acknowledge, the system can as yet only be regarded as a means for rapid separation of small amounts of protein for analytical purposes.[45] *S*-adenosylhomocysteine has been proposed as an affinity ligand for all *S*-adenosylmethionine-dependent methyl transferases,[46] and used successfully in a number of systems.[47,48] Similarly, phenylboronic acid-Sepharose has been proposed as an affinity matrix for serine proteases[49] (the serine hydroxy-group complexing with boronic acid) and dihydroxyboronyl polyacrylamide as a matrix for ADP-ribosylated proteins[50] (reacting specifically with the ribose *cis*-diol). A photochromic derivative of soybean trypsin inhibitor has been covalently coupled to agarose and used in the purification of trypsin, release being effected by exposure to light.[51] The advantage of this over conventional elution methods from the same ligand is unclear.

Choice of Matrix, Ligand, and Method of Ligand Immobilization. Both the methacrylate gels (carrying either hydroxymethacrylate[52] or epoxide[53] groups), which have been claimed to be rigid, resistant to extremes of pH, organic solvents and micro-organisms, and to have good flow properties, and also a hydrophilic co-polymer of acrylic acid and acrylamide, stabilized by agarose,[54,55] have been proposed as matrices for affinity chromatography. The increased stability of divinyl sulphone-cross-linked Sepharose over the non-cross-linked matrix was illustrated in its use as an affinity matrix for agarase isolation.[56] While many of the affinity ligands used appear to be chosen somewhat arbitrarily, in a few cases a spectrum of chemically related compounds have been investigated as to their potential as biospecific ligands. Included in this category are flavins[57] (for the purification of riboflavin-binding proteins), oestrogens[58] (for oestrogen receptor proteins), indolyl derivatives[59] (for indolyl-3-alkane α-hydroxylase), and a number of nucleotides[60] (for the isolation of *E. coli* dnaB protein). In a comparison of three ligands previously used in the isolation of acetylcholinesterase from different sources, only one was found suitable for the pig-cerebral cortex enzyme.[61] This suggests that, for these

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[45] S. Ohlson, L. Hansson, P.-O. Larsson, and K. Mosbach, *F.E.B.S. Letters*, 1978, **93**, 5.
[46] J.P.G. Mack and M.B. Slaytor, *J. Chromatog.*, 1978, **157**, 153.
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[53] J. Turková, K. Bláha, M. Malaňiková, D. Vančurová, F. Sŷec, and J. Kálal, *Biochim. Biophys. Acta*, 1978, **524**, 162.
[54] E. Brown, A. Racois, E. Boschetti, and M. Corgier, *J. Chromatog.*, 1978, **150**, 101.
[55] E. Brown, R. Joyeau, E. Boschetti, and Y. Moroux, *J. Chromatog.*, 1978, **150**, 111.
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[57] A.H. Merril and D.B. McCormick, *Analyt. Biochem.*, 1978, **89**, 87.
[58] H. Richard-Foy, C. Geynet, C. Secco-Millet, G. Redeuilh, and E.-E. Baulieu, *J. Biol. Chem.*, 1978, **253**, 8221.
[59] G. Schmer and J. Roberts, *Biochim. Biophys. Acta*, 1978, **527**, 264.
[60] E. Lanka, C. Edelbluth, M. Schlicht, and H. Schuster, *J. Biol. Chem.*, 1978, **253**, 5847.
[61] C.A. Reavill and D.T. Plummer, *J. Chromatog.*, 1978, **157**, 141.

complex ligands at least, it may not be prudent to rely solely on extrapolation from one source to another. A number of thiamine analogues have also been investigated as affinity ligands for transketolases[62] and for the pyruvate dehydrogenase complex of *E. coli*,[63] though none appeared suitable for large scale preparative work. In the latter case, the presence of magnesium gave increased specificity of binding but a lower enzyme-binding capacity of the matrix. The concentration of bound ligand was important in acetylcholine receptor purification, low ligand densities giving the best recoveries.[64]

Methods for ligand immobilization continue to be proposed and evaluated. A hydrazidation method for the coupling of ligands to CM-Sephadex was evaluated using D-arginine and D-phenylalanine as ligands and carboxypeptidase B as the protein bound. Greater than 90 % of the matrix carboxy-groups were converted to the hydrazide.[65] Aldehyde-activated polyacrylamide supports were used to bind 90 % of the offered concanavalin A and BSA and with better than 90 % of the concanavalin carbohydrate-binding activity retained. While these proteins were bound at 5—20 mg protein ml^{-1} gel in a pH and detergent-stable manner, larger proteins were coupled at lower efficiency.[66] Trichloro-*s*-triazine has been used to link proteins to Sepharose, ligands being coupled in either organic or aqueous solvents. The basis of the claimed superiority of this procedure over cyanogen bromide-mediated coupling appears to be in the amount of protein that can be bound per gram of Sepharose, since, with a range of enzymes, less than 50 % of the protein offered was bound and, with one exception, less than 20 % of the enzymic activity retained.[67] A new procedure, based on modification of the N^6-amino group of adenine, has been used to prepare immobilized ADP and ATP. The procedure could not, however, be applied to NAD^+, $NADP^+$, and their analogues.[68] Other workers continue to evaluate different methods of nucleotide attachment to matrices.[60,69] It seems likely that the claimed success or otherwise of individual procedures will be influenced by the enzyme(s) used in such investigations. As befits the most widely used procedure, the stability of cyanogen bromide-mediated coupling to ligands continues to be investigated,[70] as do the conditions required to minimize heterogeneity of ligands introduced in this manner.[71] A colorimetric method, based on the König reaction of cyanate esters with pyridine has been used to follow both activation with cyanogen bromide and the decay of active groups on Sepharose.[72] In this context, it is worth noting that ethanolamine, frequently used to block unreacted groups, has been coupled to Sepharose and used to achieve a 40-fold purification of the *E. coli* pyruvate dehydrogenase complex.[73]

[62] T. Wood and S. Fletcher, *Biochim. Biophys. Acta*, 1978, **527**, 249.
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[70] J. Lasch and R. Kvelsch, *European J. Biochem.*, 1978, **82**, 181.
[71] M.R. Rossi-Fanelli, G. Amiconi, and E. Antonini, *European J. Biochem.*, 1978, **92**, 253.
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[73] J. Visser, W. van Dongen, and M. Strating, *F.E.B.S. Letters*, 1978, **85**, 81.

The Influence of a Spacer between Ligand and Matrix. The problems associated with the use of 'spacers' were illustrated in the isolation of a light-harvesting chlorophyll a/b protein. Chlorophyll a/b protein was bound under conditions where it was monomeric and hence able to form a tetramer with unbound subunits. If no spacer was used, only low levels of binding were obtained, presumably because of steric hindrance. On the other hand, long spacer groups allowed considerable non-biospecific hydrophobic binding to occur. An optimum spacer length of two methylene groups was determined.[74] Various hexokinase isoenzymes were purified using Sepharose-bound *N*-aminoacylglucosamines. Increased specificity for particular isoenzymes was obtained by varying the length of the aminoacyl spacer. The optimum length was predictable from the free solution kinetics of the *N*-aminoacyl ligand, all of which were competitive inhibitors of hexokinase.[75] Other workers have examined the influence of spacer groups in the isolation of enterokinase[35] and methyl transferases.[46,47]

Optimum Conditions for Binding and Elution. Much of the power of affinity chromatography comes from the judicious use of binding and elution conditions. The presence of 1M sulphate considerably increased the binding of the glutamate dehydrogenase of *Halobacterium* to $NADP^+$-Sepharose.[76] Plasminogen activator was bound to lysine-Sepharose in the presence of stabilizing agents such as EDTA and benzamidine.[77] Non-histone chromatin proteins bound to immobilized histones only in the presence of 4M-urea and 2M-NaCl. After lowering the urea and salt concentrations by gradient dialysis, specific elution could be achieved either with a salt gradient or with a change in pH.[78] Both the intact and the activated forms of Factor B, a component of the alternative pathway of complement activation, could be purified on cobra venom factor-Sepharose, the form isolated depending on the presence or absence of Factor D.[79] Detergent-solubilized cardiac adenylate cyclase was not bound to ATP-Sepharose. However, after passage of the solubilized protein through dodecyl-Sepharose, on which the detergent was removed, biospecific binding to and elution from the ATP ligand was achieved.[80] *Streptococcus faecalis* lactate dehydrogenase bound to oxamate-Sepharose only in the presence of fructose-1,6-diphosphate.[81] Where several proteins might be expected to bind to a specific ligand, pre-chromatography, using conditions where the protein of interest did not bind, considerably increased the purification achieved with a subsequent specific binding step.[82,83] Similarly the ability of several *Bacilli* membrane proteins to bind to ampicillin was blocked by pretreatment of the bacteria with benzyl penicillin. Following incubation, one protein regained its ampicillin-binding capacity and was specifically

[74] M.A. Takahashi and E.L. Gross, *Biochemistry*, 1978, **17**, 806.
[75] C.L. Wright, A.S. Warsy, M.J. Holroyde, and I.P. Trayer, *Biochem. J.*, 1978, **175**, 125.
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[81] N. Kelly, M. Delaney, and P. O'Carra, *Biochem. J.*, 1978, **171**, 543.
[82] W.M. Anderson and R.R. Fisher, *Arch. Biochem. Biophys.*, 1978, **187**, 180.
[83] H.J. Eisen and W.H. Glinsmann, *Biochem. J.*, 1978, **171**, 177.

isolated (the lethal target protein).[84] Elution conditions used in the isolation of ribonucleases on immobilized nucleotides have been extensively investigated.[85,86] IMP gradient and step elution of inosinic acid dehydrogenase from GMP-Sepharose were compared, a 30% increase in specific activity of gradient over stepwise elution being found.[87] Murine immunoglobulin G subclasses have been separated on protein A-Sepharose with subtle changes in both pH[88] and salt concentration.[89] For proteins where affinity for the immobilized ligand is high, such as acetylcholine receptors[64] or steroid-binding proteins,[90,91] batch adsorption and desorption procedures are often used for both binding and elution. Prolonged incubation of the complexed ligand under dissociating conditions improves subsequent elution.[92] Where ligand affinity is high, electrophoretic desorption has also been successfully used.[39,93]

Specific Ligands.—*Anthraquinone Dyes.* A number of recent papers have examined the interaction of the above dyes, of which the most widely used is Cibacron Blue F3G-A, with various enzymes, and the concept of such interaction being a probe for a 'dinucleotide fold' super-secondary structure is now amply challenged. Aldolase binding to Cibacron Blue F3G-A does not block the active-site lysine, but only ATP and fructose-1,6-diphosphate binding sites.[94] Inhibition of F3G-A binding by various anthraquinone analogues has suggested that specific interaction with nucleotide binding sites rather than any possible 'dinucleotide fold' occurs.[95] While the holoenzyme form of bakers' yeast cytochrome b_2 is not retained on F3G-A-Sepharose, it does bind Cibacron Blue when free in solution, suggesting two binding sites, one of which, the flavin site, permits binding of the apoenzyme.[96] Interaction of Blue Dextran-Sepharose with *E. coli* polynucleotide phosphorylase was shown to require an intact polynucleotide binding-site. The bound enzyme was still active and therefore nucleoside diphosphates still had free access to the catalytic site.[97] The fractionation of a number of other nucleotide-binding enzymes (restriction endonucleases,[98] nucleosidases, and nucleoside kinases[99]) on immobilized Cibacron Blue has also been described. Several investigators have looked at other structurally related dyes and found enzyme-specific differences in binding.[100—102] One example of this is the higher affinity of $NADP^+$-

[84] H.A. Chase, P.E. Reynolds, and J.B. Ward, *European J. Biochem.*, 1978, **88**, 275.
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[96] D. Pompon and F. Lederer, *European J. Biochem.*, 1978, **90**, 563.
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[99] Y.-C. Cheng and B. Domin, *Analyt. Biochem.*, 1978, **85**, 425.
[100] A.R. Ashton and G.M. Polya, *Biochem. J.*, 1978, **175**, 501.
[101] R.S. Beissner and F.B. Rudolph, *J. Chromatog.*, 1978, **161**, 127.

dependent dehydrogenases for Procion Red HE-3B over Cibacron Blue F3G-A. Matrex 201R was preferred to Sepharose CL-4B as the matrix in this work.[102] The improved resolution achieved with Cibacron Blue F3G-A-Sepharose rather than Blue Dextran-Sepharose has also been noted.[103,104] A number of serum proteins, none with nucleotide-binding properties, have also been fractionated using immobilized Cibacron Blue, elution being performed with a pH gradient.[105] The effects of pH and magnesium concentration on the elution of deoxycytidine kinases with ATP from Cibacron Blue have been analysed in depth.[106]

Nucleic acids. A review of affinity chromatography on nucleic acids has appeared. Immobilization techniques were particularly well covered, other sections dealing with applications of immobilized nucleic acids, and factors determining the choice of matrix and method of attachment.[107] Factors affecting the binding of DNA and RNA to cyanogen bromide-activated epichlorohydrin-desulphated agarose have also been investigated. Sepharose 2B was found to be a better matrix than 4B and binding was found to be increased in the presence of formamide.[108] Preparation of dsDNA-cellulose using cyanic chloride is also claimed to give a stable ligand.[109]

Immunoglobulins. Although immunoglobulins are theoretically highly specific ligands, proteins eluted from immunoaffinity columns rarely have the purity desired. This fact was illustrated in the isolation of mouse L cell interferon, where four purification steps providing a 22-fold overall purification were required *after* a specific immunoadsorption step.[110] Investigation of non-specific binding to IgG-agarose showed it to be enhanced at low pH, low ionic strength, and low temperature, and to be decreased at high ionic strength and high temperature.[111] Other workers have suggested a washing procedure using borate buffer at pH 8.5 and containing a non-ionic detergent (0.1 % Tween 20) and 1M-NaCl as a means of reducing non-specific adsorption without affecting specific recoveries.[112] The removal of non-specifically adsorbed proteins from IgG-Sepharose was found to be lower with low pH than with high pH elution of the specifically-bound protein.[113] The problem of elution without loss of activity is still largely unresolved. Even with immediate dialysis of 3M potassium thiocyanate-eluted IgE receptors[114] or xanthine dehydrogenase[115] from immunoaffinity columns, only 12—19 % activity was retained. In the latter case this was coupled to a low, 1.2-fold, purification. Alloantigenicity of

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[115] E.S. Lyon and R.H. Garrett, *J. Biol. Chem.*, 1978, **253**, 2604.

β_2 microglobulin was also lost with elution from an immunoaffinity adsorbent at low pH or with 3.5M magnesium chloride.[116] SDS elution from an immunoaffinity column produced a 50 % loss in binding capacity of the column.[117] Inter-species cross-reacting antibodies continue to be used without any obvious increase in ease of elution of the bound protein.[118] Electrophoretic elution appears to be potentially the least harmful method of disruption of antibody–antigen interaction.[119] Many of these aspects have been covered in a review on preparative immunoadsorbtion.[120]

Hydrophobic Chromatography.—Several of the papers delivered at the First Tiselius Symposium on Modern Biochemical Separation Methods dealt with hydrophobic chromatography. Of these, three gave particular emphasis to specific groups of proteins, namely membrane proteins,[5] serum proteins,[19] and skeletal muscle enzymes.[121] Other contributions dealt with partition between polymer phases (biospecific as well as hydrophobic ligands)[122] and with modifying factors in hydrophobic chromatography. Alkyl and aromatic ligands were compared, some proteins behaving differently on ligands with similar hydrophobicities but with different structural features. These and other parameters that could be varied were discussed.[123] A further comprehensive review discussing matrices, ligands, coupling procedures, desorption techniques, factors affecting adsorption and specificity of interaction has also appeared.[124] Reasoning that hydrophobic interactions should be temperature sensitive, one group of workers has achieved some separation of proteins using temperature changes (−15 — +20 °C) to effect elution. Clearly, chromatography at sub-zero temperatures requires an anti-freeze, in this case ethylene glycol.[125] The effect of alkyl chain-length on binding affinity has been investigated with rat-liver phosphoprotein phosphatase. The enzyme was bound in 3M-NaCl and eluted with a decreasing salt gradient. In the ligand range butyl—decyl, recovery was maximized with pentyl-Sepharose, with a severe loss of protein with higher chain-lengths.[126] A number of different aryl, iso-alkyl, and n-alkyl ligands were investigated in the isolation of rabbit kidney rennin.[127] Hydrophobic chromatography has been particularly useful in the isolation of nuclear proteins as the high salt concentrations used prevent protein aggregation.[128,129]

Covalent Chromatography.—Fractionation of plasma proteins on thiol-Sepharose has been reviewed by Laurell.[130] The regeneration of organo-

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mercurial-Sepharose columns with mercuric chloride has been criticized on the grounds that the introduction of mercuric ions into many enzymes is undesirable. An alternative procedure of regeneration using 2-mercaptoethanol was proposed. Although in this the column binding-capacity was decreased, it was still sufficient for most purposes.[131] Following the removal of extrinsic membrane proteins with lithium di-iodosalicylate, sialoproteins were resolved from the erythrocyte band 3 protein on thiol-Sepharose.[132]

Biospecific Elution from Ion-exchange Resins.—Conditions for the elution of phosphoglycerate kinase from CM-cellulose have been described for the enzyme, from twenty different sources. The buffer pH was chosen with reference to the mobility of each enzyme on starch-gel electrophoresis and elution effected with the substrate. Acidic proteins with no affinity for CM-cellulose were purified using standard ion-exchange chromatographic techniques.[133] The elution of aminoacyl tRNA synthetases from phosphocellulose by their cognate tRNA's was affected in different ways by magnesium and ATP. While the tyrosyl and valyl enzymes were more specifically eluted in the presence of magnesium, and the isoleucyl enzyme in the presence of both magnesium and ATP, the threonyl enzyme was unaffected by both ligands.[134] Other examples of proteins isolated using affinity elution are given in Table 3.

Electrophoretic Procedures.—Although primarily used to prepare small amounts of protein for analytical purposes, isoelectric focusing is increasingly capable of separating significant amounts of proteins.[135] A 70 % recovery of haemoglobin A was obtained from 32 mg of a mixture of haemoglobin A and haemoglobin S. These proteins differ by only 0.2 pH units in their isoelectric points.[136] Continuous free-flow electrophoresis has also been described as a preparative procedure.[137] Electrophoretic elution from affinity ligands[39,93] has already been discussed. An electrophoretic system achieving 50-fold concentration of proteins has also been described. One significant advantage of the system is that it is non-disruptive.[138]

Solubilization of Membrane Proteins.—Numerous procedures have now been described for membrane solubilization. Differences between these are shown in the composition of proteins solubilized from *E. coli* membranes by a number of them.[139] An investigation of the ability of different detergents (anionic, zwitterionic, non-ionic, and cationic) to release α-glucosidase from *Bacillus amyloliquefaciens* membranes showed the combination of cholate and chloride to be most effective. The specific activity of the released enzyme

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ranged from 6—60.[140] The importance of monitoring both percent enzyme released and its specific activity was also shown in the EDTA-mediated release of hydrogenase from *Desulphovibrio* membranes. The highest specific activity was obtained after 15 minutes incubation, with 93% release of enzyme. After 45 minutes the amount of released enzyme had increased by 5 % but its specific activity was half of that at 15 minutes.[141] This sequential extraction of membrane proteins was also shown by the action of Tween 20 on an *Acholeplasma*.[142] A significantly higher phospholipid content was found with Tween 40-prepared membranes (1.38 mg mg^{-1} protein) than with those prepared by mechanical shearing (0.65 mg mg^{-1} protein).[143] Of seventeen studied, non-ionic detergents with a hydrophilic–lipophilic balance between twelve and fourteen were effective in solubilizing pig-kidney plasma membrane adenylate cyclase.[144] Sulphobetaines (zwitterionic substances with a generalized structure SB_n) were shown more effective ($n > 12$) than non-ionic detergents of the polyoxyethylene type (*e.g.* Nonidet P-40) in solubilizing mouse-fibroblast membranes. While less effective than SDS, sulphobetaine action did not result in protein denaturation.[145] Other procedures giving selective loss of membrane proteins have been freezing and thawing in a variety of salts[146] and phospholipase treatment of the membrane.[147] The λ phage receptor protein from *E. coli* was solubilized and further purified on Sephacryl S200 in the aprotic solvent hexamethylphosphoric triamide.[148]

Miscellaneous Procedures.—Factors that influence the precipitation of proteins by polyethylene glycol have been studied in some depth. Essentially temperature independent, precipitation of albumin was affected by pH, ionic strength, and composition of buffer, and by the size range of the polyethylene glycol used.[149] Furthermore, conditions that led to self-association of proteins produced lower solubilities of proteins in polyethylene glycol. This effect could not, however, be mimicked by chemical cross-linking.[150] A novel procedure, with possible general applicability, has been the elution of proteins from ion-exchange columns with ampholines.[151—153] Theoretical treatment of the procedure has shown that the elution position of a protein is dependent on its isoelectric point.[154] Serum components have also been fractionated on anion-exchange resins using carboxyethyl dextrans to displace bound protein.[155] Chromasomal proteins have been fractionated on hydroxy-apatite subsequent

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to binding of native chromatin. By using selected dissociating conditions, proteins could be removed leaving the DNA and non-dissociated proteins still bound.[156] In this context, the observation that increasing the salt concentration from 0.75—3 M did not increase dissociation of non-histone chromatin proteins from cultured hepatoma cells should be noted.[157] Simple modified matrices rather than complex immobilized sugars have been used in the isolation of glycosidases[158] and lectins.[159] Arginine-Sepharose has been used to concentrate esterases and esterase inhibitors from human urine, elution being achieved with 2 % ammonium hydroxide.[160] Density gradients formed by successive freezing and thawing of sucrose solutions have been analysed. While a useable sucrose gradient can be easily and reproducibly prepared, the method suffers a major disadvantage in that salt concentration gradients are also set up.[161] Fractionation at sub-zero temperatures has been used for a poly(ADP-ribose) polymerase unstable at 4 °C, aqueous–organic solvent mixtures being used to prevent freezing of the solvent.[162] An ingenious approach has been used to produce enrichment of transport-specific proteins. Following cell-membrane solubilization, proteins were incorporated into vesicles which had been preloaded with glucose. These were then incubated in a glucose-free medium and fractionated on a density gradient. Vesicles that were able to transport glucose had lowered densities and were separated from those that did not contain glucose transport proteins.[163] Papers describing multicomponent preparations of ribosomal proteins and factors involved in protein synthesis have been published.[164–167] One of these is of note in that by avoiding urea and acid treatment, ribosomal proteins with considerably different properties than those produced using denaturants were isolated.[168] Finally, a study of the inactivation of phenylmethylsulphonyl fluoride, a commonly used proteinase inhibitor, in aqueous buffers has appeared. Its half-life at 25 °C ranged from 35 minutes at pH8 to 110 minutes at pH7.[169]

3 Tabulated Summaries of Selected Purifications

The number of procedures now using affinity steps has meant that this year's tabulation is more selective than ever. One criterion used in deciding whether or not to include a specific procedure is, with a few exceptions, the publication of sufficient data (specific activities, yields *etc.*) to enable the step to be evaluated. In addition, where a single procedure has been used for a large number of enzymes (*e.g.* chloride elution of DNA polymerases from immobi-

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lized DNA) a few have been arbitrarily selected to illustrate minor variations in procedure and results achieved. Division of affinity ligands into two categories (in order to use a different tabulation) is also somewhat arbitrary. For instance, adenine nucleotides are regarded as having a widespread application whereas other nucleotides are designated specific ligands (*e.g.* UDP[170]). A similar arbitrary distinction divides affinity ligands with widespread application (*e.g.* DNA) from other chromatographic procedures (*e.g.* immunoaffinity, heparin chromatography). Once again, the purification factor achieved at a specific step is included even though it is largely dependent on the position in the total procedure at which it is used. This is well illustrated in the purification of a galactosidase from cat brain and liver, where the brain enzyme was isolated using a specific ligand followed by lectin chromatography, with purification factors of 54 and 2. With the liver enzyme the procedures were reversed, giving a 40-fold purification at the lectin step and 4-fold with the specific ligand.[171] In Table 2 all proteinases appear under proteinases and, similarly, lectins under lectins.

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Table 1 *Ligands with widespread application*

Ligand	*Protein*	*Source*	*Eluant/Comments*	*Factor*	*Ref.*
cAMP	cAMP-binding proteins	bovine kidney	various eluants for three proteins; 7M-urea for high affinity protein	×1044	172
	cAMP kinase regulatory subunit	bovine heart	5′-AMP, cAMP; stop flow to equilibrate with ligand	×166	92
AMP	adenosine 5′-phospho-sulphate sulphohydrolase	bovine-liver lysosomes	AMP	×20	173
	alcohol dehydrogenase	chicken liver	NAD^+, NAD^+ + pyrazole	×53 ×1.2	174
	aldehyde dehydrogenase	rat-liver microsomes	NAD^+	×15.6	175
	D-lactate dehydrogenase	giant barnacle depressor muscle	NAD–pyruvate adduct	×1.2	176
	malate dehydrogenase	*Schistosoma mansoni*	pH gradient	×10	177
	5′-nucleotidase	lymphocyte membranes	AMP	×30	178
	phosphofructokinase	*E. coli*	ATP; non-allosteric enzyme	×9	179
	saccharopine dehydrogenase	yeast	phosphate gradient; 55 % yield	×1.6	180
	succinate semialdehyde dehydrogenase	human brain	AMP gradient	×32 ×14	181
	threonine dehydratase	*Salmonella typhimurium*	AMP; biogradative enzyme	×127	182
ADP	NADPH–cytochrome P450 reductase	rat-liver microsomes	AMP; phenobarbitone-induced	×7	183
	glucose-6-phosphate dehydrogenase	human erythrocyte	$NADP^+$	—	184
	glucose-6-phosphate dehydrogenase	*Drosophila melanogaster*	$NADP^+$ gradient; also mouse-kidney, -liver enzymes	×356	185
	malate dehydrogenase	*Drosophila melanogaster*	$NADP^+$ gradient; as above	×200	185
	mRNA (nucleoside-2′-)-methyltransferase	vaccinia virus	NaCl gradient	×2.5	186
ATP	adenylate cyclase	heart	ATP; cAMP, PP_i, GTP did not elute	×45	80
	dnaB protein	*E. coli*	magnesium–ATP	×169	187
	dnaB protein	*E. coli*	ATP, ADP, or PP_i; not AMP or P_i	×99	60

	phosphoglycerate kinase	various sources	magnesium–ATP	×66—×3444	69
	phosphoglycerate kinase	mouse testicle, muscle	ATP	×81, ×27	188
NAD^+	aldehyde dehydrogenase	yeast	NAD^+; K^+-activated enzyme	×1.3	189
	aliphatic aldehyde dehydrogenase	*Beneckea harveyi*	NAD^+	×3	190
	NADH-degrading enzyme	*Proteus vulgaris*	NADH	×76	191
	pyridine dinucleotide transhydrogenase	bovine-heart mitochondria	NADPH	×2.2	82
	sorbitol dehydrogenase	rat liver	NAD^+	×86	192
$NADP^+$	fatty acid synthetase	*Mycobacterium smegmatis*	$NADP^+$; single-chain enzyme	×1.3	193
	glutamate dehydrogenase	*Halobacterium*	NaCl gradient; remove NADPH before binding	×12.3	20

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	ω-hydroxy fatty acid $NADP^+$ oxidoreductase	potato tuber disks	KCl, $NADP^+$	×33 ×4.9	194
	malonyl-CoA decarboxylase	rat-liver mitochondria	NaCl gradient	×2.3	195
Cibacron Blue	α-1-antichymotrypsin	human plasma	NaCl	—	196
	carbamyl phosphate synthetase-I	frog liver	bind in magnesium, elute in magnesium-free buffer	×1.8	197
	deoxycytidine kinase	various sources	magnesium–ATP/pH	×17— ×89	106
	dihydroxy-vitamin D receptor	chick intestine	KCl gradient	×7.3	198
	malate dehydrogenase	mouse-liver cytoplasm	NADH; NAD^+-dependent enzyme	×3.7	199
	orotate phosphoribosyl transferase	baker's yeast	OMP, 5-phosphoribosyl-1-pyrophosphate	×200, ×40	103
	orotidine 5′-monophosphate decarboxylase	baker's yeast	OMP or UMP	×6600	103
	phosphofructokinase	*E. coli*	ATP; non-allosteric enzyme	×73	179
	prostaglandin-Δ^{13}-reductase (15-keto)	human placenta	KCl gradient	×365	200
	prostaglandin reductase (9-keto)	human placenta	KCl gradient	×37.5	201
	prostaglandin dehydrogenase (15-hydroxy)	human placenta	KCl gradient; $NADP^+$-linked enzyme	×107	201
	pyruvate kinase	rat liver	NaCl, fructose 1,6-diphosphate	×6	202
	phosphodiesterase	*Crotalis* venom	phosphate; NAD^+ is a substrate	×57	104
	rhodopsin kinase	bovine rod	KCl gradient	×3.8	203
	shikimate dehydrogenase	*Neurosporra crassa*	NADH	×20.8	204
	shikimate oxidoreductase 3-dehydroquinate hydrolase	*Physcomitrella patens*	phosphate gradient; single chain	×21	205
	thiosulphate sulphur transferase	bovine liver	bisulphite	×6.3	206
Blue Dextran	adenylate kinase	porcine heart	NaCl; acidic enzyme	×26	207
	glucose-6-phosphate	*Methylmonas* M15	$NADP^+$ gradient	×4.6	208

	dehydrogenase				
	glycerol-3-phosphate dehydrogenase	*E. coli*	NaCl gradient, NaCl gradient + glycerol phosphate; wild type and feedback-resistant	×25, ×4.2	209
	glyoxylase I	rabbit liver	*S*-hexylglutathione	×60	210
	malate dehydrogenase	*Saccharomyces cerevisiae*	KCl or NADH	×147	211
	mercuric reductase	*E. coli*, plasmid-coded	NADPH; organomercurial hydrolase unbound	×25	212
	nitrite reductase	*Neurosporra crassa*	NADPH; NADPH-linked enzyme wash column with $NADP^+$, ATP	×27	213
	3-phosphoglycerate dehydrogenase	chicken liver	NaCl	×12	214
	polynucleotide kinase	T4	KCl, rATP	×44	215
DNA	cAMP receptor protein	*E. coli*	pH; denatured DNA used	×2	216

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deoxyribonuclease	KB cells	KCl gradient; *Herpes simplex*-induced enzyme	×139	217
DNA-dependent ATPsae II	*E. coli*	NaCl gradient	×28	218
DNA ligase	chick embryo nucleus	KCl	×9	219
DNA polymerase	*Chlamydomonas reinhardii*	NaCl gradient; ssDNA	×1036	220
DNA polymerase	*Xenopus laevis* ovary	KCl gradient; low molecular weight enzyme	×2	221
DNA polymerase	*B. Subtilis*, PBS2-induced	NaCl gradient; DNA has U not T	×16	222
DNA polymerase	*Physarum polycephalum*	KCl gradient	×8.3	223
DNA polymerase	mosquito	KCl	×12	224
DNA polymerase-β	guinea-pig liver	NaCl gradient	×21	225
DNA-unwinding protein	*Lilium* meiotic cells	NaCl	×614	226
EBV-associated nuclear antigen		NaCl, 10 % glycerol used to prevent matrix–protein interaction	×5	227
endonuclease	*B. stearothermophilus*	KCl; apurinic-site specific	×10	228
endonuclease endoR.Bsp	*B.;sphaericus*	KCl gradient; type II restriction enzyme	×12.2	229
glucocorticoid receptor		NaCl; heat-activated after first passage	×11 200	83
guanylyl transferase(mRNA)	vaccinia virus	NaCl gradient	×28	230
3-methyladenine-DNA glycosylase	*E. coli*	NaCl	×14	231
poly(adenosine diphosphate ribose) polymerase	bovine thymus	NaCl	×62	232
poly(ADP-ribose) polymerase	Ehrlich ascites cells	—	×68	233
rep protein (ATPase)	*E. coli*	NaCl	×5	234
RNA polymerase III	yeast	ammonium sulphate	×15	235
RNA polymerase III	HeLa cells	ammonium sulphate gradient; adenovirus infected and uninfected cells	×6.6, ×3	236
RNA polymerase (DNA-	*B. subtilis*	KCl gradient; separation of	×8	237

dependent)		core and holoenzyme		
RNA polymerase (DNA-dependent)	*Caulobacter*	KCl; requires magnesium to bind	×36	30
tri-iodothyronine-binding protein	rat liver	NaCl gradient	×42	238
int gene product	bacteriophage λ	—	×10.8	239

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Table 2 *Specific affinity ligands*

Protein	*Source*	*Ligand*	*Eluant/Comments*	*Factor*	*Ref*
Acetylcholinesterase	*Electrophorus electricus*	acridinium deriv.	decamethonium; ligand concentration and yield studied	—	240
	porcine brain	acridinium deriv.	decamethonium	×900	241
	pig cerebral cortex	various	edrophonium, decamethonium	×900	61
	Electrophorus electricus	pyridinium deriv.	decamethonium	×240	242
Adenosine deaminase-binding protein	human kidney	calf adenosine deaminase	glycine–HCl pH2.8	×544	243
Alkaline phosphatase	bovine intestine	phosphonic acids	binds in TRIS, elute in phosphate	×11	244
Biotin-binding protein	chicken egg yolk	biotin	biotin, 65 °C	×9	245
DD-Carboxypeptidase	*Streptococcus faecalis*	ampicillin	hydroxylamine; membrane enzyme	×6.5	246
Catechol-*O*-methyl-transferase	pig liver	2,6-dimethoxyphenol	KCl	×104	247
Catechol methyl transferase	rat heart, brain	3,4-dimethoxy-5-hydroxy phenol	phosphate gradient	×23	248
Cholinesterase	*Phaseolus vulgaris*	acridinium	NaCl	×9.6	249
'Corrinoid' enzyme	*Clostridium thermoaceticum*	pteroylglutamate	NaCl; pteroylglutamate (= folate)	×3.5	250
cAMP–nucleotide phosphodiesterase	bovine ventricle	trimethylpapaveroline	calcium gave 1 peak, EGTA gave 2 peaks	—	251
Cytochrome *b*, c_1 complex	*Neurosporra crassa*	ferricytochrome *c*	ascorbate plus NaCl	×7	252
Dexamethasone-binding protein	rat-liver cytosol	11-deoxycorticosterone	ligand bound *via* –S–S– bond, elute with protein by thiol	×1485	253
Dihydropteridine reductase	human liver	1,2-naphthoquinone	minus NADH at pH10.9	×300	254
	sheep, rat liver	methotrexate	phosphate	×19	255
DNA-Dependent RNA polymerase	chick embryo	tRNA	ammonium sulphate gradient	×1.6	256
eIF-2	rabbit reticulocyte	mRNA	KCl gradient	×100	257
Estrogen receptor	calf uterus	various estrogens	estradiol	×1730	58

Ferredoxin–$NADP^+$-reductase	spinach	ferredoxin	NaCl	×3.7	258
Ferredoxin–nitrate reductase	*Anacystis ridulans*	ferredoxin	binds to dithionite-reduced ligand, elute minus dithionite	—	259
β-Galactosidase A_2, A_3	human liver	1-thio-β-D-galactopyranoside	low recoveries (A_3–16 %)	×83, ×25	260
β-Galactosidase	rat mammary gland	β-D-thiogalacto pyranoside	lactose	×150	261
β-D-Galactosidase	jack bean meal	galactose	pH	×65	262
Galactosylhydroxylysyl-glucosyltransferase	chick embryo	UDP-glucose	NaCl	×17	263
		collagen	collagen peptides	×14	

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Glutamine synthetase	*Neurosporra crassa*	glutamate	glutamate	—	264
Glyceraldehyde-3-phosphate dehydrogenase	mouse organs	human erythrocyte ghosts	NADH; aldolase removed with fructose-1,6-diphosphate	×25—×146	265
Glycerol-3-phosphate dehydrogenase	rabbit liver	trinitrobenzene-6-phosphogluconate	NADH	×82	266
			glycerol-3-phosphate	×3.3	266
Glycineamide ribonucleotide transformylase	chicken liver	glycineamide ribonucleotide	phosphate	×8	267
GM_1 ganglioside β-galactosidase	cat brain, liver	thiogalactopyranoside	NaCl	×54, ×4	171
Gramicidin S synthetase	*B. brevis*	ornithine	KCl gradient; heavy enzyme	×9	268
	B. brevis	D-phenylalanine	free carboxyl-phosphate free amino-KCl; light enzyme	×1.5 ×9	269
cGMP-dependent protein kinase	bovine heart	cGMP	cGMP	×380	270
Guanylate cyclase	rat brain	GTP	pre-run in EDTA, bind in manganese, elute NaCl	×5.1	271
Hexokinase	various, *e.g.* rat kidney	glucosamine derivs.	glucose	×21 500	75
Hexosaminidase AB	—	2-deoxy-1-thio-β-D-galactopyranoside	*N*-acetylglucosaminolactone	×14	272
Hexosaminidase P	human serum (pregnant)	*N*-acetylglucosylamine	pH8.3	—	273
Hypoxanthine–guanine phosphoribosyltransferase	human erythrocytes	GMP	GMP	×273	274
Inhibitor protein	bovine brain	modulator protein	EGTA; inhibits calcium-activated cyclic nucleotide phosphodiesterase	×217	275
Inosinic acid dehydrogenase	*Aerobacter aerogenes*	8-amino-GMP	IMP gradient	×33	87
Interferon	mouse	poly (U)	NaCl	×92	276
Lectins					
Modeccin	*Adenia digitata*	asialo-fetuin	lactose	—	277

Lectin	black locust bark	human erythrocytes	borate	—	278
	chinook salmon ova	agarose	D-galactose or L-rhamnose	—	279
Lectin (anti-A_1)	hog peanut	*N*-acetylgalactosamine	*N*-acetylgalactosamine	—	280
Lectin	jimson weed seed	*Aspergillus niger* polysaccharide	hydrolized polysaccharide	—	278
	Patura stramonium	fetuin	*N*-acetylglucosamine	—	281
Isolectins	*Phaseolus vulgaris*	thryoglobulin	glycine–HCl pH3, 0.5M-NaCl	×3.5	282
Lectin (group B specific)	powan fish roe	α-D-galactose	D-galactose	—	283
Lectins	various fish	galactose, fucose	galactose, fucose	—	284
Lectin	*Ulex europeus*	α-L-fucoside	α-L-fucose	×40	285
Lysine-tRNA synthetase	—	lysine	lysine	×5000	286
Maldodextrin phosphorylase	*E. coli*	glycogen	NaCl gradient	×5.6	287

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α-D-Mannosidase	jack bean	benzidine	NaCl	×18	288
Methionine-tRNA synthetase	sheep liver	$tRNA^{Met}_{beef\ liver}$	selectively retarded	×37	289
	sheep lactating mammary gland	$tRNA^{Met}_{beef\ liver}$	selectively retarded	×1.6	290
N^5-Methyltetrahydrofolate homocysteine methyltransferase	*E. coli*	cobalamin	photolysis plus 0.5M-NaCl	×390	291
Methyltransferases	*Ruta graveolens* L	*S*-adenosyl-L-homocysteine	pH3; 5-methyl- and 8-*O*-enzymes	×50, ×16	48
NADPH–ferredoxin reductase	rat liver	adrenodoxin	NaCl	×383	292
Phosphatidylcholine exchange protein	bovine liver	phosphocholine deriv.	0.15 % deoxycholate	×130	293
Phosphoenolpyruvate carboxykinase	rat liver	GTP	KCl; some hydrophobic binding	×2	294
Phospholipase A_2	sheep erythrocyte	phosphatidylcholine	EDTA; membrane enzyme	×49	295
Phosphoprotein phosphatase	rat liver	protamine, histone	NaCl	×3, ×7	126
Poly (A) polymerase	rat-liver nucleus	RNA	KCl; chromatin-bound enzyme	×4.7	296
Protein kinase	rat-liver nucleus	phosvitin, casein	NaCl gradient	×173,×3	297
Proteinases					
Acid protease	rat brain	pepstatin	urea gradient	×393	298
	rat spleen	pepstatin	load pH3.5, elute pH8.6; cathepsin E-like enzyme	×52	12
Alkaline protease	rat skeletal muscle	soy bean inhibitor	HCl, pH2	×45	299
Aminopeptidase	pig uterus	leucylglycine	NaCl	×1.5	300
Atypical protease	rat mast cell	chymotrypsin inhibitor I	0.1M acetic acid, 1mM-HCl	×21	301
Carboxypeptidase N	human plasma	aminobeznoylarginine	guanidinoethylmercaptosuccinate	×117	302
Cytotoxic protease	human lymphocyte	soy bean inhibitor	pH2; 12 % recovery	×0.6	303
Dipeptidyl aminopeptidase	—	various, 4-phenylbutyl	33 % ethylene glycol, NaCl	—	304

Dipeptidyl aminopeptidase IV	pig kidney	glycylproline	NaCl gradient	—	305
Elastase	pig pancreas	alanylalanylalanine	alanine, pH4.5 or high pH; trypsin, chymotrypsin bind	×439	306
Endopeptidase	hornet larva	phenylbutylamine	chymotrypsin-like enzyme	×1.1	307
Enterokinase	human duodenal fluid	β-aminobenzamidine		×15	35
Kallikrein	human urine	aprotinin (Trasylol)	pH gradient; two enzymes	×300	308
Pepsinogen	mouse	pepstatin	load pH3.8, elute pH8.5	×4.6	309
Post-proline cleaving enzyme	—	*Z*-prolyl-D-alanine	*Z*-prolylphenylalanine	—	310

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Rennin	human	haemoglobin	pH gradient	×12	
		pepstatin	pH gradient	×21	311
		octapeptide	pH gradient	×1.9	
	rat	pepstatin	pH and salt gradient	×39	312
	human kidney	haemoglobin	separates rennin and pseudo-rennin	—	313
Trypsinogen	human pancreas	lima bean inhibitor	low ionic strength	×2.8	314
Purine nucleoside phosphorylase	human erythrocyte	formycin B	inosine	×22	315
Ribonuclease H	calf thymus	oligo(dT)	EDTA; H1 and H2 enzymes	×2, ×4	316
RNA-binding protein	rabbit reticulocyte	RNA, poly(U)	KCl, KCl plus 50 % formamide	×156 ×123	317
S-Adenosylmethionine decarboxylase	rat liver	methylglyoxalbis-(guanylhydrazone)	—	×5, ×7	318
S-Adenosylmethionine: protein-carboxyl *O*-methyltransferase	calf brain	*S*-adenosyl-L-homocysteine	*S*-adenosyl-L-methionine; 71 % yield	×377	319
Sex hormone-binding globulin	—	androstanediol	electrophoretic elution	×1118	39
Sex steroid-binding protein	immature rabbit serum	testosterone deriv.	5-α-dihydrotestosterone	×100	91
Sex steroid-binding protein	human serum (pregnant)	testosterone deriv.	dihydrotestosterone, batch elution procedure	×232	90
Spermine synthase	bovine brain	spermine	spermidine; wash putrescine	×650	320
Terminal deoxynucleotidyl-transferase	calf thymus	oligo$(dT)_{12-18}$	KCl gradient	×22	321
Transcobalamin II receptor	human placenta	rabbit transcobalamin II	complex washing and elution	×30 000	322
Tyrosine aminotransferase	rat liver	succinate	complex buffer, pH10	×259	323
UDP-glucuronyltransferase	rat liver	UDP	UDP-glucuronic acid; phenobarbitone-induced	×9.5	324
		UDP	UDP-glucuronic acid	×39	170

Urate oxidase	bovine kidney also rat liver, yeast	8-aminoxanthine	uric acid	×88	325
	rat liver	8-aminoxanthine	uric acid gradient	×10	326

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Table 3 *Miscellaneous chromatographic procedures*

Protein	*Source*	*Ligand*	*Eluant/Comments*	*Factor*	*Ref.*
Covalent chromatography					
Cathepsin B1	rabbit lung	organomercurial	cysteine	×13	327
Cathepsin B, collagenolytic cathepsin	human placenta	organomercurial	—	×2, ×2.4	328
L-Glutamine D-fructose-6-phosphate aminotransferase	mouse sublingual gland	organomercurial	dithiothreitol gradient	×60	329
γ-glutamyl cyclotransferase	rat kidney	thiol	dithiothreitol	×1.5	330
L-Pyrolidonecarboxylate peptidase	*B. amyloliquefaciens*	organomercurial	2-mercaptoethanol	×6.4	331
Heparin affinity chromatography					
α_1Antitrypsin	rabbit plasma	—	removes antithrombin III	×1.2	38
Collagenase	mouse bone	—	NaCl, calcium acetate	×24	332
DNA-dependent RNA polymerase	rat liver	—	ammonium sulphate	×11	333
	Acanthamoeba castellanii	—	ammonium sulphate; 20-fold conc.	×7	334
	Acanthamoeba	—	1M ammonium sulphate, 15—25% yield	×8.5	335
Estrogen receptor	lactating mammary gland	—	heparin	×200	336
Factor X	human plasma	—	NaCl gradient; benzamidine present	—	337
Hepatic triglyceride lipase	human post-heparin plasma	—	NaCl gradient	×1940	338
Lipoprotein lipase	human post-heparin plasma	—	NaCl gradient	×3010	338
α-L-Iduronidase	human kidney	—	NaCl gradient	×18	339
P component	human plasma	—	bind in calcium, elute citrate	×1.03	340
RNA polymerase II	*Physarum polycephalum*	—	ammonium sulphate gradient	×60	341
RNA-dependent DNA polymerase	MOPC-21	—	KCl gradient	×107	342
'Nicked' DNA-directed polymerase	MOPC-21	—	KCl gradient	×126	342
Trehalose phosphate synthase	*Mycobacterium smegmatis*	—	KCl gradient	×4	343
Hydrophobic chromatography					
Aldehyde dehydrogenase	rat-liver microsomes	aminohexyl	Triton X-100	×5.2	175
Butyryl-CoA: acetoacetate CoA transferase	*Clostridium*	hexyl	KCl gradient; lysine-fermenting bacteria	×2	344
Cellobiose oxidase	white rot fungus	phenyl	decreasing NaCl, ethylene glycol	×2.1	346
Cytochrome P450	rat-liver microsomes	laurate	detergent	×2, ×3	345

Cytochrome P450	bovine adrenal cortex	aniline	detergent; two forms-scc, 11β.	×3,×15	347
dnaG protein (primase)	*E. coli*	valyl	decreasing ammonium sulphate	×5	348
Dopamine β-hydroxylase	human plasma	octyl	ethylene glycol gradient	×17	349
3-Deoxy-D-arabino-heptulosone heptulosonate-7-phosphate synthase	*E. coli*	phenyl	decreasing phosphate gradient phenylalanine-insensitive	×3.5	350
	rat liver	phenyl	dimethylformamide	×6.4	351
1,4-β-Glucosidase	*Sporotrichum pulverulentum*	phenyl	decreasing NaCl, ethylene glycol	×2	352
α-Galactosidase	human placenta	butyl	NaCl	×4	353
Glutamate dehydrogenase	human brain	phenyl	decreasing phosphate, Triton	×14	354

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Glucocerebrocidase	human spleen	octyl, decyl	ethylene glycol; normal, Gaucher	×2, ×5 ×3, ×5	355 355
Glycogen phosphorylase *b*	bovine uterus	aminohexyl	ammonium sulphate	×5	356
Glycogen synthase	*Neurosporra crassa*	aminopropyl	NaCl gradient	×4.2	357
Interferon	human fibroblasts	phenyl	ethylene glycol	—	358
Nucleoside diphosphatase	rat liver	alanyl	decreasing ammonium sulphate; other ligands studied	×2.6	359
Plasmin	human plasma	aminohexyl	retarded	×1.25	360
Tyrosine hydroxylase	rat-caudate nuclei	phenyl	decreasing ammonium sulphate	×5.8	361
Immunoaffinity chromatography					
Aminopeptidase	pig intestine	—	—	×6	362
β_2Microglobulin	human serum	—	3M-potassium thiocyanate	×2.4	363
Cholesteryl ester transfer protein	human serum	—	3M-sodium thiocyanate; 38 % yield	×6.5	364
Dipeptidylpeptidase IV	pig intestine	—	low ionic strength	×48	32
Fibrillary acidic protein	bovine glial cells	—	1M-acetate, 5M-urea, 0.8M-NaCl pH2.5	—	365
β-Glucuronidase	human placenta	—	6M-urea, 60 % yield	×360	366
	mouse	—	8M-urea; 60—80 % recovery	—	367
i-Antigen	*Paramecium tetraurelia*	—	Glycine–NaOH, pH10.4	—	368
Interferon	mouse L cells	—	pH3.0, acetate buffer	×15.7	110
	mouse	—	0.1M-citrate, 1M-NaCl, pH2	×6.7	276
Phosphothioredoxin	*E. coli*	—	4M-guanidine, 0.1M-acetic–HCl,pH1	—	369
Pregnancy zone protein	human	—	3M-magnesium chloride, 0.6M-NaCl, pH4.8	—	370
Lectin affinity chromatography					
Acid phosphatase	human liver	Con A	D-mannose	×4.2	371
Alkaline phosphatase	rat ascites hepatoma	Con A	α-methyl-D-mannoside	×3.2	372
α_1-Antiproteinase	canine plasma	Con A	α-methyl-D-mannoside	×2	373
α_1-Antitrypsin	rabbit plasma	Con A	α-methyl-D-mannoside	×2.5	38
	human plasma	Con A	α-methyl-D-mannoside	×1.4	374
Collagen galactosyltransferase	chick embryo	Con A	α-methyl-D-mannoside + 50 % ethylene glycol; 1 % recovery	×160	375
Dopamine-β-hydroxylase	human plasma	Con A	α-methyl-D-glucoside	×47	349
α_1-Foetoprotein	foetal calf serum	Con A	α-methyl-D-glucoside	×2.4	376
α-Galactosidase	human placenta	Con A	α-methyl-D-mannoside + 1M-NaCl	×160	353
β-Galactosidase A_2, A_3	human liver	Con A	α-methyl-D-mannoside	×22	260
Galactosylhydroxylysyl glucosyltransferase	chick embryo	Con A	α-methyl-D-glucoside + 20 % ethylene glycol	×77	263

GM_1 ganglioside β-galactosidase	cat brain, liver	Con A	α-methyl-D-mannoside	×2, ×40	171
Hexosaminidase AB	—	Con A	—	×22	272
Interferon	human fibroblast	Con A	α-methyl-D-mannoside + 50 % ethylene glycol	—	358
Lipases	human plasma	Con A	α-methyl-D-glucoside	×3	337
Neutral procollagen *N*-protease	chick embryo tendon	Con A	α-methyl-D-mannoside	×36	377
Nucleoside diphosphatase	rat liver	Con A	α-methyl-D-glucoside gradient	×5.4	359
Sphingomyelinase	human placenta	Con A	α-methyl-D-mannoside, 2 °C	×100	378
			22 °C	×180	378
Thyrotropin	bovine pituatory	Con A	α-methyl-D-mannoside	×6	379
Tyrosinase	human melanoma	Con A	α-methyl-D-mannoside; particulate enzyme, trypsin treated	×10	380

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5′-Nucleotidase	lymphocyte membrane	*Lens cul.*	α-methyl-D-mannoside	×6.3	178
thy-1 Antigen	mouse brain	*Lens cul.*	α-methyl-D-mannoside	×27	381
Tumour antigen	rat carcinoma	*Lens cul.*	α-methyl-D-mannoside	×28	382
Sendai virus fusion protein		*Lens cul.*	α-methyl-D-mannoside pH8.3 + deoxycholate after mannoside pH6.8	—	383
Affinity elution					
N-acetylgalactosaminyl transferase	human plasma	Sepharose	UDP; variable adsorption	×100 000	21
Aldolase	*Ceratitis capitata*	P-cellulose	fructose-1,6-diphosphate	×33	384
	rabbit liver	P-cellulose	fructose-1,6-diphosphate	×14	385
Formyltetrahydrofolate synthetase	*Peptococcus aerogenes*	P-cellulose	ATP; after salt elution	×2	386
Fructose-1,6-bisphosphatase	mouse small intestine	P-cellulose	AMP, fructose-1,6-diphosphate	×171	387
Pyruvate kinase	rat	P-cellulose	phosphoenolpyruvate; type R enzyme	×23.9	388
Threonyl-tRNA synthetase	*Saccharomyces carlsbergensis*	P-cellulose	*Candida utilis* $tRNA_{thr}$ gradient	×8.7	389

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Kirschenbaum has continued his labours with two further tabulations of molar absorptivities of proteins at selected wavelengths.[390,391] In addition, a tabulation of isoelectric points of proteins (400 selected from 3000 cited between January 1970 and March 1976) has appeared.[392] Presumably these could find value as internal reference points in isoelectric focussing gels.

Protein Determination.—Substances that interfere with protein determination by the Lowry method continue to be investigated. Zwitterionic buffers have been shown to interfere with colour development and also to produce colour themselves.[393] In addition, reagents used in polyacrylamide gel formation and which are extracted along with proteins cause interference and cannot easily be removed by filtration, centrifugation, or dialysis.[394] The effect of desilylation and Smith degradation of glycoproteins on colour development with Lowry reagents has also been investigated.[395] Other workers have looked at the effect of sodium iodide on protein determination[396] and at the effect of alkali and detergent concentrations on a microbiuret protein assay.[397] A simple linear transform, $A = P \times (aP + b)^{-1}$ where A = absorbance and P = protein concentration, has been shown for the Folin–Lowry method.[398] In addition, numerous new or modified procedures for protein determination have been suggested. These are listed in Table 4.

Electrophoretic Procedures.—In a detailed investigation of continuous zone and multiphasic zone electrophoresis it was concluded that the only advantage that multiphasic gels provided was that of concentration of large samples. An identical resolving power was found for the two systems.[399] Acrylamide–methyl acrylate co-polymers and dimethyl sulphoxide–water buffer mixtures have been used for electrophoretic separations at temperatures as low as −30 °C.[400] The importance of controlled deaeration of polyacrylamide gels, particularly at low temperatures, low pH, or where phenyl buffers are used has been emphasized.[401]

Isoelectric Focusing. Warnings about ampholine–protein interaction giving rise to multiple bands continue to appear.[402—404] In addition, the heterogeneity seen on isoelectric focusing of hypoxanthine phosphoribosyltransferase was

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Table 4 *Methods for protein determination*

Basis of procedure	*Range*	*Purpose*	*Comments*	*Ref.*
Lowry	up to 50 μg	SDS-solubilized protein	high concentration Folin reagent	*a*
	50—600 μg ml^{-1}	stable colour development	$\pm$2% reproducibility claimed	*b*
	?	eliminate 2-mercaptoethanol interference	heat treatment	*c*
	?	eliminate thiol interference	uses EDTA, tartrate replaced	*d*
	?	membrane lipoprotein determination	SDS in alkali, tolerates sucrose, EDTA	*e*
	?	automated	non-ionic detergents used	*f*
	1—400 μg ml^{-1}	automated	decreased Folin reagent concentration	*g*
Biuret	?	use in detergents	sodium replaces potassium tartrate	*h*
Turbimetric	25—2500 μg	—	—	*i*
[^{3}H]-FDNB	100—1000 ng	high sensitivity	no thiol, varies with protein	*j*
Fluorescamine	125—10000 ng	high sensitivity	in Triton X-100, alkaline hydrolysis	*k*
Fluorescamine or *o*-phthaldehyde	?	serum proteins	—	*l*
o-Phthaldehyde	10 ng	high sensitivity	requires alkaline hydrolysis	*m*
Bromophenol Blue	10—80 μg	resists detergent interference	binds under acid conditions	*n*
Coomassie Blue G250	0.5—50 μg	sensitivity	conditions for observance of Beer's law	*o*
	sub μg	sensitivity	based on colour change on binding	*p*
Coomassie Blue R250	0.05—3 mg ml^{-1}	sensitivity	dye eluted from stained spot	*q*

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found in part to be substrate dependent.[405] A theoretical analysis of isoelectric focusing of interacting systems has shown that two well-resolved peaks can be formed, either transiently or at equilibrium, and therefore lead to mistaken identification of isoelectric variants.[406] Conditions for eliminating both cathodic[407] and anodic[408] drift, seen with pH gradients set up in polyacrylamide gels, have been described. A novel procedure has been proposed for concentration of samples prior to isoelectric focusing, up to 4 ml of a protein solution being adsorbed onto 0.1 ml phenyl-Sepharose, and the washed Sepharose being applied at the loading point to the gel.[409] Streaking of membrane proteins on isoelectric focusing gels was reduced by applying the sample in Bio-Gel P60.[410] Focusing on ultra-thin gels (0.12 mm) supported by a cellophane backing has been claimed to give increased resolution.[411] Problems with CO_2 absorption[412] might however be critical here. Two procedures giving increased resolution for analysis in alkaline pH ranges have been described.[413,414] Cascade electrofocusing was shown to give better resolution than isotachophoresis, resolution being maintained when zones were mobilized and eluted by cascade stacking.[415] Alkyl polyoxyethylene alcohols have been removed from focusing gels by addition of anionic or cationic detergents and then reapplication of a voltage gradient across the gel. Mixed micelles formed migrated to the anode or cathode.[416]

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. During the year, a number of proteins with anomalous mobilities in SDS were reported. In the case of a *Rhodospirillum* membrane protein this was claimed to be the result of high SDS binding (4 gm gm^{-1} protein). Nothing unusual was observed in its amino-acid composition however.[417] On the other hand, leghaemoglobin, binding the normal 1.4 gm gm^{-1} protein, gave a molecular weight which was 20 % too low. A suggested explanation for this was that increased binding of SDS at the hydrophobic end of the molecule might produce a preferred orientation and therefore decreased frictional coefficient.[418] The mobilities of tobacco mosaic virus[419] and cucumber green mosaic virus[420] coat proteins varied in the presence and absence of urea. Here again, unusual SDS binding ratios were observed. The use of covalently cross-linked protein oligomers as molecular weight markers has been criticized, cross-linked proteins having

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higher mobilities and leading to over-estimation of molecular weights by 5—15 %.[421] A two-dimensional procedure by which proteins with anomalous mobilities can be detected has also been described.[422] An analysis of molecular weight determination using SDS and gradient gels has suggested that it is not accurate for molecular weights below 13 000.[423] A simple procedure using Bromophenol Blue to give a visible indication of gradient uniformity has been described.[424] Other factors affecting protein mobility and resolution in SDS include the buffer and incubation conditions, illustrated by the behaviour of the PAS-1 component of the erythrocyte membrane,[425] and amino-acid composition or sequence, illustrated by single amino-acid substitutions producing altered mobilities of the α-crystallin subunit.[426] Increased separation of poliovirus polypeptides was obtained by performing electrophoresis in a buffer pH gradient. However this was accompanied by a considerable loss of sharpness of the bands.[427] Incorporation of a thiol into the running buffer (thus preventing protein-thiol oxidation) has been suggested as a means for increasing band sharpness.[428] A thiol, thioglycolate, has also been used to preserve tryptophan in preparative SDS gels. Blocked *N*-termini, frequently found in proteins eluted from gels, could be unblocked by incubation at high pH.[429] A method proposed for band detection in preparative gels, using tyrosyl and tryptophanyl phosphorescence at low temperatures, avoids protein fixation and subsequent difficulties with elution. In this procedure, cysteamine was the thiol incorporated into the running buffer as a protective agent.[430] Fixed bands have been eluted from gels after homogenization of the gel in 66% acetic acid. After gel filtration on Sephadex G25 to remove impurities contained in the gel, again in 66 % acetic acid, 50—80 % recovery of protein was found.[431] Two procedures, using the ion-retardation resin AG 11A8[432] and Bio-beads SM-2,[433] have been described for the removal of SDS from proteins. Both gave good recoveries of protein although with the latter only proteins excluded from the gel could be used, lower molecular weight proteins being adsorbed.

Miscellaneous Electrophoretic Procedures. Triton X-100–urea–acetic acid polyacrylamide gels have been claimed to separate polypeptides differing only by a single neutral amino-acid substitution,[434] and the system used to resolve haemoglobin subunits.[435] Incorporation of polyacrylamide into cellulose acetate membranes has been claimed to give increased versatility of the

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membrane.[436] Electrophoresis of membrane proteins in 13M formic acid has also been described, one claimed advantage being the avoidance of noxious solvents.[437]

Two-dimensional Electrophoretic Separations of Proteins. Two-dimensional systems have become immensely powerful methods not only for analysis of complex mixtures of proteins but also as preparative methods for analytical quantities of material which can then be further characterized. One illustration of how such gels can be used was shown in the screening of cell extracts for genetic variants. In the initial separation on SDS-polyacrylamide gels, dansylated cognate protein was added to a ^{14}C-grown cell-culture extract. Location of the dansylated marker enabled that band to be recognized following focusing in the second dimension, and hence, possible genetic variants to be identified.[438] Specific bands can also be located using an immune replica technique.[439] Since these systems are increasingly likely to be used as preparative procedures for analytical purposes, a listing of some selected from the year's literature is given in Table 5. Many are based on procedures originally described by Panyim and Chalkley,[440] Howard and Traut,[441] and O'Farrell.[442]

Immunochemical Techniques.—The number of adaptions of crossed electrophoresis (electrophoresis in agarose in the first dimension and then into specific precipitin, usually antibody, again in agarose, in the second dimension) continues to grow with the inclusion of isoelectric focusing as the separation procedure in the first dimension.[443] Electrophoresis from the first dimension through a strip containing phenyl-Sepharose into antibody has also been used. Behaviour of proteins in this system can be extrapolated from their behaviour on hydrophobic interaction chromatography with the same ligand (and *vice versa*).[444] When electrophoresis in SDS has been used in the first dimension, the insertion of an agarose strip containing Triton X-100 between the first dimension strip and the antibody-containing gel has given improved results.[445,446] Identity between proteins has been shown using split-gel polyacrylamide-gel electrophoresis followed by immunodiffusion,[447] and sequence homology between proteins shown by immunochemical cross-reactivity of the *denatured* antigens.[448] Immunoprecipitation has also been used to show the existence of a precursor of cytochrome oxidase.[449]

[436] M.J. Frenkel and R.J. Blagrove, *Analyt. Biochem.*, 1978, **84**, 583.
[437] L.C. Mokrasch, *Analyt. Biochem.*, 1978, **88**, 263.
[438] B.S. Singer, H. Morrissett, and L. Gold, *Analyt. Biochem.*, **85**, 224.
[439] F. Cabral, M. Solioz, Y. Rudin, G. Schatz, L. Clavilier, and P.P. Slonimski, *J. Biol. Chem.*, 1978, **253**, 297.
[440] S. Panyim and R. Chalkley, *Arch. Biochem. Biophys.*, 1969, **130**, 337.
[441] G.A. Howard and R.R. Traut, Methods in Enzymology, Academic Press, London, 1974, Vol. 30, p. 526.
[442] P.H. O'Farrell, *J. Biol. Chem.*, 1975, **250**, 4007.
[443] R. Schmidt-Ullrich and D.F.H. Wallach, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4949.
[444] O.J. Bjerrum, *Analyt. Biochem.*, 1978, **90**, 331.
[445] F.H. Kirkpatrick and D.J. Rose, *Analyt. Biochem.*, 1978, **89**, 130.
[446] L.T. Lee, J.E. Deas, and C. Howe, *J. Immunol. Methods*, 1978, **19**, 69.
[447] M.E.H. Bruns, A. Fausto, and L.V. Avioli, *J. Biol. Chem.*, 1978, **253**, 3186.
[448] M.M. Zakin, J.-R. Garel, A. Dautry-Varsat, G.N. Cohen, and G. Boulet, *Biochemistry*, 1978, **17**, 4318.
[449] G. Ries, E. Hundt, and B. Kodenbach, *European J. Biochem.*, 1978, **91**, 179.

Table 5 *Two-dimensional separation systems*

First dimension	*Second dimension*	*Comments*	*Ref.*
IEF	SDS	nuclease treat before loading at acidic end of focusing gel	*a*
IEF	SDS	membrane proteins, references other membrane procedures	*b*
IEF	SDS	modified lysis procedure	*c*
SDS	SDS–urea–Triton CF10	cell surface proteins	*d*
Triton X-100–urea	SDS	histones	*e*
Urea	SDS	RNA polymerase	*f*
Acid–urea	SDS	nuclear proteins	*g*
	acid–urea–lubrol-WX	histones	*h*
	SDS	histones	*i*
Acid–urea–Triton X-100	SDS	ribosomal proteins	*j*
urea–pH8.6–8 % acrylamide	urea–pH4.5–18 % acrylamide	ribosomal proteins	*k*
Cellulose acetate	SDS	good for reduction before SDS	*l*
SDS	SDS	membrane protein complex, heat between dimensions	*m*
CTAB	SDS	chromosomal proteins, avoids need for DNA removal	*n*
Agarose–acrylamide	acid–urea	chromosomal proteins, inactive DNAse in agarose-activate with magnesium before acid–urea dimension	*o*

(*a*) M. L. Privalsky and E. E. Penhoet, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3625. (*b*) R. W. Rubin and C. Milikowski, *Biochim. Biophys. Acta*, 1978, **509**, 100. (*c*) D. B. Archer, A. W. Rodwell, and E. S. Rodwell, *Biochim. Biophys. Acta*, 1978, **513**, 268. (*d*) M. Imada, *Analyt. Biochem.*, 1978, **89**, 292; M. Imada, P. Hsieh, and N. Sueoka, *Biochim. Biophys. Acta*, 1978, **507**, 459. (*e*) A. Savic and D. Poccia, *Analyt. Biochem.*, 1978, **88**, 573. (*f*) J. Jendrisak and T. J. Guilfoyle, *Biochemistry*, 1978, **17**, 1322. (*g*) L. F. Levinger, C. W. Carter, K. K. Kumaroo, and J. L. Irvin, *J. Biol. Chem.*, 1978, **253**, 5232. (*h*) Y. M. Bhatnagar and A. R. Bellvé, *Analyt. Biochem.*, 1978, **86**, 754. (*i*) J. K. W. Mardian and I. Isenberg, *Biochemistry*, 1978, **17**, 3825. (*j*) W. L. Hoffman and R. M. Dowben, *Analyt. Biochem.*, 1978, **89**, 520. (*k*) S. Roberts and C. D. Ashby, *J. Biol. Chem.*, 1978, **253**, 288. (*l*) R. J. Blagrove and M. J. Frenkel, *Analyt. Biochem.*, 1978, **87**, 287. (*m*) C. Bordier, W. F. Loomis, J. Elder, and R. Lerner, *J. Biol. Chem.*, 1978, **253**, 5133. (*n*) V. V. Shmatchenko and A. J. Varshavsky, *Analyt. Biochem.*, 1978, **85**, 42. (*o*) N. L. Batus, S. C. Albright, R. D. Todd, and W. T. Garrard, *J. Biol. Chem.*, 1978, **253**, 2568.

Peptide Mapping.—A number of proteins have been mapped on polyacrylamide gels following either limited proteolytic digestion[450—452] or cyanogen bromide digestion.[453] Peptide mapping of cyanogen bromide digests has also been performed using isoelectric focusing in polyacrylamide.[454] Systems for peptide mapping using reversed-phase h.p.l.c. have been described.[455—457] While peptide mapping on paper is probably more versatile, the expense and space involved in operating the required facilities has meant that many laboratories have turned to thin layer techniques. There appears to be as many systems described as there are laboratories working with them. However some of the more elegant-looking that were used during the year are listed in Table 6.

Detection of Peptides and Proteins.—Charge-dependent staining of proteins using various dyes has been suggested as a means of identifying specific proteins prior to fixation. Elution of the active protein could then be achieved.[458] Metachromicity of Coomassie Blue R-250, staining red with some peripheral nerve proteins, has also been observed.[459] Characteristic stains using fluorescent biotin[460] (for biotin-binding proteins), Fluorescein-[461,462] or dehydrogenase-[463] coupled lectins (for glycoproteins) and specific antibodies plus ^{125}I-labelled protein A[464] are others that have been used. Protein-binding dehydrogenase-coupled lectins were visualized by formazan dye precipitation. Chloramine T catalysed iodination of proteins in gels followed by autoradiography has also been used to 'stain' proteins,[465] although the sensitivity achieved (0.2—10 μg) is little better than that found with dyes. A new stain, ferrous bathophenanthroline sulphonate, has been claimed to give a linear staining intensity over the range 1—60 μg protein.[466] Controlled trichloracetic acid precipitation of small peptides following isoelectric focusing has circumvented the problem of ampholine precipitation and, coupled to densitometry, detection over the range 1—30 μg achieved.[467] Fluorographic procedures for detection of ^{3}H and ^{14}C on thin layer plates[468] and *X*-ray intensifying screens

[450] F.P. Guengerich, *Biochem. Biophys. Res. Comm.*, 1978, **82**, 820.
[451] T.C. Hall, Y. Ma, B.A. Buchbinder, J.W. Pyne, S.M. Sun, and F.A. Bliss, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3196.
[452] S. Saheki, K. Saheki, and T. Tanaka, *F.E.B.S. Letters*, 1978, **93**, 25.
[453] C. Marriq, A. Stein, M. Rolland, and S. Lissitzky, *European J. Biochem.*, 1978, **87**, 275.
[454] T. Bibring and J. Baxandall, *Analyt. Biochem.*, 1978, **85**, 1.
[455] W.S. Hancock, C.A. Bishop, R.L. Prestidge, and M.T.W. Hearn, *Analyt. Biochem.*, 1978, **89**, 203.
[456] W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, and M.T.W. Hearn, *J. Chromatog.*, 1978, **153**, 391.
[457] W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, and M.T.W. Hearn, *Science*, 1978, **200**, 1168.
[458] R. Rüchel, A.E. Retiet, and C. Richter-Landsberg, *Analyt. Biochem.*, 1978, **90**, 451.
[459] S. Micko and W.W. Schlaepfer, *Analyt. Biochem.*, 1978, **88**, 566.
[460] J.A. Swack, G.L. Zander, and M.F. Utter, *Analyt. Biochem.*, 1978, **87**, 114.
[461] S. Hoffman and D. McMahon, *J. Biol. Chem.*, 1978, **253**, 278.
[462] C.M. West, D. McMahon, and R.S. Molday, *J. Biol. Chem.*, 1978, **253**, 1716.
[463] G. Avigad, *Analyt. Biochem.*, 1978, **86**, 443.
[464] R. Bigelis and K. Burridge, *Biochem. Biophys. Res. Comm.*, 1978, **82**, 322.
[465] A.R. Christopher, M.L. Nagpal, A.R. Carroll, and J.C. Brown, *Analyt. Biochem.*, 1978, **85**, 404.
[466] G. Graham, R.S. Nairn, and G.W. Bates, *Analyt. Biochem.*, 1978, **88**, 434.
[467] P.L. Storring and R.J. Tiplady, *Biochem. J.*, 1978, **171**, 79.
[468] W.M. Bonner and J.D. Stedman, *Analyt. Biochem.*, 1978, **89**, 247.

Table 6 *Thin-layer peptide maps*

Plate	*First dimension*	*Second dimension*	*Comments*	*Ref.*
Silica	ethanol–conc. ammonia, 7 : 3	electrophoresis, pH3.5	iodinated proteins	*a*
Cellulose	electrophoresis, pH3.6	butan-1-ol–acetic–water 4 : 1 : 5	—	*b*
Silica	chloroform–methanol–ammonia 2 : 2 : 1	electrophoresis, pH3.5 or 6.5	fluorescamine staining	*c*
Silica	n-propanol–ammonia 7 : 3	electrophoresis, pH3.5 or 6.5	—	*c*
Cellulose	electrophoresis, pH6.5	n-butanol–acetic–water–pyridine, 15 : 3 : 12 : 10	pre-electrophoresis before spotting	*d*
Cellulose	n-butanol–acetic–water–pyridine, pH4.7	electrophoresis	digests of proteins eluted from gel	*e*
Silica	chloroform–methanol–30 % ammonia 2 : 2 : 1	electrophoresis, pH3.5	—	*f*
Cellulose	electrophoresis, pH3.5 or 6.5	n-butanol–acetic–water–pyridine, 15 : 3 : 12 : 10	digests of proteins eluted from gel	*g*

All electrophoresis is performed in pyridine–acetic acid buffers

(*a*) M. J. Dunn, R. B. Kemp, and A. H. Maddy, *Biochem. J.*, 1978, **173**, 197. (*b*) A. T. Andrews, *European J. Biochem.*, 1978, **90**, 59, 67. (*c*) R. E. Stephens, *Analyt. Biochem.*, 1978, **84**, 116; R. E. Stephens, *Biochemistry*, 1978, **17**, 2882. (*d*) D. L. Brautigan, S. Ferguson-Miller, G. E. Tarr, and E. Margoliash, *J. Biol. Chem.*, 1978, **253**, 140. (*e*) J. I. Rushbrook and R. A. Harvey, *Biochemistry*, 1978, **17**, 5339. (*f*) L. F. Shoes, N. R. Shine, and F. Talamantes, *Biochim. Biophys. Acta*, 1978, **537**, 336. (*g*) M. A. Hutchinson, T. Hunter, and W. Eckhart, *Cell*, 1978, **15**, 65.

used in the enhanced autoradiography of ^{32}P and ^{125}I [469] have both been investigated.

Amino-acid Composition.—Three procedures have been suggested for the determination of amino-acid compositions of proteins eluted from gels. Of these, one required that cells were grown on ^{14}C-containing amino-acids,[470] one used a double-label procedure with ^{3}H-FDNB and ^{14}C-labelled amino-acid standards,[471] and the third used *o*-phthaldialdehyde.[472] Phenylalanine and tryptophan determination using second derivative spectroscopy has also been described.[473]

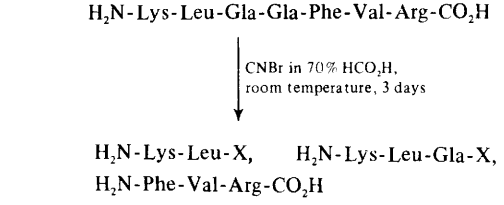
Molecular Weight Determination.—A scanning electron microscopy investigation of the effects of salt and pH on Sepharose has shown considerable morphological changes in the beads from the structure seen at low ionic strength and neutral pH.[474] Chromatography on Sephacryl S-200 superfine in 6M guanidine hydrochloride has been used to determine molecular weights in the range 1000—30 000 with a 5 % accuracy.[475] Gel filtration of sialoglycoproteins before and after sialidase treatment has suggested that a charge exclusion effect might give anomalous elution positions of some glycoproteins.[476] Thin layer chromatography of proteins on Sephadex G-200 in detergent-containing buffers has shown Triton X-100 to have an anomalous effect on the matrix.[477] A study of the effect of molecular asymmetry on the characterization of proteins on agarose gels has also appeared.[478] Procedures for partial specific volume and sedimentation coefficient measurement in sucrose density gradients, based on a comparison of behaviour in non-isotopically labelled and deuteriated water have been described,[479] as has a method for molecular weight determination utilizing a table top, high speed, air-turbine centrifuge.[480] Finally, an evaluation of active-enzyme centrifugation has shown several possible sources of error, the most serious being that a slow change in enzymic activity could be interpreted as a change in size of the enzyme.[481]

[469] R. Swanstrom and P.R. Shank, *Analyt. Biochem.*, 1978, **86**, 184.
[470] H. Kaplan, D.C.H. Cheng, G. Oda, and M. Kates, *Canad. J. Biochem.*, 1978, **56**, 517.
[471] F. Cabral and M.M. Gottesman, *Analyt. Biochem.*, 1978, **91**, 548.
[472] D.G. Drescher and K.S. Lee, *Analyt. Biochem.*, 1978, **84**, 559.
[473] C. Balestrieri, G. Colonna, A. Giovane, G. Irace, and L. Servillo, *European J. Biochem.*, 1978, **90**, 433.
[474] A.W.-S.M. Lin and D.O. Castell, *Analyt. Biochem.*, 1978, **86**, 345.
[475] M. Belew, J. Fohlman, and J.-C. Janson, *F.E.B.S. Letters*, 1978, **91**, 302.
[476] J.A. Alhadeff, *Biochem. J.*, 1978, **173**, 315.
[477] J.F. Collawn, D.J. Cox, L.M. Hamlin, and W.W. Fish, *J. Chromatog.*, 1978, **157**, 227.
[478] I. Axelsson, *J. Chromatog.*, 1978, **152**, 21.
[479] R.D. O'Brien, C.A. Timpone, and R.E. Gibson, *Analyt. Biochem.*, 1978, **86**, 602.
[480] M.A. Bothwell, G.J. Howlett, and H.K. Schachman, *J. Biol. Chem.*, 1978, **253**, 2073.
[481] D.J. Llewellyn and G.D. Smith, *Arch. Biochem. Biophys.*, 1978, **190**, 483.

PART 1B: Protein Sequencing Methods *by M. Rangarajan*

1 Chain Cleavage and Separation of Peptides

Cleavage at γ-Carboxy-glutamic Acid Residues.—During the determination of the sequence of bovine factor $IX_{a\alpha}$ it was observed that cyanogen bromide (CNBr) cleaved not only a methionyl peptide bond, but also peptide bonds in the region rich in γ-carboxy-glutamic acid residues.[1] By amino-acid and sequence analysis, the sites of cleavage were identified as being *C*-terminal to γ-carboxy-glutamic acid residues. This was further confirmed by treating a heptapeptide of known sequence with CNBr in 70 % formic acid at room temperature for three days when three peptides were generated (Scheme 1).



Gla = γ-carboxyglutamic acid, X = unknown derivative of Gla

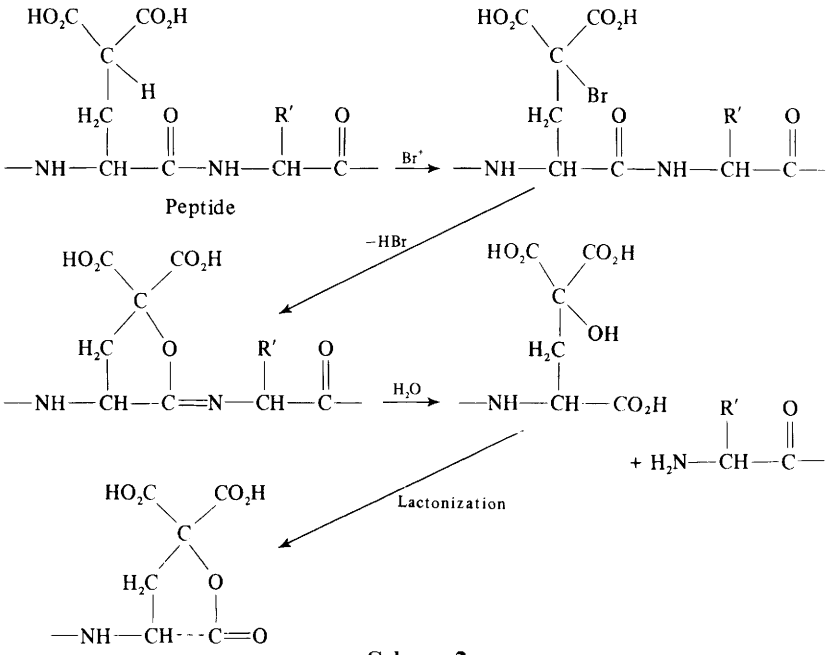
Scheme 1

Similar results were obtained when the peptide was treated with two equivalents of *N*-bromosuccinimide (NBS) for 30 minutes at room temperature. BNPS-skatole cleaved the peptide at identical sites as CNBr and NBS, but more than 10 equivalents of reagent were required for 5 h at room temperature to complete the reaction. During the cleavage of the peptide bond, γ-carboxy-glutamic acid is converted to a derivative whose structure is as yet unknown. A hypothetical mechanism[1] for the cleavage reaction is given in Scheme 2. The γ-carboxy-glutamic acid is first brominated at the γ-carbon and the peptide bond cleaved subsequently *via* its iminolactone.

The new *C*-terminal residue may be a lactonized form of γ-hydroxy-γ′-carboxy-glutamic acid. These results indicate that brominating agents like NBS and BNPS-skatole cleave γ-carboxy-glutamyl bonds. The splitting of the bond with CNBr may be due either to the reagent itself or to bromine already existent in the reagent. The identification of the sites of anomalous cleavage by CNBr should aid in sequencing proteins that are subjected to vitamin K-dependent modification of glutamic acid residues.

Cleavage at Cysteine Residues.—The selective reaction of cysteinyl residues in peptides and proteins with 2-nitro-5-thiocyanobenzoic acid was reported some years ago by Jacobson *et. al.*,[2] and Degani and Patchornik.[3] This reagent selectively cyanylates cysteinyl residues, and under alkaline conditions the peptide bond *N*-terminal to cysteine is cleaved (Scheme 3).

[1] K. Katayama and K. Titani, *F.E.B.S. Letters*, 1978, **95**, 157.
[2] G.A. Jacobson, M.H. Schaffer, G.R. Stark, and T.C. Vanaman, *J. Biol. Chem.*, 1973, **248**, 6583.
[3] Y. Degani and A. Patchornik, *Biochemistry*, 1974, **13**, 1.

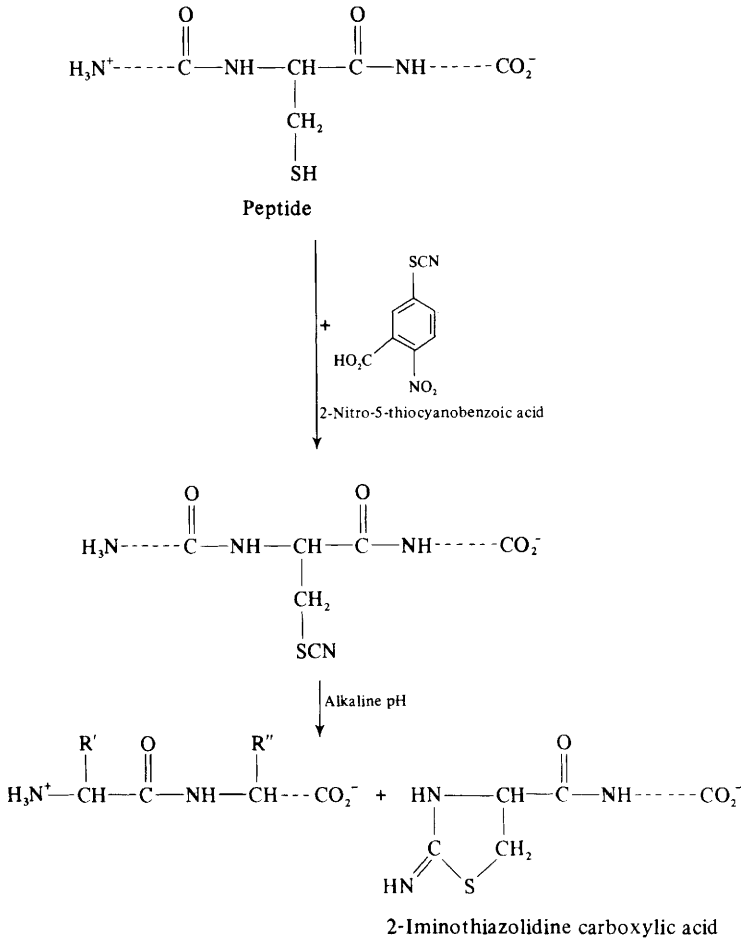


Scheme 2

The released *C*-terminal peptide is blocked at its *N*-terminus by a thiazolidine ring and hence is not susceptible to Edman degradation procedures. Only the peptide with the original *N*-terminus can yield any sequence information by stepwise degradation. A method to unmask these *N*-blocked peptides by Raney nickel desulphurization has now been described[4] which may enable more sequence information to be obtained from a digest than was previously possible. Treatment of *N*-blocked peptides with excess Raney nickel at pH 7.0 and 50 °C, for 6—10 h desulphurizes the acyliminothiazolide peptide in greater than 90 % yield to give alanine at the *N*-terminus. Control experiments on amino-acids showed that cysteine was converted to alanine and methionine to α-amino-n-butyric acid by this treatment, but that no other amino-acids were affected. Raney nickel desulphurization was used successfully on cyanylated and cleaved β-chains of guinea-pig haemoglobin. This technique promises to be useful in the selective cleavage of proteins at cysteinyl residues thereby generating large peptides for sequence analysis.

Cleavage of Pyrrolidone Carboxylic Acid (Pyroglutamic Acid) from the *N*-Terminus.—Pyrrolidone carboxylic acid (PCA) occurs at the *N*-terminus of several proteins and is believed to be formed by the cyclization of glutaminyl residues. Sanger and Thompson[5] found that PCA-containing peptides could arise spontaneously during the isolation and proteolysis of peptides bearing glutamine at the *N*-terminus. The presence of PCA means that such peptides

[4] S. Otieno, *Biochemistry*, 1978, **17**, 5468.
[5] F. Sanger and E.O.P. Thompson, *Biochem. J.* 1953, **53**, 366.



Scheme 3

cannot be subjected to direct sequence analysis using the Edman reaction. A few methods have been described for the removal of PCA. Takahashi and Cohen[6] used diborane in tetrahydrofuran or tetramethylurea to reduce the pyrrolidone ring of PCA to a prolyl residue. The method had disadvantages in that diborane also reduced carboxy-groups and peptide bonds and that only 35—46 % of PCA was converted to proline. An enzymatic method was described[7,8] using pyrrolidonyl peptidase isolated from *Pseudomonas fluorescens* and *Bacillus subtilis* which could release successfully PCA from the *N*-terminus of peptides and proteins. However, the isolation procedures for the enzymes were lengthy, and the purified enzymes were unstable. Podell and

[6] S. Takahashi and L.A. Cohen, *Biochemistry*, 1969, **8**, 864.
[7] R.F. Doolittle and R.W. Armentrout, *Biochemistry*, 1968, **7**, 516.
[8] A. Szewczuk and M. Mulczyk, *European J. Biochem.*, 1969, **8**, 63.

Abraham[9] have now used commercially available calf-liver pyroglutamate amino peptidase for the removal of PCA from the *N*-terminus of five immunoglobulin polypeptide chains. The enzyme is stable for an indefinite period when stored lyophilized at –20 °C. Deblocking is performed at 4 °C and pH 8 in buffer containing dithiothreitol.

Enzyme Cleavage at Proline Residues.—Two enzymes with a high specificity for proline-containing substrates were purified from lamb kidney: (i) post-proline cleaving enzyme, an endopeptidase, could cleave peptide bonds *C*-terminal to proline,[10] and (ii) post-proline dipeptidyl aminopeptidase, an exopeptidase with a specificity for splitting bonds *C*-terminal to proline.[11] A detailed comparison of the enzymatic properties and substrate specificities of the two enzymes is described.[12] Both are serine proteases and besides cleaving proline-containing substrates are also capable of splitting a peptide bond *C*-terminal to alanine at a slow rate. Neither protease is able to cleave the Pro–Pro bond. Post-proline dipeptidyl amino peptidase was found to be a strong catalyst for transpeptidation reactions.

2 Fluorescamine, *o*-Phthalaldehyde, and Ninhydrin

A sensitive and specific method has been developed for the detection of tryptophan, *N*-terminal tryptophan, and other 3-(2-aminoethyl)-indoles on thin-layer (t.l.c.) plates.[13] Tryptophan and its derivatives are treated with fluorescamine followed by perchloric acid when they produce a yellowish-orange fluorescence under u.v. light. Most compounds could be detected below the 100 pmole level. Tryptophan and peptides containing tryptophan can also be detected on t.l.c. plates after spraying with 70 % perchloric acid.[14] A specific, strong yellow-orange fluorescence develops in a few minutes. The fluorescence is also obtained with hydrochloric, sulphuric, and phosphoric acids, but is most stable when developed with perchloric acid. About 40—850 pmoles of 3-substituted indole derivatives can be detected using this specific fluorogenic reaction.

Ohtsuki and Hatano[15] have analysed several halo- and nitro-derivatives of tyrosine and phenylalanine using a single column amino-acid analyzer in the presence of all the amino-acids usually found in protein hydrolysates. The buffers used were those originally described by Moore *et al.*[16] and the analysis was complete in 11 h. Welch[17] has described in detail the incorporation of a commerically available gradient former (LKB Ultrograd) into a fully automated single column amino-acid analyzer which analyzes one protein hydrolyzate in 4 h. The operation of the gradient elution and sample-loading systems

[9] D.N. Podell and G.N. Abraham, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 176.
[10] M. Koida and R. Walter, *J. Biol. Chem.*, 1976, **251**, 7593.
[11] T. Yoshimoto and R. Walter, *Biochim. Biophys. Acta*, 1977, **485**, 391.
[12] T. Yoshimoto, M. Fischl, R.C. Orlowski, and R. Walter, *J. Biol. Chem.*, 1978, **253**, 3708.
[13] H. Nakamura and J.J. Pisano, *J. Chromatog.*, 1978, **152**, 153.
[14] H. Nakamura and J.J. Pisano, *J. Chromatog.*, 1978, **152**, 167.
[15] K. Ohtsuki and H. Hatano, *J. Chromatog.*, 1978, **148**, 536.
[16] S. Moore, D.H. Spackman, and W.H. Stein, *Analyt. Chem.*, 1958, **30**, 1185.
[17] R.W. Welch, *J. Chromatog.*, 1978, **152**, 528.

are given in detail. An air-stable ninhydrin system is used for colour development. Excellent resolution of protein hydrolyzates is obtained and there is scope for introducing improved resins or a more sophisticated data-handling system into the existing system. Some useful information on the cleaning of fluid lines in amino-acid analyzers is given by Gurusiddaiah and McFadden.[18] The presence of stannous chloride in most ninhydrin solutions causes a fine white precipitate to accumulate in the analyzer fluid lines and cuvettes. This interferes in the quantitation of amino-acids. However, the lines can be cleared by washing with dilute acetic acid (20—25 %).

Protein bands on polyacrylamide gels which have been fixed and stained with Coomassie Blue have been extracted with sodium dodecyl sulphate (SDS), hydrolyzed with 4N methanesulphonic acid and analyzed in the automatic amino-acid analyzer using *o*-phthalaldehyde (*o*-PA) and fluorimetry for detection.[19] SDS and Coomassie Blue do not interfere with the amino-acid determination and contamination by ammonia from gels is low. *o*-PA was preferred to fluorescamine because of its stability in aqueous solutions. Amino-acid analysis of bands containing 1—5 μg of protein can be obtained using this method. At low levels of protein, the determination of cysteine poses problems. Proline, after oxidation with chloramine T, gives low fluorescence with *o*-PA. Tryptophan and the hydroxy-amino-acids serine and threonine give recoveries of 60—70 % and 90 %, respectively. Human carbonic anhydrase C was analyzed by this method and its composition was found to be similar to that obtained by other methods requiring larger amounts of protein.

3 Amino-acid Analysis

A sensitive fluorometric method for the quantitative determination of sub-microgram quantities of arginine is reported.[20] A slight excess of 9,10-phenanthrenequinone is mixed with the sample at high pH (2M-NaOH) followed by acidification (1.2M-HCl) to produce a fluorescence. Arginine, both free and in intact protein, can be determined in this way. The commonly occurring amino-acids did not interfere with the analysis, nor did the commonly used buffers and organic solvents. No fluorescence was observed in the presence of pyridine and ethylenediamine. This assay is approximately 1000-fold more sensitive than the Sakaguchi method[21] but the various proteins tested showed widely differing rates of reaction with 9,10-phenanthrenequinone. This would require calibration curves to be constructed for each protein tested.

A double-labelling procedure for the amino-acid analysis of proteins is described.[22] A mixture of 18 ^{14}C-labelled amino-acids is treated with ^{3}H-1-fluoro-2,4-dinitrobenzene (FDNB), when each amino-acid gives a dinitrophenyl (DNP) derivative having a fixed ^{3}H : ^{14}C ratio. Addition of an unlabelled protein to the mixture of ^{14}C-amino-acids followed by hydrolysis

[18] S. Gurusiddaiah and B.A. McFadden, *Analyt. Biochem.*, 1978, **88**, 332.
[19] D.G. Drescher and K.S. Lee, *Analyt. Biochem.*, 1978, **84**, 559.
[20] R.E. Smith and R. MacQuarrie, *Analyt. Biochem.*, 1978, **90**, 246.
[21] G. Tomlinson and T. Viswanatha, *Analyt. Biochem.*, 1974, **60**, 15.
[22] H. Kaplan, D.C.H. Cheng, G. Oda, and M. Kates, *Canad. J. Biochem.*, 1978, **56**, 517.

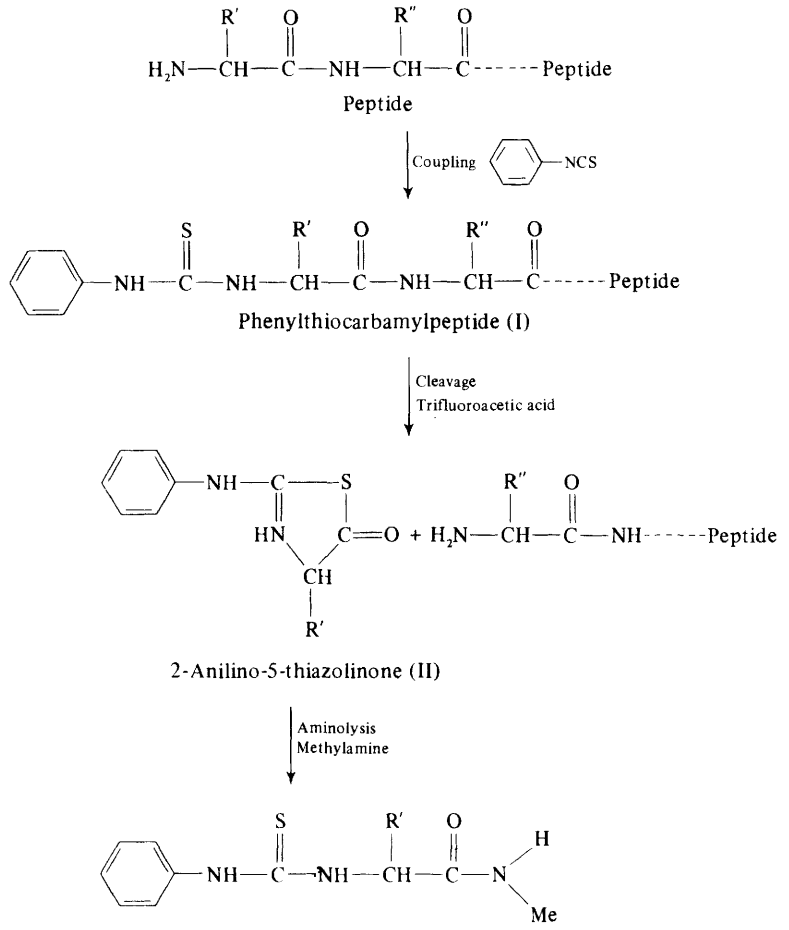
and reaction with ^{3}H-FDNB would increase the ^{3}H : ^{14}C ratio of each DNP-derivative in proportion to the amount of that amino-acid present in the hydrolyzate. The ^{14}C-amino-acid mixture is added to the unlabelled protein prior to hydrolysis so that any destruction of amino-acids during hydrolysis is automatically corrected. The incorporation of an internal standard (β-alanine) permits the determination of absolute amounts of amino-acids present. Complete reaction with ^{3}H-FDNB is not required since the extent of reaction will not alter the ^{3}H : ^{14}C ratio in the resulting DNP derivative. Reliable results were obtained with insulin and lysozyme. Bacteriorhodopsin from *Halobacterium cutirubrum* gave values consistent with those reported for *H. halobium*.[23] This method has advantages over the similar procedure of Brown and Perham[24] in which ^{14}C-amino-acids are added to a protein hydrolyzate and then treated with ^{3}H-dansyl chloride.

4 End-group Determination

In the manual dansyl-Edman degradation procedure, fluorescent dansyl-dipeptide spots frequently appear on the t.l.c. plates and often make interpretation of chromatograms difficult. The resistant peptide bonds usually involve two hydrophobic residues or a hydrophobic and a prolyl residue. Increasing the hydrolysis time does not cleave the peptide bonds, but reduces the yields of several other dansyl-amino-acids. Sutton and Bradshaw[25] have dansylated 18 dipeptides containing combinations of leucine, isoleucine, valine, alanine, and proline and chromatographed them on t.l.c. polyamide plates using the solvent systems usually employed in the dansyl-Edman procedure.[26] Their report contains diagrammatic representations of the separation of these derivatives and will be very useful to those who sequence peptides by the dansyl-Edman procedure. The separation of DNS-amino-acids usually requires three or four solvent systems. Bertrand *et al.*[27] have achieved this separation using only two solvents. The separation of the pairs DNS-aspartic and DNS-glutamic acids, and DNS-leucine and DNS-isoleucine depends on the careful application of sample at the origin. Metrione[28] has determined the R_f values of all the DNS-amino-acids in solvent systems commonly used. He has also developed new solvents to separate basic, acidic, and hydroxy-amino-acid derivatives.

The identification of tryptophan, asparagine, and glutamine during sequence analysis of peptides requires additional steps such as the hydrolysis of DNS-tryptophan peptides with mercaptoethanesulphonic acid[29] and measurements of electrophoretic mobilities for amide assignments. It was shown that when 2-anilino-5-thiazolinones (Scheme 4, II) from the Edman degradation cycle were treated with methylamine, they were converted to the corresponding phenylthiocarbamyl-methylamides(PTC-methylamides, Scheme 4, III) which could be identified by t.l.c. or h.p.l.c. without any interference from back-

[23] D. Oesterhelt and W. Stoeckenius, *Nature*, 1971, **233**, 149.
[24] J.P. Brown and R.N. Perham, *European J. Biochem.*, 1973, **39**, 69.
[25] M.R. Sutton and R.A. Bradshaw, *Analyt. Biochem.*, 1978, **88**, 344.
[26] B.S. Hartley, *Biochem. J.*, 1970, **119**, 805.
[27] O. Bertrand, Y. Kroviarski, and P. Boivin. *J. Chromatog.*, 1978, **147**, 435.
[28] R.M. Metrione, *J. Chromatog.*, 1978, **154**, 247.
[29] J.R. Giglio, *Analyt. Biochem.*, 1977, **82**, 262.



Scheme 4

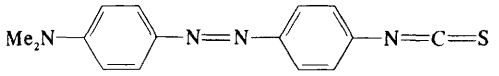
ground material.[30—32] The inclusion of the aminolysis step in the dansyl-Edman method was investigated.[33] No drastic modifications of the established method were found to be necessary. Aminolysis of thiazolinones was usually performed before their extraction into organic solvents as this gave better yields of PTC-methylamides. Tryptophan was not destroyed nor were the amides of aspartic acid and glutamic acid deamidated. Used in conjunction with the

[30] J.K. Inman and E. Appella, in 'Solid-Phase Methods in Protein Sequence Analysis', ed. R.A. Laursen, Pierce Chemical Company, Illinois, U.S.A., 1975, p. 241.

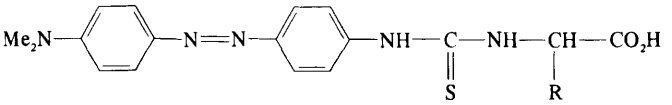
[31] J.K. Inman and E. Appella, in *Methods in Enzymology*, ed. C.H.W. Hirs and S.N. Timasheff, 1977, **47**, 374.

[32] E. Appella, J.K. Inman, and G.C. DuBois, in 'Solid-Phase Methods in Protein Sequence Analysis,' ed. A. Previero and M.-A. Coletti-Previero, North-Holland Publishing Company, Amsterdam, New York, Oxford, 1977, p. 121.

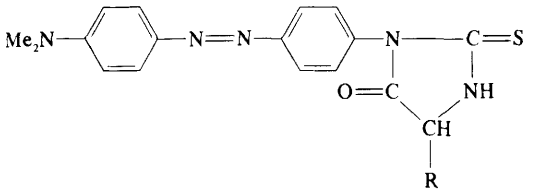
[33] H. Jornvall, J.K. Inman, and E. Appella, *Analyt. Biochem.*, 1978, **90**, 651.



(IV)



(V)



(VI)

Scheme 4 (*contd.*)

dansyl-Edman method, it can be a powerful technique for identifying tryptophan, asparagine, and glutamine. If the dansyl method reveals an aspartic acid, or glutamic acid, or a 'blank' on the t.l.c. plate, it is suggested that the thiazolinone formed in the following Edman cycle should be subjected to aminolysis to establish the identity of the amino-acid in the sequence. Any ambiguity in the dansylation step could be solved in most cases by the aminolytic reaction. The only by-product formed in the dansylation step as a result of aminolysis was DNS-methylamine, which did not interfere in the t.l.c. of DNS-amino-acids. The method was applied successfully to several di-, tri-, and tetra-peptides and a 31-residue tryptic peptide from an immunoglobulin. The limit of detection of PTC-amino-acid methylamides is similar to that of phenylthiohydantoin amino-acids.

An extremely sensitive method for the stepwise degradation of peptides made use of a coloured isothiocyanate, 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate (DAAITC, IV).[34—36] Because of the limited solubility of DAAITC in organic solvents, the quantitative coupling of an *N*-terminal amino group could only be achieved using high temperatures (75 °C, 1.5 h). However, a novel micro-sequencing method is described which makes use of a double coupling technique[37] in which quantitative coupling of the *N*-terminal group is obtained by first coupling with DAAITC followed by a second coupling reaction with phenylisothiocyanate (PITC). After acid cleavage and conversion, only the 4-*NN*-dimethylaminoazobenzene thiohydantoin (DAA-TH) amino-acids are identified by t.l.c. on polyamide plates. The technique

[34] J.Y. Chang, E.H. Creaser, and K.W. Bentley, *Biochem. J.*, 1976, **153**, 607.
[35] J.Y. Chang, *Biochem. J.*, 1977, **163**, 517.
[36] J.Y. Chang, E.H. Creaser, and G.J Hughes, *F.E.B.S. Letters*, 1978, **78**, 147.
[37] J.Y. Chang, D. Brauer, and B. Wittmann-Liebold, *F.E.B.S. Letters*, 1978, **93**, 205.

avoids the use of high temperatures, does not require special instrumentation, and should be generally applicable for micro-sequence analysis of peptides and proteins. From the recoveries of the coloured DAA-TH amino-acids, it is estimated that about 25—50 % of *N*-terminal groups react with DAAITC in the first coupling. Extraction of by-products and excess reagents after the coupling step is necessary as the rate of hydrolysis of DAAITC to 4-*NN*-dimethylaminoazobenzene is high. Experiments performed with nmole quantities of several proteins showed that the method has a 10—20-fold increase of sensitivity over the conventional manual technique.

Several ribosomal proteins have *N*-monomethyl-amino-acids at their *N*-termini.[38—40] The unusual phenomenon of 'pre-overlapping' *i.e.* overlapping by a succeeding amino-acid instead of a preceding residue observed in the Edman degradation reaction of ribosomal protein S11[40] was explained as being due to the presence of an iso-peptide bond in its *N*-terminal sequence. The reaction of DAAITC and PITC with several methylamino-acids has been investigated.[41] The reaction of DAAITC with amino-groups can be followed visually as a series of colour changes from DAAITC (purple) through 4-*NN*-dimethylaminoazobenzene-thiocarbamylamino-acid (V, blue) to the thiohydantoin, (VI, red). With methylamino-acids however, DAAITC did not yield the expected blue product, but gave a red colour characteristic of the thiohydantoin. Treatment with acid did not change its colour or its behaviour on t.l.c. U.v. absorption and mass spectrometry showed that a thiohydantoin ring had formed in the coupling buffer. Proteins with methylamino-acids at their *N*-termini gave similar results although the rate of conversion to thiohydantoin was different for each. For protein S11, 92 % of the *N*-terminal *N*-methyl-alanine was cleaved as the thiohydantoin in the first coupling reaction. As a result, the second amino-acid residue (lysine) also reacted with DAAITC and PITC in the coupling reaction of the first cycle and appeared after the first cleavage step. The expected residue therefore appeared one step earlier during the following degradations. These results explain the 'pre-overlapping' which is observed.

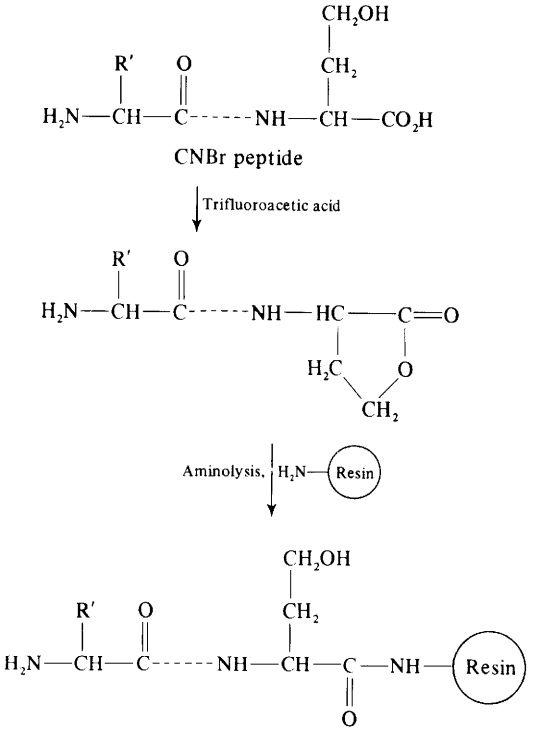
5 Spinning Cup Sequencing

It is often impossible to sequence short peptides, especially hydrophobic ones, by automated stepwise degradation because of loss of material during extraction with organic solvents. This has been overcome by the addition of Polybrene (1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide), a quaternary ammonium compound, to the spinning cup.[42] Klapper *et al.*[43] have sequenced over 50 peptides from various origins and of various lengths to their *C*-terminus by the incorporation of Polybrene in the spinning cup. The mechanism of action of Polybrene is still unclear but at least 2—3 mg are necessary for efficient action irrespective of the amount of peptide used. To

[38] R. Chen, J. Brosius, B. Wittmann-Liebold, and W. Schafer, *J. Mol. Biol.*, 1977, **111**, 173.
[39] B. Wittmann-Liebold and R. Pannenbecker, *F.E.B.S. Letters*, 1976, **68**, 115.
[40] R. Chen and U. Chen-Schmeisser, *Proc. Nat. Acad. Sci.*, 1977, **74**, 4905.
[41] J.Y. Chang, *F.E.B.S. Letters*, 1978, **91**, 63.
[42] G.E. Tarr, J.F. Beecher, M. Bell, and D.J. McKean, *Analyt. Biochem.*, 1978, **84**, 622.

reduce the artifacts, the first Edman degradative cycle is performed without the addition of PITC. After this preliminary 'wash', sequencing proceeds in a normal manner. It is now used routinely in the laboratory of the authors[43] and is a powerful aid in sequencing peptides.

It is possible to sequence hydrophobic tryptic peptides containing lysine, right up to their *C*-terminii, by the reaction of the ε-amino-groups with aromatic sulphonic acid isothiocyanates,[44—46] which makes them more hydrophilic and prevents losses during organic solvent extraction. Horn and Laursen[47] previously described a way of attaching CNBr peptides to resins containing amino-groups which involved lactonization of terminal homoserine with trifluoroacetic acid followed by aminolysis of the lactonized peptide with amino resin (Scheme 5).



Scheme 5

The technique of aminolysis of lactonized peptide has been used by Braunitzer and Pfletschinger[48] for making CNBr peptides more hydrophilic. Following lactonization with trifluoroacetic acid, aminolysis is performed with diamines

[43] D.G. Klapper, C.E. Wilde, III, and J.D. Capra, *Analyt. Biochem.*, 1978, **85**, 126.
[44] G. Braunitzer, B. Schrank, and A. Ruhfus, *Z. physiol. Chem.*, 1970, **351**, 1589.
[45] G. Braunitzer, B. Schrank, A. Ruhfus, S. Petersen, and U. Petersen, *Z. physiol. Chem.*, 1971, **352**, 1730.
[46] G. Braunitzer, B. Schrank, S. Petersen, and U. Petersen, *Z. physiol. Chem.*, 1973, **354**, 1563.
[47] M.J. Horn and R.A. Laursen, *F.E.B.S. Letters*, 1973, **36**, 285.
[48] G. Braunitzer and J. Pfetschinger, *Z. physiol. Chem.*, 1978, **359**, 1015.

(ethylenediamine or hexamethylenediamine) or with histamine, in dimethylformamide, dimethylsulphoxide, or 6M guanidine hydrochloride as solvent. The free amino-groups in the peptide are then treated either with 4-(isothiocyanato)benzenesulphonic acid or 7-(isothiocyanato)naphthalene-1,3,5-trisulphonic acid to make them hydrophilic. The authors recommend the use of 4-(isothiocyanato)benzenesulphonic acid when a hydrophobic program is used,[49—51] and 7-(isothiocyanato)naphthalene-1,3,5-trisulphonic acid if a 1N Quadrol program is used. CNBr peptides which had been treated in this way were sequenced to their *C*-terminus with good yields of PTH-homoserine which also indicates the end of the sequence.

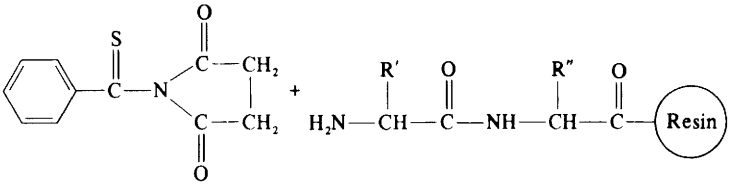
6 Solid-phase Sequencing

Short peptides have been successfully coupled to solid supports for sequencing. With large peptides, however, good results are obtained only by using hydrophilic or rigid supports containing suitable functional groups at their surfaces.[52—54] Peptides containing lysine have been coupled to aminated polyacrylamide supports using phenylene di-isothiocyanate (DITC).[55] Coupling of the support with DITC increases its aromatic and hydrophobic character and makes the subsequent reactions in aqeuous solution less efficient. On the other hand, coupling can be performed by pre-activating the protein with DITC followed by addition of resin. Pre-activation is not always possible because of the difference in solubility between the protein and DITC. The synthesis of a hydrophilic isothiocyanate resin to which lysine-containing peptides can be coupled in aqueous medium is described.[56] This involves treating polyacrylyl chloride with a large excess of sulphonated *p*-phenylenediamine, when a monoacylated derivative is formed. The free amino-groups are then converted to isothiocyanate groups by treatment with carbon disulphide and dicyclohexylcarbodi-imide. Proteins were coupled to these resins in water or in urea solutions with attachment yields of 50—90 % and successfully subjected to sequencing in the solid-phase sequenator.[57]

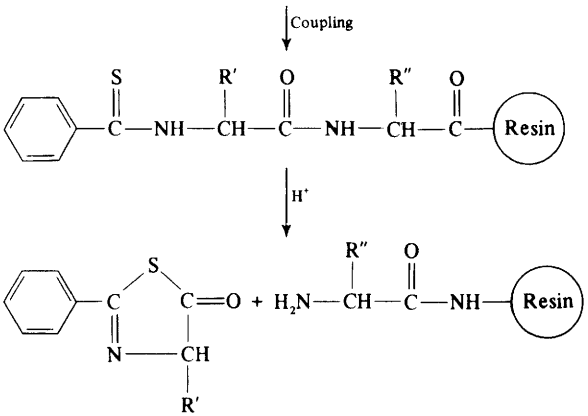
Carboxy-groups in proteins and peptides can be used as sites of attachment to solid supports.[58,59] However, they have not been widely used because of low yields and lengthy blocking procedures.[60] A convenient method is described for activating carboxy-groups and their attachment to insoluble resins.[61]

[49] G. Braunitzer and B. Schrank, *Z. physiol. Chem.*, 1970, **351**, 417.
[50] H.D. Niall, H.T. Kentmann, D.H. Copp, and J.T. Potts, *Proc. Nat. Acad. Sci.*, 1969, **64**, 771.
[51] M.A. Hermodson, L.H. Ericsson, K. Titani, H. Neurath, and K.A. Walsh, *Biochemistry*, 1972, **11**, 4493.
[52] E. Wachter, W. Machleidt, H. Hofner, and J. Otto, *F.E.B.S. Letters*, 1973, **35**, 98.
[53] E. Atherton, J. Bridgen, and R.C. Sheppard, *F.E.B.S. Letters*, 1976, **64**, 173.
[54] J.-C. Cavadore, J. Derancourt, and A. Previero, *F.E.B.S. Letters*, 1976, **66**, 155.
[55] R.A. Laursen, M.J. Horn, and A.G. Bonner, *F.E.B.S. Letters*, 1972, **12**, 62.
[56] J.-C. Cavadore and B. Vallet, *Analyt. Biochem.*, 1978, **84**, 402.
[57] R.A. Laursen, *European J. Biochem.*, 1971, **20**, 89.
[58] R.A. Laursen, *J. Amer. Chem. Soc.*, 1966, **88**, 5344.
[59] A. Previero, J. Derancourt, M.-A. Coletti-Previero, and R.A. Laursen, *F.E.B.S. Letters*, 1973, **33**, 135.
[60] G.A. Mross and R.F. Doolittle, *Fed. Proc.*, 1971, **30**, 1241.
[61] H.-M. Lee and J.F. Riordan, *Analyt. Biochem.*, 1978, **89**, 136.

Activation involves formation of mixed anhydride in the presence of trifluoroacetic acid and trifluoroacetic anhydride. The activated peptides are then attached to triethylenetetramine polystyrene resin and subjected to solid-phase sequencing. The absence of a PTH-derivative at the end of a cycle indicates the presence of an aspartic acid or a glutamic acid in the sequence. If PTH-aspartic acid or PTH-glutamic acid is detected at any step in the cycle, it indicates the presence of asparagine or glutamine in the sequence. The authors point out that activation of the β-carboxy-group of aspartic acid could result in the formation of a cyclic imide by displacement of trifluoracetic acid. If this were to occur, the Edman degradation would not proceed beyond this point. Several peptides would have to be sequenced in order to study this side-reaction. A thioacylating agent, *N*-thiobenzoyl-succinimide has been synthesized and is used as an alternative to PITC in solid-phase sequencing.[62] Thioacylation of peptides possesses certain advantages over the Edman degradation. It requires only a short cyclization step, and a phenylthiazolinone is released as the final product of the reaction which can be identified by t.l.c., or by g.c. after trimethylsilylation, or as the amino-acid by acid hydrolysis (Scheme 6).



N-Thiobenzoylsuccinimide



2-Phenylthiazolinones

Scheme 6

Thioacylating agents are ideal for use in solid-phase sequencing as they have a low solubility and a low reactivity towards amino-groups in aqueous solution, and solid-phase sequencing permits the coupling reaction to be performed in

[62] J.-C. Cavadore, *Analyt. Biochem.*, 1978, **91**, 236.

non-aqueous media. Several small peptides attached to aminated polyacrylamide or polystyrene resins were subjected to stepwise degradation by thioacylation and the reagent was shown to be quite efficient as an alternative to PITC.

7 Microsequencing

Microsequencing procedures usually involve modification of (i) the chemistry and instrumentation of the automated Edman degradation, or (ii) the techniques available for the identification and quantitation of PTH amino-acids. One way of reducing the amounts of starting material required for sequence analysis is by the addition of polymeric carriers to the spinning cup which reduces loss of peptide during washing and solvent extraction.[42,43,63] The number of solvent extraction steps can be reduced by lowering the concentration of coupling buffer and this has permitted partial sequencing of proteins at the 5—25 nmol level.[64,65] The sensitivity of detection of PTH amino-acids can be increased by incorporating radioactive label either in the amino-acids or in the PITC used for coupling.[57,66—71] This method is not generally applicable and proves to be very expensive. An improved method for microsequencing is described[72] involving the modification[73,74] of a commercial sequenator at modest cost. This improved the vacuum and reagent–solvent delivery systems so that the cleaved amino-acid derivatives were almost free of contaminants. A second reaction vessel is inserted between the spinning cup and the fraction collector to provide automated conversion of thiazolinones to PTH amino-acids; yields are significantly higher. Polybrene is added to the spinning cup to retain small amounts of polypeptide, the normal Quadrol program is used with slight modification, and the PTH amino-acids are analysed using h.p.l.c. which allows them to be unambiguously identified. Results obtained with this method show that it is far superior to other methods in terms of the amounts of protein used and the number of identifiable cycles possible. Using 200 pmol of myoglobin, 47 residues could be determined; with 5 nmol of κ light-chain 4050, 77 residues could be identified. As a general rule, the authors recommend 100 pmol of protein for extended (> 40 cycles) *N*-terminal analyses and less material for shorter analyses. The sensitivity of the technique can be further improved by repurifying all the reagents and solvents used.

8 PTH Amino-acids

H.p.l.c. seems to have replaced t.l.c. and g.c. as the most favoured technique for

[63] H.D. Niall, J.W. Jacobs, J. van Rietschoten, and G.W. Tregear, *F.E.B.S. Letters*, 1974, **41**, 62.
[64] A.W. Brauer, M.N. Margolies, and E. Haber, *Biochemistry*, 1975, **14**, 3029.
[65] W.G. Crewther and A.S. Inglis, *Analyt. Biochem.*, 1975, **68**, 572.
[66] J. Silver, *Methods in Enzymology*, ed. C.W. Hirs and S.N. Timasheff, 1978, **47**, 247.
[67] B. Ballou, D.J. McKean, E.F. Freedlender, and O. Smithies, *Proc. Nat. Acad. Sci.*, 1976, **73**, 4487.
[68] Y. Burstein and I. Schecter, *Proc. Nat. Acad. Sci.*, 1977, **74**, 716.
[69] S. Oroszlan, T. Copeland, M. Summers, and G. Smythers, in ref. 30, p. 179.
[70] J.W. Jacobs and H.D. Niall, *J. Biol. Chem.*, 1975, **250**, 3629.
[71] J. Bridgen, *F.E.B.S. Letters*, 1975, **50**, 159.
[72] M.W. Hunkapiller and L.E. Hood, *Biochemistry*, 1978, **17**, 2124.
[73] B. Wittmann-Liebold, *Z. physiol. Chem.*, 1973, **354**, 1415.
[74] B. Wittmann-Liebold, H. Graffunder, and H. Kohls, *Analyt. Biochem.*, 1976, **75**, 621.

the separation of PTH amino-acids. Several laboratories have developed new solvent systems or have tried to improve existing procedures to achieve a better separation of all the PTH amino-acids. The less toxic and cheaper methanol is used instead of acetonitrile in eluting buffers at room temperature, for the separation and quantitation of PTH amino-acids in 16 minutes,[75] 20 minutes,[76] and 32 minutes.[77] PTH-Val and PTH-Met could not be separated in this way. Micro-h.p.l.c. has been developed for the separation of PTH amino-acids.[78] The column used is only about 1 % the volume of an ordinary h.p.l.c. column which reduces the working costs, but it does not have any other advantages. The sensitivity of detection is not improved and the analysis time is not shortened. The separation of PTH amino-acids using an efficient isocratic reversed-phase system at 50 °C is described.[79] PTH-Met is not separated from PTH-Pro. When sequencing radioactively labelled proteins, it is often necessary to pool the radioactive peaks of PTH amino-acids from the h.p.l.c. column for liquid scintillation counting. This is not feasible if aqueous solvents are used. Devillers-Thiery and Blobel[80] have tried to improve published procedures for PTH-separation using only organic solvents for their elution. A few pairs of PTH-derivatives co-eluted, and separation was achieved in 20 minutes. Another h.p.l.c. system is described[81] in which pmol quantities of PTH amino-acids could be analysed using a two-gradient elution step and detected using u.v. absorbance at 254 nm and 313 nm.

Bhown *et al.*[82] have developed a computer-controlled h.p.l.c. system to identify ^{35}S-labelled PTH-derivatives from a solid-phase sequencer. Each radioactive derivative from the sequencer is added to a standard PTH-amino-acid mixture and analysed by h.p.l.c. The output of the h.p.l.c. detector is connected to the computer *via* an interface. The computer in turn activates a fraction collector and a rotary valve which directs the eluate stream to waste or to collect. The computer is programmed to monitor signal levels and changes in slope with the onset of each peak and to index the valve function and fraction collector stepping (Figure 1). The radioactive PTH-derivatives generated from 700 pmol of lysozyme in the solid-phase sequencer were tested with this system. It was found to be extremely reliable, the background was low and there was no spillover of counts into the adjacent peak of the chromatogram.

A sensitive method of identifying PTH-Arg on fluorescent polyamide plates makes use of the reaction of guanidino groups with phenanthrene-quinone.[83] PTH-Arg appears as a yellow-green fluorescent spot on a fluorescent blue-violet background when irradiated with long-range u.v. light. It was possible to detect as little as 50 pmol of PTH-Arg on polyamide sheets.

[75] R. Zeeuws and A.D. Strosberg, *F.E.B.S. Letters*, 1978, **85**, 68.
[76] A.S. Bhown, J.E. Mole, A. Weissinger, and J.C. Bennett, *J. Chromatog.*, 1978, **148**, 532.
[77] J. Elion, M. Downing, and K. Mann, *J. Chromatog.*, 1978, **155**, 436.
[78] J. van Beeumen, J. van Damme, and J. de Ley, *F.E.B.S. Letters*, 1978, **93**, 373.
[79] M. Abrahamsson, K. Groningsson, and S. Castensson, *J. Chromatog.*, 1978, **154**, 313.
[80] A. DeVillers-Thiery and G. Blobel, *Analyt. Biochem.*, 1978, **87**, 66.
[81] M.N. Margolies and A. Brauer, *J. Chromatog.*, 1978, **148**, 429.
[82] A.S. Bhown, J.E. Mole, W.L. Hollaway, and J.-C. Bennett, *J. Chromatog.*, 1978, **156**, 35.
[83] R.S. Reed and C.M. MacKay, *Analyt. Biochem.*, 1978, **84**, 324.

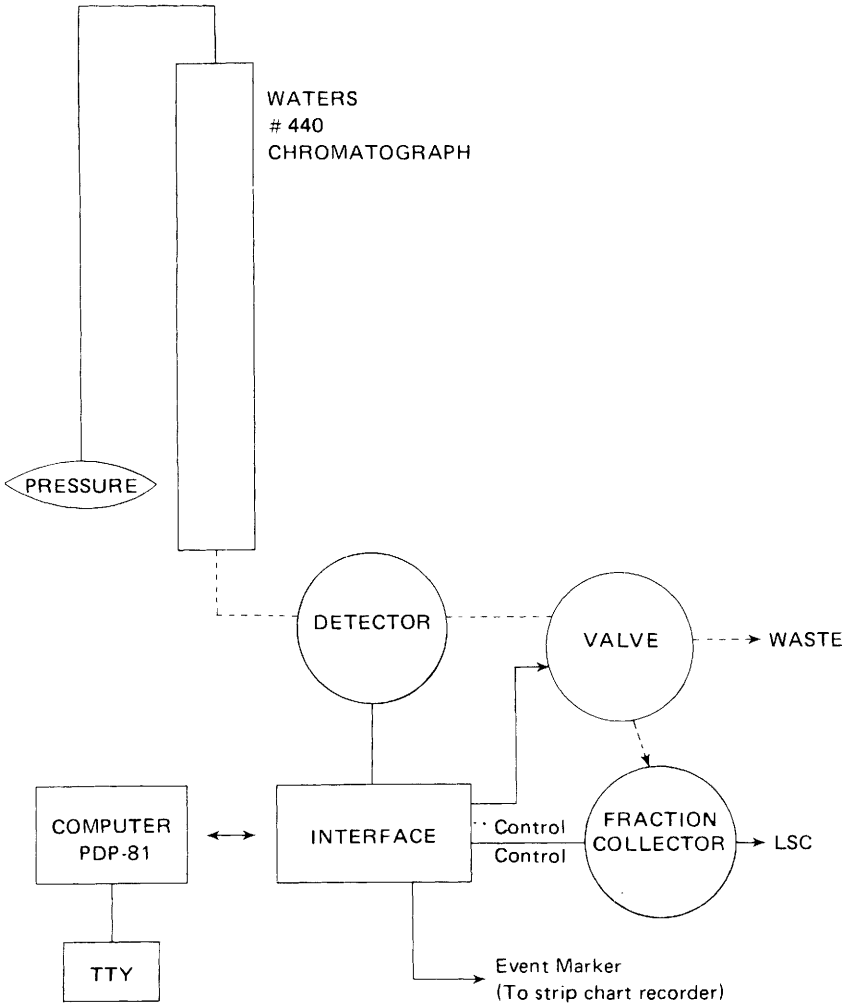


Figure 1 *Schematic representation of computer-controlled h.p.l.c. system* (Reproduced with permission from *J. Chromatog.*, 1978, **156**, 35).

9 *C*-Terminal Sequencing

Parham and Loudon[84] have reported a solid-phase method for the removal of *C*-terminal residues of peptides in good yield. The *C*-terminal amino-acid is released as an aldehyde leaving behind a shorter peptide amide still attached to the solid support. The same authors[85] found that treatment of a peptide amide attached to a solid support with 1,1-bis(trifluoroacetoxy)iodobenzene under

[84] M.E. Parham and G.M. Loudon, *Biochem. Biophys.Res. Comm.*, 1978, **80**, 7.
[85] M.E. Parham and G.M. Loudon, *Biochem. Biophys. Res. Comm.*, 1978, **80**, 1.

aqueous acidic conditions brings about a Hofmann-type degradation and converts the peptide amide to a primary amine. Hydrolysis of the amine in neutral aqueous buffer cleaved the *C*-terminal amino-acid as an aldehyde, leaving behind a peptide amide which was one residue shorter attached to the solid support. Repetition of the Hofmann-type degradation cycle would yield sequence information from the *C*-terminal end. Repetitive yields of 80 % were obtained with hexapeptides. At present, it is not possible to identify the aldehydes cleaved off the peptide. Sequence information can be obtained only from difference amino-acid analysis.

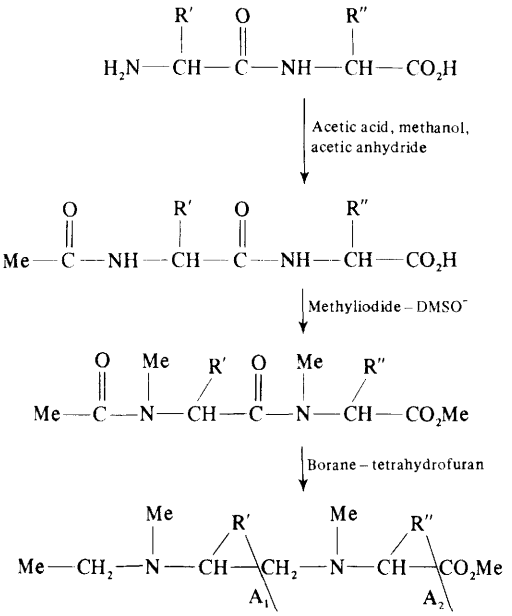
10 Mass Spectrometry

When sequencing peptides by electron impact mass spectrometry (m.s.) of *N*-acylated peptide esters, leucine and isoleucine cannot be distinguished from each other. However, if the *N*-terminus is blocked with trifluoroacetyl-perdeuteriated leucine, McLafferty rearrangement occurs with the elimination of perdeutero-isobutene from the common *N*-terminal blocking group (M^+–64), and the loss of isobutene or butene from the side chain of leucine or isoleucine (M^+–56), respectively.[86] Several peptide methyl ester derivatives containing leucine and isoleucine, and whose *N*-terminus was blocked with trifluoroacetyl-perdeutero leucine were subjected to electron impact m.s. The ratio of the peaks $\frac{M^+-56}{M^+-64}$ (McLafferty ratio) was recorded for each derivative. The ratio varies with the length of peptide and with the position of leucine or isoleucine in the peptide chain. For a pair of peptides where one contains leucine and the other isoleucine, the McLafferty ratio is always lower for the Ile-containing peptide. For use in protein sequencing, it may be necessary to divide the peptide in two so that one may be blocked with perdeuterio leucine and the other with perdeutero-isoleucine in order to get the McLafferty ratio. The method needs to be investigated further before it can be applied routinely to sequence peptides derived from proteins.

New peptide amine derivatives have been described[87] which are obtained from permethylated derivatives by reduction with borane-tetrahydrofuran (Scheme 7). These derivatives are more volatile than the corresponding permethylated ones, and hence a lower probe temperature can be used.

The technique was applied to di-, tri-, tetra-, and octa-peptides; sequence information was obtained using 10—100 nmol of material. Data have been collected on the g.c. and m.s. behaviour of over 120 dipeptides.[88] They were analysed as their *NO*-perfluoropropionyl methyl ester derivatives by g.c.–m.s. using both electron impact and chemical ionization. Of the 400 possible dipeptides containing all the 20 protein amino-acids, about 200 were trimethylsilylated and analysed by g.c.–m.s.[89] From the variety of samples studied, it should be possible to predict the g.c. behaviour of the other 200 dipeptides which should aid in their unambiguous identification.

[86] R. Waern and H. Falter, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 448.
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Scheme 7

11 New Strategies in Protein Sequencing

A skeletal model of the 51 000 mol.wt. monomer of yeast hexokinase B was built from a 2.5Å resolution electron density map in an attempt to derive an approximate amino-acid sequence.[90] There have been many previous occasions when sequence data were obtained from a multiple isomorphous replacement electron density map.[91–93] In these cases the maps were well phased to 2.3 or 2.0Å resolution and the *X*-ray sequence was 50—60 % correct,[94] while in the case of hexokinase the estimated sequence was only about 25—30 % correct. Anderson *et al.*,[95] however, have carried out a crystallographic refinement of the hexokinase monomer at 2.1Å resolution which improves the atomic co-ordinates and the amino-acid sequence obtained from the 2.5Å map. The amino-acid composition of the refined model accounts for 72 % of the expected residues while the other 28 % occur in the composition as alanines or serines. Most of the amino-acids missing from the composition are long side-chain residues such as Lys and Glx. The major

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factor that limits the usefulness of crystallographic refinement techniques for amino-acid sequencing is the overall crystal temperature factor, *i.e.* the amount of disorder in the crystal. For example, in the case of rubredoxin which has well-ordered protein crystals, with a crystallographic refinement at 1.5Å resolution it was possible to derive the amino-acid sequence.[96] In the case of hexokinase, the temperature factor limits the refinement to 2.1Å and thus a sequence which is only 60—70 % correct can be obtained. The improvements in the sequence deduced by *X*-ray methods should reduce the effort required to determine a chemical sequence. Peptides about 8 or 9 residues long can be located in the refined sequence, which means that overlapping by chemical methods is no longer necessary.

Primary Structures

Table 1 *Amino-acid sequences reported during 1978*

Protein	*Source*	*Comments*	*Ref.*
Enzymes			
Acid protease	*Rhizopus chinensis*	sequence of 2 peptides containing Asp reactive with diazo-acetyl-DL-NLe methyl ester, 6 residues, and an Asp reactive with 1,2-epoxy-3-(*p*-nitrophenoxy) propane, 6 residues	97
Actinidin	*Actinidia chinensis*	sequence of tryptic peptides, a total of 220 residues. Considerable degree of homology between actinidin and papain	98
Alcohol dehydrogenase	*Drosophila melanogaster* N-11	blocked *N*-terminus determined by m.s. to be *N*-acetyl-Ser	99
	chicken liver	sequence of 6 tryptic peptides containing CysH	100
Aminoglycoside acetyltransferase (3)-I	*E. coli* plasmid RIP 135	*N*-terminal, 21 residues	101
Anthranilate-5-phosphoribosyl-1-pyrophosphate phosphoribosyltransferase	*Erwinia carotovora*	*N*-terminal, 39 residues	102
Anthranilate synthetase component II	*Pseudomonas putida*	complete sequence, 197 residues	103

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Protein	*Source*	*Comments*	*Ref.*
Aspartate amino transferase	pig-heart mitochondria	sequence around the active centre Lys residue involved in the formation of an aldimine bond with the co-enzyme pyridoxal-5-phosphate, 25 residues	104
Aspartokinase I-homoserine dehydrogenase I	*E. coli* K12	*N*-terminal sequence of dehydrogenase fragment produced by limited subtilisin digestion, 25 residues. Sequence of a CNBr fragment isolated from the entire protein, 59 residues	105
Ca^{2+}-transporting ATPase	rabbit skeletal muscle	partial sequence of tryptic peptides and sequences around 17 CysH residues	106
Carbonic anhydrase II	rabbit erythrocytes	complete sequence, 259 residues	107
Cationic trypsinogen	human pancreas	sequence of the activation peptide, 2 residues and *N*-terminal sequence of cationic trypsin	108
Citrate-lyase acyl carrier protein	*Klebsiella aerogenes*	complete sequence, 78 residues	109
Cytochrome oxidase	beef heart	complete sequence of one of the polypeptides, 47 residues	110
Dihydrofolate reductase	*Lactobacillus casei* amethopterin resistant	complete sequence, 162 residues	111
	E. coli B methotrexate resistant	complete sequence, 159 residues	112
Fructose 1,6-bis phosphatase	rabbit liver	sequence of 2 peptides at site of cleavage by lysosomal membrane fraction, 5 and 14 residues	113
β-Galactosidase	*E. coli*	complete sequence, 1021 residues	114

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Protein	*Source*	*Comments*	*Ref.*
β-Galactosidase	*E. coli*	identification of the Met residue labelled by an active-site directed inhibitor as residue 500 in the sequence	115
β-Glucosidase A	bitter almond meal	sequence of 2 peptides labelled with 6-bromo-3, 4,5-trihydroxy cyclohex-1-ene oxide, 4, and 16 residues	116
Glutaminase-asparaginase	*Acinetobacter glutaminasificans*	*N*-terminal, 60 residues, *C*-terminal, 16 residues	117
	Pseudomonas 7A	*N*-terminal, 26 residues	
Glyceraldehyde-3-phosphate dehydrogenase	rat skeletal muscle	*N*-terminal, 30 residues	118
Glycogen phosphorylase	rabbit muscle	*N*-terminal, 349 residues including the sites of allosteric control	119
	rabbit muscle	sequence of 2 CNBr fragments, 78 and 163 residues	120
	rabbit muscle	*C*-terminal, 492 residues. This completes the sequence of 841 residues	121
'Group-specific' protease (GSP)	rat small intestines	complete sequence, 224 residues. Approximately 33 % identity with chymotrypsin, trypsin, and elastase	122
Inorganic pyrophosphatase	yeast	complete sequence, 285 residues	123
β-Ketoadipate enol-lactone hydrolase	*Acinetobacter calcoaceticus*	*N*-terminal, 30 residues	124
2-Keto-3-deoxy-gluconate-6-phosphate aldolase	*Pseudomonas putida*	sequence of peptide containing active-site glutamic acid, 7 residues	125

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Protein	*Source*	*Comments*	*Ref.*
Maltodextrin phosphorylase	*E. coli*	sequence of pyridoxal-5-phosphate binding site, 21 residues	126
Mangano superoxide dismutase	*E. coli* B	complete sequence, 205 residues	127
Mitochondrial F_1-ATPase, β subunit	beef-heart mitochondria	sequence of tryptic peptide containing a Tyr at a nucleotide-binding site modified by an affinity label, 16 residues	128
Muconolactone Δ isomerase	*Pseudomonas putida*	*N*-terminal, 50 residues	124
	Acinetobacter calcoaceticus	*N*-terminal, 50 residues	
Penicillinase	*E. coli* plasmid R6K	partial sequence, 263 residues	129
Pepsinogens I II	Japanese monkey (*Macaca fuscata*) gastric mucosa	sequence of peptide which contains the site of attachment of carbohydrate, 9 residues	130
3-Phosphoglycerate kinase	yeast	*N*-terminal and *C*-terminal residues of 4 CNBr peptides	131
Phospholipase A_2	bovine	complete sequence, 123 residues, 71 % of the residues from bovine, porcine, equine sources are conserved	132
Plasminostreptin	*Streptomyces anti fibrinolyticus*	complete sequence, 109 residues, exhibits homology with the pancreatic secretory trypsin inhibitors from mammals	133
Rhodanese	bovine liver	*N*-terminal, 79 residues, and *C*-terminal, 12 residues	134
	bovine liver	complete sequence, 293 residues	135, 136
Ribulosebis phosphate carboxylase-oxygenase	spinach	sequence of 2 tryptic peptides which contain the essential Lys, 13 and 20 residues	137

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Protein	*Source*	*Comments*	*Ref.*
Ribulosephosphate carboxylase–oxygenase	spinach	sequence of tryptic peptides containing the 2 CysH residues and a Lys modified by the affinity label, bromoacetylethanol amine phosphate, 13 residues	138
Ribonuclease	red kangaroo (*Macropus rufus*) pancreas	complete sequence, 122 residues	139
	red-necked wallaby (*Macropus rufogriseus*)	tentative sequence has been derived, 122 residues	
Serine protease	rat skeletal muscle	*N*-terminal, 51 residues	140
Staphylococcal protease	*Staphylococcus aureus*	*N*-terminal, 213 residues. The *C*-terminal 43 residues have not been sequenced and most of these are Asp, Asn, and Pro	141
	Staphylococcus aureus strain V8	sequence of *C*-terminal tryptic peptide, 43 residues	142
Sucrase-isomaltase complex (SI)	rabbit small intestine	*N*-terminal sequence of the isomaltase subunit, 37 residues	143
Sulphite oxidase	rat liver	*N*-terminal sequence of 3 CNBr fragments of the haeme-binding region of the protein	144
Superoxide dismutase	human	partial sequence of tryptic and chymotryptic peptides	145
GMP-synthetase	*E. coli*	sequence of a peptide containing an essential CysH residue, 51 residues	146
Tonin	rat submaxillary gland	*N*-terminal, 34 residues	147
Trimethylamine dehydrogenase	methylotropic bacterium W 3A1	sequence of active-centre peptide containing the flavin co-enzyme, 12 residues	148

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Protein	*Source*	*Comments*	*Ref.*
Trypsinogens I II	human pancreatic juice	*N*-terminal, 9 residues are identical. They differ in the activation peptides released during transformation to trypsins	149
Tyrosinase	*Neurospora crassa*	Complete sequence, 407 residues. Single CysH residue at position 96 is covalently linked *via* a thioether bridge to a His residue at position 94	150
Zymogen	porcine pancreas	*N*-terminal, 14 residues	151
Hormones			
Adrenocorticotropin 53 (corticotropin)	ostrich (*Struthio camelus*)	complete sequence, 39 residues	152
Cholecystokinin peptides	sheep brain	complete sequence of 2 peptides, 8 residues	153
Follicle-stimulating hormone α-subunit	equine pituitary glands	complete sequence, 82 residues, it is shorter by 10—14 amino-acids at the *N*-terminus and has several other substitutions compared to human FSH α and bovine thyroid-stimulating hormone α	154
Follicle-stimulating hormone β-subunit	equine pituitary	complete sequence, 118 residues, it is analogous to human FSH β-subunit	155
Follitropin (follicle-stimulating hormone) β-subunit	porcine	complete sequence, 107 residues, it is shorter than the human chain at both the *N*- and *C*-terminal ends	156
Growth hormone structural variant	human pituitary	*N*-terminal, 23 residues	157
MSEL-neurophysin	whale (*Balaenoptera physalus*)	complete sequence, 95 residues	158
Neurophysin-I (bNP-I)	bovine pituitary	complete sequence, 93 residues	159
Neurotensin	bovine small intestine	complete sequence, 13 residues	160

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Protein	*Source*	*Comments*	*Ref.*
Parathyroid hormone	human	complete sequence, 84 residues	161
Preprotein form of α-subunit of chorio-gonadotropin	human placenta	*N*-terminal sequence of *in vitro* synthesized protein, 24 residues	162
Pre-prolactin	rat	*N*-terminal sequence of *in vitro* synthesized protein, 29 residues	163
Somatomedin B (mediators of the *in vivo* action of growth hormone somatotropin)	human plasma	complete sequence, 44 residues	164
Muscle Proteins			
Actin	*Physarum poly-cephalum*	complete sequence, 375 residues. Closely related to mammalian actin	165
Actin	bovine	*N*-terminal, 18 residues. The sequences show 6 unique *N*-terminal peptides in one mammalian species. The sequences contain common features which are also retained in the actin of the lower eucaryote *Physarum polycephalum*	166
M_1 (α)	skeletal muscle		
M_2 (α)	cardiac muscle		
SM 1	smooth muscle aorta		
SM 2	smooth muscle aorta		
C_1 (β)	brain		
C_2 (γ)	thymus		
Parvalbumin (less acidic components)	coelacanth (*Latimeria chalumnae*) muscle	complete sequence, 111 residues. The two components are identical except that one of them has an acetylated *N*-terminus	167
pI 5.44			
pI 4.95			
Troponin I	chicken breast muscle, leg muscle	complete sequence, 182 residues. The two proteins are identical	168
Troponin C	frog (*Rana esculenta*) skeletal muscle	complete sequence, 162 residues	169
Troponin T components	chicken breast muscle, leg muscle	partial sequences of tryptic peptides	170
α-Tropomyosin	rabbit skeletal muscle	complete sequence, 284 residues	171, 172

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Protein	*Source*	*Comments*	*Ref.*
Tropomyosin	horse platelets	*N*-terminal, 11 residues and *C*-terminal, 5 residues	173
Tropomyosin	horse platelets	partial sequence, residues 183 to 238	174
Histones			
Histone H2B	sea urchin (*Parenchinus angulosus*) embryo	partial sequence of 3 CNBr peptides, a total of 53 residues	175
	trout (*Salmo trutta*) testes	complete sequence, 123 residues. Comparison with calf thymus H2B revealed 8 changes out of 125 residues	176
Histone H2B	erythrocytes of *Xenopus laevis*	*N*-terminal, 84 residues	177
	Crocodilus niloticus	92 residues	
	Gallus domesticus	89 residues	
Histone $H2B_{(3)}$	sea urchin (*Parechinus angulosus*) sperm	complete sequence, 148 residues	178
Histone H2A	sea urchin (*Psammechinus miliaris*) gonads	complete sequence, 125 residues	179
Histone H2B (two variants) $H2B_{(1)}$ *Psammechinus* $H2B_{(2)}$ *Psammechinus*	sea urchin (*Psammechinus miliaris*) sperm	partial sequence, 50 residues out of 134 and 73 residues out of 142 assigned	180
Toxins			
Cytotoxins A A-II A-III A-IV	marine worm (*Cerebratulus lacteus*)	*N*-terminal, 12 residues and *C*-terminal, 2 residues of all three toxins	181
Diphtheria toxin, fragment B	*Corynebacterium diphtheriae*	partial sequence analysis of 6 CNBr fragments which represent the entire fragment B	182

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Protein	*Source*	*Comments*	*Ref.*
Neurotoxin V	scorpion (*Leiurus quinquestriatus quinquestriatus*	complete sequence, 66 residues	183
Secapin	European honey bee (*Apis mellifera*)	complete sequence, 24 residues	184
Melittin		complete sequence, 19 residues	
Peptide 401 (MCD peptide)		complete sequence, 22 residues	
Toxin I	*Anemonia sulcata* (sea anemone)	complete sequence, 46 residues. Homologous to toxin II from *Anemonia sulcata* and to anthopleurin A, a toxin from *Anthopleura xanthogrammica.*	185
Toxin II	*Anemonia sulcata* (sea anemone)	location of the three disulphide bridges	186
Toxins a	sea snake (*Astrotia stokesii*)	complete sequence, 60 residues	187
b		complete sequence, 70 residues	
c		complete sequence, 72 residues	
Toxins CM-2	Egyptian cobra (*Naja haje haje*) venom	complete sequence, 61 residues	188
CM-11		complete sequence, 65 residues	
CM-12		complete sequence, 62 residues	
Toxins CM-5	Egyptian cobra (*Naja haje haje*) venom	complete sequence, 71 residues	189
CM-6		complete sequence, 61 residues	
CM-10a		complete sequence, 61 residues. These toxins reveal a high degree of homology with the corresponding neurotoxins from *Naja haje annulifera* and *Naja nivea* venoms	
Toxins CM-7	Egyptian cobra (*Naja haje haje*) venom	complete sequence, 60 residues	190
CM-8		complete sequence, 60 residues	
CM-9		complete sequence, 60 residues	

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Protein	*Source*	*Comments*	*Ref.*
CM-10b		complete sequence, 60 residues. Each one contains 4 intra-chain disulphide bridges	
Ribosomal Proteins			
S5	*E. coli* K, A19	complete sequence, 166 residues	191
S7	*E. coli* B K	complete sequence, 153 residues and 177 residues. The difference of 24 residues occurs at the *C*-terminal end of the chain	192
S8	*E. coli*	complete sequence, 129 residues. Differences in the *C*-terminal half of the protein have been found with the previously reported sequence	193
S17	*E. coli*	complete sequence, 83 residues	194
S19	*E. coli*	complete sequence, 91 residues	195
L1	*E. coli* K	complete sequence, 233 residues	196
L3	*E. coli* MRE 600	complete sequence, 209 residues. Contains an N^5-methyl-Gln residue at position 150	197
L7/L12	*Artemia salina*	sequence of *C*-terminal tryptic peptide, 26	198
	rat liver	32 residues	
L13	*E. coli* K	complete sequence, 142 residues	199
L14	*E. coli* K19	complete sequence, 120 residues	200
L19	*E. coli*	complete sequence, 114 residues	201
L31	*E. coli*	complete sequence, 62 residues	202

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Protein	*Source*	*Comments*	*Ref.*
Large ribosomal subunit protein B-L9	*Bacillus subtilis*	complete sequence, 122 residues. Comparison with *E. coli* L7/L12 shows a relatively high degree of homology, 53 % of amino-acids are identical in both proteins	203
Ribosomal protein 44	*Saccharomyces cerevisiae* 60S subunit	complete sequence, 103 residues. An unusual amino-acid was observed at positions 40 and 54. It is related to mono-methyl-lysine	204
Globins			
Haemoglobins:			
Haemoglobins	trout I trout IV	sequence of pentapeptide from blocked *N*-terminus by m.s.	205
α-Chain	trout (*Salmo irideus*)	complete sequence, 144 residues	206
α-Chain	badger (*Meles meles*)	complete sequence, 141 residues	207
α-Chain β-Chain	slender loris (*Loris tardigradus*)	complete sequence, 141 and 146 residues	208
Major β-Chain IIβ	rat	complete sequence, 146 residues	209
δ-Chain	human	complete sequence, 146 residues	210
Abnormal haemoglobins:			
Coventry	human	β141, Leu is deleted	211
Detroit	human	β95, Lys→Asn	212
Presbyterian	human	β108, Asn→Lys	213
Strasbourg	human	β23, Val→Asp (revised sequence)	214
Ty Gard	human	β124, Pro→Gln	215
Yatsushiro	human	β60, Val→Leu	216

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Protein	*Source*	*Comments*	*Ref.*
Myoglobin (major component)	humpback whale (*Megaptera novaeangliae*)	complete sequence, 153 residues	217
Myoglobin (major component)	dall porpoise (*Phocoenoides dalli dalli*)	complete sequence, 153 residues	218
	common porpoise (*Phocoena phocoena*)	reinvestigation of primary structure. 2 residues have been altered	
Myoglobin (major component)	finback whale (*Balaenoptera physalus*)	complete sequence, 153 residues	219
Myoglobin	pilot whale (*Globicephala-melaena*)	complete sequence, 153 residues	220
Myoglobin	spiny ant-eater (*Tachyglossus aculeatus aculeatus*)	complete sequence, 153 residues	221
Cytochromes:			
b_5	bovine-liver microsomes	sequence of segment at the *C*-terminal end of the protein which is bound to membranes, 43 residues	222
c	blowfly (*Lucilia cuprina*)	complete sequence, 107 residues	223
c	*Schizosaccharo-myces pombe*	complete sequence, 108 residues	224
c	*Hippopotamus amphibius*	complete sequence, 104 residues	225
Ferredoxin I	*Pseudomonas ovalis*	complete sequence, 106 residues	226
Ferredoxin	*Porphyra umbilicalis* (red alga)	complete sequence, 98 residues	227
Hemerythrin	sipunculid worm (*Themiste dyscritum*)	complete sequence, 113 residues	228
Molybdenum–iron protein	*Azotobacter vinelandii*	*N*-terminal sequence of α and β subunits, 19 residues. *C*-terminal sequence of α and β subunits, 3 and 5 residues	229

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Protein	*Source*	*Comments*	*Ref.*
Phosphothioredoxin	*E. coli*	partial sequence of phospho-peptide, 5 residues. The phospho-amino-acid linkage was identified as a thiol-phosphate	230
C-Phycocyanin	*Oscillatoria agardhii*	partial sequence of α and β subunits, 34 and 28 residues. 3 chromophoric tryptic peptides from the α subunit have been sequenced	231
	thermophilic cyano-bacterium (*Mastigocladus laminosus*)	complete sequence of the two subunits α and β, 162 and 172 residues	232
C-Phycocyanin	*Synechococcus* sp. 6301 (*Anacystis nidulans*)	complete sequence, 170 residues. Sites of the phycocyanobilins are at CysH 81 and 152	233
	Synechococcus sp. 6301 (*Anabaena variabilis*)	partial sequence of *C*-terminal peptide of α-subunit, 33 residues	
C-Phycocyanin	*Porphyridium aerugineum*	sequence of 2 bilin-containing peptides from β-subunit, 7 and 10 residues	234
R-Phycocyanin	*Porphyridium cruentum*	partial sequence of 2 bilin-containing peptides from β-subunit, 7 and 18 residues	
Phycoerythrocyanin	*Anabaena variabilis*	partial sequence of a bilin-containing peptide from β-subunit, 38 residues	
C-Phycocyanin	*Anacystis nidulans* (*Synechococcus* sp. 6301)	sequence around the sites of attachments of the bilin prosthetic group, α-subunit, 14 residues and β-subunit, 37 residues, and 8 residues	235
Plastocyanin	spinach	*N*-terminal, 44 residues	236
Lectins:			
Lectin α-Chain β-Chain	*Lens culinaris*	complete sequence of α-chain, 52 residues, and *N*-terminal sequence of β-chain, 42 residues	237
Ricin	*Ricinus communis* (castor beans)	*N*-terminal sequence of A and B chains, 7 and 17 residues	238

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Protein	*Source*	*Comments*	*Ref.*
Ricinus communis agglutinin (RCA)		*N*-terminal sequence of A and B chains, 17 and 19 residues	
Structural Proteins:			
Collagen α1	chick skin	reinvestigation of the sequence of a CNBr peptide α1-CB7. Sequence of 271 residues has been corrected	239
Collagen α1(I)	chick skin	sequence of *C*-terminal peptide, 91 residues. Shows a sequence identity of 90 % with the corresponding segment of calf-skin collagen	240
Collagen Type II, α1 (II) chain	bovine nasal cartilage	*N*-terminal sequence of a CNBr peptide, 111 residues. This sequence is found in the centre, from 552–661	241
Collagen Type III	human liver	sequence of 5 CNBr peptides which occur consecutively, a total of 329 residues	242
α-Crystallin A Chain (minor component)	rat lens	complete sequence, 195 residues. Differs from the normal major A chain by having an insertion of 22 amino-acids between residues 63 and 64. It is also called αA^{Ins}	243
Kerateine-A, low sulphur protein, type II segments	wool	sequence of peptides from the highly helical fragments obtained by chymotryptic and tryptic digestion	244
	wool	complete sequence, 109 residues. Most of the cationic residues occur in pairs	245
Kerateine-A, low sulphur protein, type I helical segment	wool	complete sequence, 103 residues. The anionic residues occur in pairs	246
Viral Proteins:			
λ-Repressor	bacteriophage λ	complete sequence, 236 residues	247
Filamentous virus X_f major coat protein	*Xanthomonas oryzae*	complete sequence, 44 residues	248

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Protein	*Source*	*Comments*	*Ref.*
Foot and mouth disease virus coat proteins (3 proteins)	foot and mouth disease virus	*N*-terminal, 32, 20, and 40 residues. The 3 proteins are referred to as VP_{Gly}, VP_{Asp}, and VP_{Thr} after their *N*-terminal amino-acid	249
Gene F protein	bacteriophage ϕX174	complete sequence, 426 residues. A few of the amino-acids have been fitted in after the nucleotide sequence was known	250
Gene K protein	bacteriophage G4	complete sequence, 56 residues by micro-sequencing	251
Influenza haemagglutinin heavy chain	virus Hong Kong variant A/Memphis/102/72	*C*-terminal, 68 residues	252
Major capsid protein (P23), amber fragment of P23	bacteriophage T4	sequence of an amber fragment am B272, 151 residues. Protein P23 is cleaved to the polypeptide P23* during head assembly. This morphogenesis-specific cleavage takes place at the *N*-terminal region of P23 and results in a mol.wt. loss of 10 000. Am B272 covers the cleavage site as well as about 30 % of the whole protein	253
Phage λ receptor protein (1am B protein)	phage λ	*N*-terminal, 7 residues	254
Small outer capsid protein (Soc)	bacteriophage T4	complete sequence, 85 residues. Does not contain any CysH or Met	255
Tumour antigens (T) Large T Small t	simian virus 40	*N*-terminal, 25 residues, 33 residues	256
Blood Clotting Proteins:			
Blood-coagulation Factors X_1 and X_2 Light chains	bovine	*N*-terminal, 42 residues	257

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Protein	*Source*	*Comments*	*Ref.*
Fibrinogen	bovine	partial sequence of 3 disulphide linked CNBr peptides	258
Fibrinogen α-chain	human	sequence of peptide containing the only intra-chain disulphide loop in α-chain, 38 residues	259
Fibrinogen	human	arrangement of 6 disulphide bonds in the middle part of the molecule	260
Fibrinogen β-chain	human blood	sequence of 15 CNBr fragments, a total of 462 residues	261
Fibrinopeptide A	chicken blood	complete sequence, 15 residues. Shows considerable homology with mammalian peptide A.	262
Fibrin α-chain	human	*N*-terminal, 224 residues	263
	human	sequence of a disulphide-containing internal peptide obtained by plasmin digestion, 67 residues	264
	human	sequence of 2 CNBr peptides at *C*-terminal part of α-chain, 134 residues	265
Protein C, light chain	bovine plasma	complete sequence, 155 residues. The *N*-terminal Vitamin K-dependent part showed an extensive homology to both prothrombin and factor X, whereas the rest of the chain showed a strong homology to factor X but little similarity to prothrombin	266
β-Thromboglobulin (platelet-specific protein)	human platelets	complete sequence, 81 residues	267
Complement Proteins:			
Subcomponent C1q, B chain	human	*N*-terminal, 108 residues. The inter-chain disulphide bonds between A and B chains have been located	268

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Protein	*Source*	*Comments*	*Ref.*
Anaphylatoxin (C3a)	rat serum	complete sequence, 78 residues. Shows 70 % homology with human and porcine anaphylatoxins	269
Anaphylatoxin (C5a)	human serum	complete sequence, 74 residues. 9 % of its residues are half-cystine	270
HLA Antigens and Antibodies:			
Antibodies to type II pneumococcal polysaccharide 4432, 311	outbred rabbits hyper-immunized with type II pneumo-coccal polysaccharide	complete sequence of L chain variable region, 112 residues	271
Antibody 3T72 heavy chains	rabbit	sequence of variable-domain fragments, residues 10—122	272
Anti-*p*-azophenyl-arsonate, H and L chains, variable regions	A/J mice suppressed for a cross-reactive idiotype	*N*-terminal, 20 residues H chains and 13 residues, L chains	273
Anti-micrococcus lysodeikticus, L chain	rabbit	complete sequence, 211 residues	274
Anti-pneumococcal type III poly-saccharide 3368, L chain	rabbit	*N*-terminal sequence of variable region, 111 residues	275
Anti-streptococcal group A-variant K9-335 K9-338	rabbit	sequence of 2 variable regions of 2 L chains, 110 residues each. First demonstration of two identical L chain variable regions in rabbit antibodies raised against the same antigen	276
Galactan-binding proteins, L chains	mouse myeloma BALB/c	*N*-terminal, 108 residues of 3 L chains	277
Histocompatibility antigens HLA-A2 HLA-B7	human lymphoblastoid cell line	*C*-terminal sequence of glycoprotein p44 CNBr fragment, 31 residues for each	278

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Protein	*Source*	*Comments*	*Ref.*
Immunoglobulin light chain precursors κ-type and λ-type	mouse	*N*-terminal sequence of *in vitro* synthesized protein, 19—22 residues. They differ in up to 73 % of the sequence, but share a number of features, (i) high percentage of hydrophobic residues and (ii) comparable size	279
Immunoglobulin I_gG_{2a} variant, H chains	mouse myeloma	sequence of F_c region	280
Immunoglobulin I_gG Hil, L chain	human serum	complete sequence, 212 residues	281
Immunoglobulin I_gG2	human myeloma PIG Gm (n or 23) negative	sequence of pFć fragment obtained by pepsin digestion, 112 residues	282
Immunoglobulin Win light chain (λ)	Human myeloma	complete sequence, 216 residues	283
Immunoglobulin I_gG_1, κ, heavy chain	mouse myeloma MOPC 21	sequence of the first 6 CNBr fragments, 303 residues. This completes the derivation of the sequence of 1 chain of 440 residues	284
Mutant immunoglobulins, heavy chains of	murine plasmacytoma MOPC 21	characterization of all the CNBr fragments and comparison with wild type.	285
IF 2		There is an internal deletion of 96 residues from residue 121—215. No disulphide bonds between H and L chains	
IF 1		There is a deletion of the *C*-terminal 83 residues from 358—440	
a-Negative immunoglobulin H chain, V region	rabbit hyperimmunized with Group A *Streptococci*	*N*-terminal, 43 residues and residues 65 to 124 have been sequenced	286
Inulin-binding myeloma protein, H chains, E 109, A4, and U 61	mouse myeloma BALB/c	sequence of variable regions, 113 residues	287
$V_{\kappa\text{-}21}$ kappa chains	mouse myeloma BALB/c	complete sequence of variable region, 107 residues	288
A 22			
T 111			
CB 101			
CB 32		partial sequence, 88 residues	

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Protein	*Source*	*Comments*	*Ref.*
κ-Chain	mouse myeloma MPC 11	complete sequence, 225 residues. When compared to other mouse chains it was found to have 12 extra residues at its *N*-terminus	289
α-1-Microglycoprotein	human with plasma cell leukemia	*N*-terminal, 21 residues	290
Myeloma protein Tro (monoclonal I_gG-I immunoglobulin)	human	sequence of 4 CNBr fragments	291
Myeloma protein Tro	human	sequence of tryptic peptides	292
Myeloma protein Tro	human	complete sequence of H chain	293
Phosphorylcholine-binding protein, k-chain	mouse myeloma	complete sequence of variable region, 112 residues	294
Transplantation antigen A_gB4	rat of A_gB4 haplotype	*N*-terminal, 28 residues. Homologous to those of mouse, human, and guinea-pig	295
Miscellaneous:			
Antifreeze glycopeptide 8	antarctic fish *Trematomus borchgrevinki*	sequence of 3 glycopeptides present in fraction 8, 14 residues	296
Antifreeze glycoprotein	arctic fish *Boreogadus saida*	complete sequence, 14 residues. Similar to the antifreeze glycoproteins present in antarctic fish	297
α-1-Antitrypsin S Z	human plasma	*N*-terminal, 19 residues. Sequence of CNBr fragment which contains the mutation areas of the molecule, 109 residues	298
Apoferritin	rat liver	*N*-terminal, 3 residues	299
Apolipoprotein Apo A-I	human plasma	complete sequence, 243 residues. Significantly different from that reported previously	300

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Protein	*Source*	*Comments*	*Ref.*
Brain-specific protein, S-100 PAP I-b protein	bovine brain	complete sequence, 91 residues. Dimer of two identical polypeptides. The acidic cluster of amino-acids has a high sequence homology with the calcium binding sites of troponin C and parvalbumin	301
Ca^{2+}-dependent regulator protein	rat testis	partial sequence, a total of 135 residues have been fitted in the sequence of 148 amino-acids. Shows 50 % homology with troponin C from rabbit skeletal muscle. The regulator protein is 11 residues shorter than troponin C	302
β-Casein	human milk	ordering of 4 CNBr peptides and 4 tryptic peptides	303
Colicin E 3 subunit A	*E. coli* W 3110 (E3)	sequence of an active fragment T2A obtained from subunit protein A, 97 residues	304
Conalbumin	egg-white	*N*-terminal sequence of *in vitro* synthesized protein, 19 residues	305
Dimeric α-amylase inhibitor	wheat (*Triticum aestivum*) kernel	*N*-terminal, 23 residues	306
DNA-binding proteins NS 1 NS 1	*E. coli* ribosomes	complete sequence, 90 residues in each. The proteins have identical amino-acids at 62 out of 90 positions. They showed no homologies with calf thymus histones H1, H2a, H2b, H3, and H4	307
Elongation factor G	*E. coli*	sequence of fragment containing the exposed –SH group which apparently participates in the formation of the GTP-binding centre, 69 residues, the CysH being at position 55	308
α-Fetoprotein	murine	*N*-terminal, 24 residues. *C*-terminal residue also identified	309

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Protein	*Source*	*Comments*	*Ref.*
Glycophorin A^{mn}	human erythrocytes	complete sequence, 131 residues. Contains 16 oligosaccharide units attached to the *N*-terminal third of the molecule	310
Glycophorin A from M and N homozygotes	erythrocyte membrane	*N*-terminal, 31 residues. The two peptides differ at positions 1 and 5	311
Glycophorin B	erythrocyte membrane	*N*-terminal, 35 residues	
H-$2K^b$ Glycoprotein (MHC-alloantigen)	mouse H-2 major histocompatibility complex	*N*-terminal sequence of 5 CNBr peptides, 23—39 residues in each	312
HMG 1 (non-histone chromosomal protein)	calf thymus	*C*-terminal, 41 residues. Contains only Asp and Glu	313
HMG-20 (high mobility group)	calf thymus	*N*-terminal, 20 residues. Identical to the protein ubiquitin whose function is not yet known	314
Insulin-like growth factor I (IGF-I)	human serum	complete sequence, 70 residues. Homologous to pro-insulin. Positions 1—29 are homologous to insulin B chain, and positions 42—62 to insulin A chain. Positions 30—41 shows no homology to pro-insulin C peptide	315
Insulin-like growth factor II (IGF-II)	human plasma	complete sequence, 67 residues. Shows a 62 % homology with IGF-I and with pro-insulin	316
Iturine A	*Bacillus subtilis*	cyclic peptido-lipid structure. Contains 7 amino-acids with the Ser linked to the amino-fatty acids through a peptide bond	317
lac Repressor-β-galactosidase protein (chimeric protein)	*E. coli* deletion mutant	sequence of a CNBr peptide containing the fusion joint, 77 residues. The site of joining is from residue 355	318

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Protein	*Source*	*Comments*	*Ref.*
		of lac repressor to residue 24 of β-galactosidase indicating that the last 4 residues at the *C*-terminus of lac repressor and the first 23 residues at the *N*-terminus of β-galactosidase are not essential for the activities of these two proteins	
B 116 lac Repressor (an altered lac repressor)	*E. coli*	*N*-terminal, 26 residues. An additional 16 amino-acids are added to the *N*-terminal end of wild-type repressor	319
Major copper-binding protein	human foetal liver	partial sequence of CNBr cleaved protein, 7 residues	320
β_2-Microglobulin	guinea-pig urine	*N*-terminal, 18 residues of major form	321
Normal cross-reacting antigen (NCA)	human spleen	*N*-terminal, 26 residues	322
Outer cell envelope membrane major protein	*E. coli* B/r	sequence of CNBr fragments	323
Ovalbumin	hen's egg	sequence around the 4 —SH groups and around the 2 half-cystines	324
	hen's egg	*N*-terminal, 16 residues. This sequence differs at positions 3 and 4 from a previously published sequence	325
Pili proteins	*Neisseria gonorrhoeae* strains B, F62, 33, and 7122	*N*-terminal, 29 residues	326
	Neisseria meningitidis strain 13090	*N*-terminal, 29 residues	
	E. coli	*N*-terminal, 24 residues	
Pre-α-lactalbumin	sheep	*N*-terminal sequence of *in vitro* synthesized protein, 19 residues	327

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Protein	*Source*	*Comments*	*Ref.*
Pre-β-lactoglobulin	sheep mammary glands	*N*-terminal sequence of *in vitro* synthesized protein, 18 residues	328
Pre-ovomucoid	hen	*N*-terminal sequence of *in vitro* synthesized protein, 26 residues	329
Protein H-2K^q	adult mice spleen	*N*-terminal, 21 residues	330
Protein HU (DNA-binding protein)	*E. coli*	*N*-terminal, 37 residues, *C*-terminal, 11 residues	331
Protamine (clupeine)	herring	sequence of 3 peptides at the phosphorylation sites, 13, 8, and 8 residues	332

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PART 1C: Chemical modification of Proteins *by A. Dell*

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

Table 1 *Chemical modification of proteins*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Abrin	*Abrus precatorius*	various reagents		B chain can be selectively modified	1
Acetate kinase	*Veillonella alcalescens*	*p*-chloromercuribenzoate, *N*-ethylmaleimide	Cys	inhibition	2
Acetoacetyl-CoA thiolase	porcine	chloromethylketone derivatives of fatty acids	Cys	synthesis of reagents described	3
Acetylcholinesterase	bovine	various reagents		Cys, Tyr, Trp, His, Glu, Asp, Arg, Met modified	4
Acetylcholinesterase	*Electrophorus electricus*	[^{3}H]di-isopropyl fluorophosphate	Ser	active-site labelling	5
Acetylcholinesterase	electric eel	various *N*-hydroxysuccinimide esters of substituted amino-acids	Tyr	reagents assessed for specificity as acylating agents	6
Acetylcholinesterase	electric eel	pyrenebutyl methylphosphono-fluoridate	Ser	fluorescent active-site label	7
Acetylcholinesterase	*Electrophorus electricus*	benzenemethane sulphonylfluoride	Ser	active-site label	8
Acetylcholinesterase	electric eel, rat, chicken	*O*-ethyl-*S*^{2}-di-isopropylaminoethyl methylphosphonothionate	Ser	specific active-site reagent	9
Acetylcholine receptor	*Torpedo californica*	dimethylsuberimidate, 3,3′-dithiobispropionimidate	Lys	pentameric structure?	10
Acetylcholine receptor	*Torpedo californica*	4-(*N*-maleimido)benzyltri-methylammonium iodide		theoretical study of affinity labelling	11
Acetylcholine receptor	*Torpedo californica*	[^{3}H]bis(azido)ethidium chloride–photoirradiation		photolabelling of receptor in membranes more specific than purified receptor	12
Acetylcholine receptor	*Torpedo californica*	*N*-ethylmaleimide, iodoacetamide	Cys	S–S bonds between 65 000 dalton subunits	13
Acetylcholine receptor	*Torpedo californica*	1.$NaBH_4$–crosslinker 2.photo-irradiation	1.Cys	affinity directed cross-linking within membrane	14
Acetylcholine receptor	*Torpedo californica*	bromoacetyl [*Me*-^{3}H]choline		affinity label for α-chain only	15
Acetylcholine receptor	*Torpedo californica*	*N*-(3-pyrene)maleimide	Cys	fluorescent probe	16
Acetylcholine receptor	*Torpedo ocellata*	4-(*N*-maleimido)benzyl[^{3}H]tri-methylammonium iodide		terbium binding-sites labelled	17
Acid protease	*Endothia parsitica*	glutaraldehyde	Lys	cross-linking of crystals	18
Acid protease	rat	various sulphydryl reagents	Cys	essential Cys?	19

Actin	rabbit	*N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine dansyl chloride	Lys	nanosecond fluorescence depolarization study	20
F-Actin	rabbit	*p-NN'*-phenylenebismaleimide	Cys, Lys	single cross-link	21
Actin	rabbit	cyclohexane-1,2-dione	Arg	Arg-28, 95, 147 modified	22
Actin	rabbit	*N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine	Cys-373	fluorescence study	23
Actin	rabbit	5-iodoacetamidofluorescein	Cys	labelled actin incorporated into living cells	24
F-Actin	rabbit	dansyl aziridine	Cys-373	fluorescence study	25
G-Actin	rabbit	*N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide	Cys-373	Cys-373 is 30Å from nucleotide binding-site	26

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Activator protein	bovine	tetranitromethane	Tyr	Tyr-138 in unique environment	27,28
		N-acetylimidazole	Tyr, Lys		
Activator protein	bovine	^{125}I	Tyr	monoiodination achieved	29,30
Activator protein	bovine	*N*-chlorosuccinimide–Ca^{2+}	Met	Met-71, 72, 76, in Ca^{2+} sites?	31
S-Adenosylmethionine decarboxylase	rat	$NaB[^3H]_4$		covalently bound pyruvate	33
Adenylate cyclase	mouse	mercaptoethanolamine, cystamine	Cys	activation of enzyme	34
Adenylate kinase	porcine, rabbit	[^{14}C]phenylglyoxal	Arg-97	identified by peptide mapping	35
β-Adrenergic receptor	turkey	*N*-[2-hydroxy-3-(1-naphthyloxy)-propyl]-*N'*-bromoacetylethylene-diamine		affinity label	36
Aequorin	*Aequorea forskalea*	DTNB	Cys	loss of luminescent activity	37
Agglutinin	wheat germ	photoirradiation–trichloro-ethanol	Trp	Trp modified after excitation	38
Alanine aminotransferase	porcine	β-chloroalanine		suicide substrate	39
D-Alanine carboxypeptidase	*E. coli*	*p*-chloromercuribenzoate, DTNB	Cys	release of penicillin inhibited	40
Alanine transport carrier	*Bacillus subtilis*	*p*-chloromercuribenzoate	Cys	inhibits binding	41
Albumin	bovine	various spin-labelled maleimides	Cys	spin-label studies at various pH's	42,43
Albumin	bovine	lactoperoxidase–thiocyanate–hydrogen peroxide	Cys, Tyr, Trp, His	reaction mechanism studied	44
Albumin	bovine	dansyl chloride	Lys	protein precipitation study	45
Albumin	bovine	*p*-benzoylbenzyl bromide	Lys	modification carefully controlled	46
Albumin	human	[^{14}C]bilirubin–3-(3-dimethyl-aminopropyl)-1-ethylcarbodi-imide	Lys-240	involved in bilirubin binding	47
Albumin	human	^{125}I, ^{77}Br, [^{125}I]-labelled *N*-succinimidyl-3-(4-hydroxy-phenyl)propionate		sites of labelling determined	48
Albumin	human	2,6-dinitro-4-trifluoromethyl-benzene sulphonate	Lys	2 Lys highly reactive	49
Alcohol dehydrogenase	horse	iodoacetic acid	Cys-46	Trp fluorescence of modified protein studied	50
		ethyl acetimidate	Lys		
Alcohol dehydrogenase	horse	methyl isonicotinimidate	Lys	modified protein crystallized	51
Alcohol dehydrogenase	horse	various bromoacetamido acids	Met, Cys	co-enzyme directed alkylation	52

Alcohol dehydrogenase	horse	N^6-[*N*-(6-aminohexyl)carbamoyl]-methyl-NAD–carbodi-imide	carboxyl	covalently bound functioning co-enzyme	53
Alcohol dehydrogenase	horse	formaldehyde–$NaBH_4$	Lys	kinetic study	54
Alcohol dehydrogenases	*Pseudomonas putida*	*p*-chloromercuribenzoate	Cys	inhibits enzymes	55
Aldehyde dehydrogenase	ovine	1-[^{14}C]disulphiram	Cys	covalent binding	56
Alkaline mesentericopeptidase	*Bacillus mesentericus*	various peptide chloromethyl ketones	His	selective reaction of essential His	57

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Alkaline phosphatase	calf	1. methylacetimidate 2. diazonium-1*H*-tetrazole	1. Lys 2. His	Lys first protected before His-reagent added	58
D-Amino-acid oxidase	porcine	D-propargylglycine		suicide substrate	59
D-Amino-acid transaminase	*Bacillus sphaericus*	β-bromo-D-alanine, β-bromo-pyruvate–D-amino-acid		key intermediate trapped	60
Aminoacyl-tRNA synthetase	*E. coli, Bacillus stearothermophilus*	[^{14}C]-2′,3′-dialdehyde derivative of ATP–$NaBH_4$	Lys	affinity labelling	61
δ-Aminolevulinic acid synthase	*Rhodopseudomonas spheroides*	pyridoxal 5′-phosphate–$NaBH_4$	Lys	ε-*N*-pyridoxyl-lysine identified after acid hydrolysis	62
D-1-Amino-2-propanol: NAD^+ oxidoreductase	*E. coli*	*p*-mercuribenzoate, *N*-ethylmaleimide	Cys	both molecular forms inhibited	63
cAMP-adenosine binding protein	mouse	DTNB	Cys	one highly reactive Cys/subunit	64
Angiotensin converting enzyme	rabbit	various reagents		essential Tyr, Arg, carboxyl	65
Anthranilate synthetase	*Pseudomonas putida*	iodoacetamide, L-2-amino-4-oxo-5-chloro-pentanoic acid	Cys-79	selective alkylation	66
Antithrombin III	bovine	*O*-methylisourea	Lys	study of heparin action	67
α_1-Antitrypsins	human	DTNB–various thiols	Cys	one reactive thiol	68
α_1-Antitrypsin	human	cyclohexane-1,2-dione	Arg	no effect on inhibitory activity	69, 70
		citraconic anhydride, acetic anhydride, trinitrobenzene sulphonic acid, formaldehyde	Lys	loss of inhibitory activity	
Apoleghemoglobin a	soybean	photoirradiation–sensitizers	His, Trp	selective oxidation using porphyrins	71
Apolipoprotein A-II	human	DTT	cystine	sedimentation studies	72
Arginase	human	*p*-hydroxymercuribenzoate	Cys	active dimers formed	73
Arginine decarboxylase	*E. coli*	DTNB	Cys	dissociation of decamer studied	74
Arginine deiminase	*Mycoplasma arthritidis*	DTNB formamidinium acetate	Cys	half-site reactivity affinity reagent	75
L-Asparaginase	*E. coli*	2-*O*-methoxypolyethylene glycol-4,6-dichloro-*S*-triazene	Lys	active enzyme which has lost antigenicity produced	76
Aspartate transaminase	porcine	KNCO	Lys-258	^{19}F-n.m.r. study	77
Aspartate transaminase	porcine	*N*-(1-oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)maleimide	Cys-390	e.s.r. study of active site	78

Aspartate transaminase	porcine	β-chloro-L-alanine	Lys	labelled peptides identified	79
Aspartate transaminase	porcine	1,5-difluoro-2,4-dinitrobenzene	Lys, Cys, Tyr	bifunctional reagent	80
Aspartate transaminase	porcine, chicken	DTNB, *N*-ethyl 2,3-[^{14}C]maleimide	Cys-166	labelled chymotryptic peptide sequenced	81
Aspartate transaminase	rat	$NaB[^3H]_4$		reduction of co-factor–apoprotein linkages	82
Aspartate transaminase	porcine	*N*-ethylmaleimide	Cys-390	rate of alkylation studied	83
Aspartate transcarbamylase	*E. coli*	tetranitromethane	Tyr	labelled tryptic peptide isolated	84, 85

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Aspartate transcarbamylase	*E. coli*	pyridoxamine phosphate 2-mercuri-4-nitrophenol	Cys	distance between catalytic sites on different subunits determined	86
Aspartate transcarbamylase	*E. coli*	tartaryl diazide		stabilization of relaxed state	87
Aspartokinase I-Homoserine dehydrogenase	*E. coli*	iodoacetic acid	Cys	unique Cys modified	88
ATPase F1	bovine	*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline	carboxyl	1 mol per active site inactivates	89
ATPase	bovine	2-mercaptopropionylglycine	Cys	increase in intensity of band 4	90
ATPase	bovine	[^{14}C]phenylglyoxal	Arg	essential Arg protected by efrapeptin	91, 92
ATPase F1	bovine	*p*-fluorosulphonyl[^{14}C]benzoyl-5′-adenosine	Tyr	labelled tryptic peptide sequenced	93
ATPase	canine	Cu^{2+}	Cys	cross-linking does *not* require *o*-phenanthroline	94
ATPase	electric eel	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Tyr	essential Tyr	95
ATPase	lamb	*N*-ethylmaleimide	Cys	study of phosphorylation	96
ATPase F1	*Micrococcus*	6-[(3-carboxy-4-nitrophenyl) thio]- and 6-[(2,4-dinitrophenyl) thio]-9-β-D-ribofuranosyl-purine 5′-triphosphate		covalent binding exclusive to subunits: Cys not involved	97
ATPase F1	*Micrococcus luteus*	8-azido-adenosine 5′-triphosphate–photoirradiation		80 % of label bound to β-subunits	98
ATPase	porcine	[^{3}H]-2-nitro-5-azidobenzoyl-ouabain–photoirradiation		labels large polypeptide and a proteolipid component	99
ATPase	rabbit	*N*-ethylmaleimide	Cys	labelling precludes use of ligand directed trypsinolysis	100
ATPase	rabbit	DTNB, *N*-ethylmaleimide	Cys	at least two classes of SH	101
ATPase	rabbit	*N*-ethylmaleimide, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)maleimide	Cys	effect of lipids on protein motion studied	102, 103
ATPase	rabbit	*N*-(oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)iodoacetamide	Cys	ligand induced changes in reactivity observed	104
ATPase	rabbit	*S*-mercuric *N*-dansylcysteine	Cys	12 reactive thiols per 10^5 daltons	105
ATPase	*Salmo gairdneri*	6,6′-dithiobis(inosinylimido-diphosphate	Cys	no protection by substrate	106

ATPase F1	*Thermophilic bacterium*	DTNB	Cys	3 α-subunits	107
Bacteriorhodopsin	*Halobacterium halobium*	iodine	Tyr	modification of 1 to 2 Tyr inhibits photocycling	108
Bisphosphoglyceromutase/ phosphatase	human	trinitrobenzenesulphonate	Lys	essential Lys	109
α-Bungarotoxin	*Bungarus multicinctus*	^{125}ICl	Tyr	mono, di-, tri-iodinated species purified	110
Carbonic anhydrase B	human	bromo-1-[^{13}C]acetic acid	His-200	^{13}C-n.m.r. study	111
Carbonic anhydrase B	human	iodo 1- and 2-[^{13}C]acetate	His-200	^{13}C-n.m.r. study	112
Carboxylesterases	rat	bis-*p*-nitrophenylphosphate	Ser	specificity of reaction determined	113

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Carboxypeptidase A	bovine	1. diazotized 5-amino-1*H*-tetrazole 2. tetranitromethane	Tyr	1. Tyr-248 modified 2. Tyr-198 is then reactive	114
Carboxypeptidase A	bovine	diazotized arsanilic acid	Tyr-248	kinetic study	115,116
Carboxypeptidase B	porcine	tetranitromethane	Tyr	reactivity affected by Zn	117,118
Cardiotoxin $V^{II}1$	*Naja melanoleuca*	*N*-chlorosuccinimide	Met-24, 26	loss of toxicity	119
Cardiotoxin	cobra	tetranitromethane	Tyr	selective nitration of Tyr-11, 22	120
α-Casein	bovine	various reagents	His, Trp, Met	1 His essential for clotting; Trp, Met not involved	121
Cathepsin B	human	iodoacetic acid, 2,2′-di-pyridyl disulphide	Cys	essential Cys	122
Chloramphenicol acetyl-transferase	*E. coli*	various sulphydryl reagents	Cys	Cys near chloramphenicol binding-site	123
Chorismate mutase–Pre-phenate dehydratase	*E. coli*	DTNB	Cys	prevents Phe binding	124
Chymopapains A,B	*Carica papaya*	2,2′-dipyridyl disulphide	Cys	method for detecting chymopapain	125
α-Chymotrypsin	bovine	phenacyl bromide	Met-192	high pH modified enzyme is not modified at Cys	126
α-Chymotrypsin	bovine	diazonium salts of D-Phe	Tyr-146	catalytic properties modulated by the different reagents	127
α-Chymotrypsin	bovine	1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide–$NH_2(CH_2)_nNH_2$	carboxyl	effect of length of cross-link on thermostability studied	128
Citrate synthase	porcine	*S*-(4-bromo-2,3-dioxobutyl)-CoA		affinity label for enzymes that bind acetyl CoA	129
Cloacin DF 13	*Enterobacter cloacae*	various reagents	Tyr, Trp, Asp, Glu	all modifications resulted in loss of ability to kill bacteria	130
Cloacin complex	*Enterobacter cloacae*	fluorescamine	Lys	cloacin's interaction with membranes studied	131
Coat protein	Tobacco mosaic virus	various maleimide spin-labels	Cys	e.s.r. study	132
Coat protein	Tobacco mosaic virus	^{125}I	Tyr-139,2	Tyr-139 most reactive in disk	133
Collagen	chicken	$NaB[^3H]_4$		osteopetrotic bone has changed cross-link pattern	134
Collagen	bovine	$NaB[^3H]_4$		dihydroxy- and hydroxy-lysino-norleucine in ration 9 : 1	135
Collagenase	*Achromobacter iophagus*	bromoacetone	His	essential His	136
Collagenase	*Achromobacter iophagus*	vinylpyridine	Cys	each subunit a single polypeptide	137

Complement C1s	human	[^{3}H]-*m*-[*o*-(2-chloro-5-fluoro-sulphonylphenylureido)-phenoxybutoxy]benzamidine		affinity label	138
Concanavalin A	jack bean	hexamethylene di-isocyanate	Lys	mono-, di-, tetra-valent derivatives stable	139
Connectin	chicken	$NaB[^3H]_4$		connectin, collagen and elastin have similar cross-linking	140
Coupling factor	*Rhodospirillum rubrum*	phenylglyoxal, butane-2,3-dione	Arg	essential Arg	141

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Coupling factor 1	spinach	[^{3}H]pyridoxal 5′-phosphate–$NaBH_4$	Lys	inhibits phosphorylation in membrane bound CF1	142
Creatine kinase	rabbit	2-mercuri-4-nitrophenol	Cys	role of reactive thiol studied	143
α-Crystallin	bovine	various sulphydryl reagents	Cys	3 classes of sulphydryls	144
Cyclic nucleotide binding proteins	bovine	8-azido[^{32}P]cAMP or 8-azido-[^{3}H]cGMP–photoirradiation		cytosol nuclear proteins compared	145
Cytochrome b_5	bovine	*N*-bromosuccinimide	Trp	Trp-108 or 109 identified as fluorescent residue	146
Cystathionase	rat	pyridoxal 5′-phosphate	Lys	bound co-factor not a photo-sensitizer of protein	147
Cytochrome *c*	horse	4-chloro-3,5-dinitrobenzoate	Lys	8 singly substituted derivatives isolated	148
Cytochrome *c*	horse	formaldehyde–$NaBH_4$	Lys	methylation after complex formation with cytochrome *c* peroxidase	149
Cytochrome *c*	horse	ethylthioltrifluoroacetate, trifluoromethylphenylisocyanate	Lys	effect of specific modification on reduction of enzyme studied	150
Cytochrome *c*	horse	*N*-5-azido-2-nitrobenzoyl-succinimide or ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate–photoirradiation	Lys	crosslinking of cytochrome *c* to bound proteins	151
Cytochrome *c*	horse	[^{3}H]acetic anhydride, formaldehyde–$KB[^3H]_4$	Lys	competitive labelling	152,153
Cytochrome *c*–cytochrome *c* oxidase	bovine	dithiobis-succinimidyl propionate, *NN*′-bis(3-succinimidyloxy carbonylpropyl)tartarate	Lys	subunit interactions studied	154
Cytochrome *f*	*Raphanus sativus*	DTNB	Cys	sulphydryl is electron donor	155
Cytochrome P450	*Pseudomonas putida*	*N*-ethylmaleimide	Cys	(succinimido-Cys)$_4$-protein described	156
Cytochrome *c* oxidase	bovine	arylazidocytochrome *c*–photo-irradiation		covalent cross-links formed	157
Cytochrome *c* oxidase	bovine	[^{14}C]phenylisothiocyanate	Lys	specific labelling of essential Lys?	158
Cytochrome *c* oxidase	bovine	dimethylsuberimidate	Lys	study of inactivation	159
Cytochrome oxidase	yeast	*N*-[^{14}C]ethylmaleimide arylazidocytochrome *c* derivatives–photoirradiation	Cys	subunit II identified polypeptide II bonded to cytochrome *c*	160

Cytochrome b_5 reductase	rabbit	*p*-hydroxymercuribenzoate	Cys	strong inhibition	161
NO-Diacetylmuramidase	*Chalaropsis*	glycinamide or taurine/1-ethyl-3-(dimethylaminopropyl)carbo-di-imide	carboxyl	essential Asp, Glu	162
DNA-binding protein	*E. coli*	various reagents		effect on DNA binding studied	163
DNA gyrase	*E. coli*	2′,3′-dialdehyde derivative of α-[^{32}P]ATP	Lys	novobiocin inhibits reaction	164
DNA polymerase-α	calf	*N*-ethylmaleimide	Cys	enzyme D very sensitive	165
DNA polymerase	mosquito	*N*-ethylmaleimide, *p*-mercuri-benzoate	Cys	inhibits enzyme	166

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Elastin	porcine	$NaBH_4$		merodesmosine and lysinonor-leucine obtained on hydrolysis	167
Elongation factor Tu	*E. coli*	N^{ϵ}-bromoacetyl-Lys-tRNA		functional covalent complex	168
Elongation factor Tu	*E. coli*	*N*-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidin-3-yl)maleimide	Cys	reactive Cys not essential for aminoacylated tRNA binding	169
Elongation factor G	*E. coli*	[^{14}C]-labelled derivatives of GTP		GTP binding site located	170
Elongation factor G	*E. coli*	p-azidobenzaldehyde–$NaB[^3H]_4$–photoirradiation	Lys	EF-G interacts with both ribosomal subunits	171
Elongation factor Tu	*E. coli*	formaldehyde–$NaB[^3H]_4$	Lys	competitive labelling	172
Elongation factors	*Thermus thermophilus*	*p*-chloromercuribenzoate	Cys	sulphydryl essential for interaction with ribosomes	173
Endopeptidase	*Vespa crabro*	phenylmethanesulphonylfluoride, carbobenzoxy-Phe chloroketone	Ser	similar catalytic properties to chymotrypsin	174
Enolase	yeast	butane-2,3-dione	Arg	1 essential Arg/subunit	175,176
Epoxide hydrase	rat	various reagents		essential His modified by 2-bromo-4′-nitroacetophenone	177
Estradiol 17β-dehydro-genase	human	4-bromo 2′-[^{3}H]acetamidoestrone methyl ester	Lys, Cys	Cys protected by $NADP^+$	178
Factor IX	bovine	CNBr, *N*-bromosuccinimide	Gla	cleavage at Gla demonstrated	179
Factor X	bovine	methanesulphonylfluoride, di-isopropylfluorophosphate	Ser	active-site labelling	180
Factor X_a	bovine	di-isopropylfluorophosphate	Ser	active-site labelling	181
Factor XII	bovine	di-isopropylfluorophosphate	Ser	active-site labelling	182
Fatty acid synthetase	goose	various reagents	Cys, Ser	essential Cys, Ser?	183
Fatty acid synthetase	rat	1,3-[^{14}C]di-isopropylfluoro-phosphate	Ser	two thioesterase domains	184
Ferredoxin–$NADP^+$ reductase	*Bumilleriopsis filiformis*	butane-2,3-dione	Arg	essential Arg	185
		dansyl chloride	Lys	essential Lys	
Fibrin	human	monodansyl cadaverine		labelling of cross-link acceptor sites	186
Fibrinogen	human	2-hydroxy-5-nitrobenzyl bromide	Trp	no cross-linking	187
		hydrogen peroxide	Trp, Met, Tyr	intramolecular cross-linking	
Fibrinogen	human	photoirradiation–Methylene Blue	His, Trp	loss of coagulability	188

Flavoprotein	egg white	tetranitromethane	Tyr	essential Tyr protected by riboflavin	189
		dimethyl(2-hydroxy-5-nitrobenzyl)-sulphonium bromide	Trp	essential Trp not protected by riboflavin	
α-Foetoprotein	rat	iodoacetic acid, 8-[*N*-(iodo-acetylaminoethyl)amino]naph-thalene-1-sulphonic acid	Cys	no Cys in estrogen site	190
Follitropin	human	*N*-bromosuccinimide	Trp	Trp domain near subunit–subunit contact	191
Fructose bisphosphate aldolase	rabbit	dihydroxyacetone phosphate–$NaBH_4$	Lys-227	Cibacron Blue dye does not interact with Lys-227	192

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
α-L-Fucosidase	monkey	ethanolamine–ethyldimethyl aminopropyl carbodi-imide	carboxyl	essential carboxyl	193
		p-chloromercuribenzoate	Cys	inhibits	
β-Galactosidase	*E. coli*	iodo[^{14}C]acetate	Cys	Cys not essential	194
β-Galactosidase	*E. coli*	β-D-galactopyranosyl[^{14}C] methyl *p*-nitrophenyltriazene	Met-500	labelled Met identified by field desorption mass spectrometry	195,196
Glucagon	porcine	methyl iodide	Met	^{13}C-n.m.r. study of adduct with $^{13}CO_2$	197
Glucagon receptor		N^{ε}-4-azido-2-nitrophenyl-glucagon–photoirradiation		photoaffinity label	198
Glucose 4-epimerase	*Saccharomyces fragilis*	DTNB	Cys	essential Cys	199
Glucose synthase	*E. coli*	[^{3}H]pyridoxal 5′-phosphate–$NaBH_4$	Lys	2 labelled peptides sequenced	200
β-Glucosidase A	almond	6-bromo-3,4,5-trihydroxy-cyclo[2-^{3}H]hex-1-ene oxide	Asp	2 labelled tryptic peptides sequenced	201
Glutamate dehydrogenase	*Bacillus megaterium*	[^{14}C]phenylglyoxal,butane-2,3-dione	Arg	essential Arg	202
		pyridoxal-5′-phosphate	Lys	inactivation	
Glutamate dehydrogenase	bovine	5′-*p*-fluorosulphonylbenzoyl guanosine		new affinity label for guanosine nucleotide sites	203
Glutamate dehydrogenase–aminotransferase	bovine	dimethyl 3,3′-dithiobis-propionimidate	Lys	evidence for complex of two enzymes	204
Glutamate dehydrogenase	bovine	*p*-chloromercuribenzoate, methyl mercuric chloride	Cys-319	catalysis study	205
Glutamate dehydrogenase	bovine	3-[2′-^{14}C]acetyl-2-nitroestrone	Lys	substrate deacylated before labelling enzyme	206
Glutamate dehydrogenase	bovine	iodo 2-[^{14}C]acetyldiethylstil-bestrol	Cys-89	affinity label for estrogen binding-site	207
Glutamate dehydrogenase	bovine	1-oxyl-2,2,6,6-tetramethyl-4-oxypiperidine–$NaBH_4$	Lys-126	active site Lys	208
		N-(1-oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)iodoacetamide		several groups react; one not sulphydryl	
Glutaminase	rat	L-2-amino-4-oxo-5 -chloro 5-[^{14}C]-pentanoic acid		binds to glutamate specific site	209
Glutamine synthetase	*E. coli*	pyridoxal 5′-phosphate–$NaBH_4$	Lys	3 bound groups	210
Glutamate synthase	*Bacillus megaterium*	dimethylsuberimidate	Lys	cross-linked structures of 200 000 dalton formed	211

Glutamate synthase	*Bacillus megaterium*	[^{14}C]phenylglyoxal,butane-2,3-dione	Arg	Arg not essential	202
		pyridoxal 5′-phosphate	Lys	inactivation	
Glutamic acid decarboxylase	*E. coli*	*R*-(–)-4-aminohex-5-ynoic acid		stereospecific inhibition	212
γ-Glutamyl cyclotransferase	rat	DTT–iodoacetic acid	Cys	active modified enzyme isolated	213
γ-Glutamylcysteine synthetase	human	cystamine	Cys	inactivates	214
γ-Glutamyl transpeptidase	rat	phenylmethanesulphonyl fluoride		inactivates enzyme	215
γ-Glutamyl transpeptidase	rat	6-diazo-5-oxo-L-1,2,3,4,5-[^{14}C] or 6-[^{14}C]norleucine		isolated large subunit has active-site which is masked in native enzyme	216,217

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
γ-Glutamyl transpeptidase	rat	6-diazo-5-oxonorleucine, azaserine		protected by L-serine and borate	218
Glutathione peroxidase	rat	[^{14}C]iodoacetate, ethylenimine, iodoacetamide	SeCys	SeCys is in active site	219,220, 221
Glutathione reductase	human	ethoxyformic anhydride	His	essential His	222
Glutathione reductase	yeast	1-(2-chloroethyl)-3-(1-[^{14}C]-cyclohexyl)-1-nitrosourea, 1,3-bis(2-[^{14}C]-chloroethyl)-1-nitrosourea		stoicheiometric inactivation	223
Glyceraldehyde-3-phosphate dehydrogenase	rabbit	fluorescein isothiocyanate	Lys	evidence for interaction with aldolase	224
Glyceraldehyde-3-phosphate dehydrogenase	rabbit	phenanthrene 9,10-oxide, 4-carbo-t-butoxybenzene oxide, 4-carboxybenzene oxide	Cys	active-site reagents	225
Glyceraldehyde-3-phosphate dehydrogenase	rat	1-fluoro-2,4-dinitrobenzene	Cys	half-of-the-sites reactivity	226
Glyceraldehyde-3-phosphate dehydrogenase	rat	butane-2,3-dione	Arg	one essential Arg/subunit	227
Glyceraldehyde-3-phosphate dehydrogenase	spinach	tetrathionate, iodo[$^{14}C_2$]-acetic acid	Cys	2 Cys essential	228
Glyceraldehyde-3-phosphate dehydrogenase	yeast, sturgeon, rabbit	acetyl phosphate, pH8.5	Lys-183	Lys-183 in area of subunit contact	229
Glycerophosphate dehydrogenase	rabbit	diazotized-3-aminopyridine adenine dinucleotide	Cys	essential Cys labelled	230
Glycogen phosphorylase *b*	rabbit	P^1,P^2-bis(5′-pyridoxal) diphosphate/NaBH_4	Lys-573	one Schiff base not reduced	231
Glycohydrolase	calf	butane-2,3-dione Woodward's Reagent K	Arg carboxyl	essential Arg partial inactivation	232
Guanine deaminase	porcine	DTNB	Cys	essential Cys	233
Guanylate cyclase	rat	various sulphydryl reagents	Cys	evidence for vicinal dithiols	234
Haemoglobins	*Artemia salina*	iodoacetic acid	Cys	subunit determinations	235
Haemoglobin	human	2-methoxy-5-nitrotropone	α-NH_2	increased oxygen affinity	236

Haemoglobin	human	bromotrifluoroacetone–NaB^3H_4	Cys-β93	specific modification shown by mapping	237
Haemoglobin	human	*p*-mercuribenzoate	Cys	flash photolysis study	238
Haemoglobin	human	*N*-(1-oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)maleimide	Cys	librational motion of 'immobilized' label	239
Haemoglobin	human	*N*-(1-oxyl-2,2,6,6-tetramethyl piperidin-4-yl)iodoacetamide	Cys-β 93	study of tertiary structure variability	240
Haemoglobin	*Rana catesbeiana*	iodo[^{14}C]acetamide	Cys	β-chain Cys preferentially alkylated	241
Hexokinase PII	yeast	butane-2,3-dione, phenylglyoxal	Arg	4.2 Arg modified inactivates	242
High density lipoprotein	human	dimethylsuberimidate	Lys	state of self association studied	243

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Histamine *N*-methyl transferase	guinea pig	2′,3′-dialdehyde derivatives of *S*-adenosylhomocysteine and *S*-adenosylmethionine	Lys	affinity labels	244
Histidine decarboxylase	*Lactobacillus*	2-bromo 1-[^{14}C]acetamidopyridine		affinity label	245
Histone H3	calf	DTNB	Cys	rate of reaction studied	246,247
Histones	calf	1.hydrogen peroxide 2.iodo-acetamide	Cys	S–S bridges linking histones	248
Histones	chicken	formaldehyde	Lys	sedimentation study	249
Histones	rat	methylacetimidate, dimethyl-suberimidate, dimethyladipimate	Lys	monofunctional reagent cross-links protein	250
Histones	rat	photoirradiation		H2A and H2B preferentially cross-link to DNA	251
Histones	rat	dithiobis-succinimidyl propionate		cross-links identified	252
Histone H3	trout	*N*-ethyl[^{3}H]maleimide	Cys	reactivity used to probe structure	253
Homoserine dehydrogenase	*Rhodospirillum rubrum*	iodo 1-[^{14}C]acetate, *p*-chloro-mercuri[^{14}C]benzoate	Cys	essential Cys buried	254
Hypoxanthine–guanine phosphoribosyltransferase	human	dimethylsuberimidate, dimethyl-adipimidate, glutaraldehyde	Lys	tetramer?	255
ω-Hydroxyfatty acid: NADP oxidoreductase	*Solanum tuberosum*	phenylglyoxal	Arg	Arg essential for NADPH binding	256
		pyridoxal 5′-phosphate–$NaBH_4$	Lys	Lys essential for 16-oxohexadeca-noate binding	
Δ^5-3β-Hydroxysteroid dehydrogenase	bovine	various sulphydryl reagents	Cys	reactive thiol in vicinity of steroid binding-site?	257
20β-Hydroxysteroid dehydrogenase	*Streptomyces hydrogenans*	21-2′-[^{3}H]bromoacetylamino-progesterone	His	dicarboxymethyl His identified	258
Immunoglobulin M	mouse	iodo 2-[^{14}C]acetate	Cys	free sulphydryl	259
Initiation factor IF3	*E. coli*	fluorescein isothiocyanate	Lys,Cys	study of binding to 30*S* subunits	260
Initiation factors	rabbit, mouse	oxidized [^{3}H]methyl labelled mRNA–sodium cyanoboro-hydride	Lys	specific cross-linking	261
Interferon	human	^{125}I	Tyr	loss of activity	262
		[^{3}H]dansyl chloride	Lys	activity retained	
Interferon	human	*N*-ethylmaleimide	Cys	Cys not essential	263
		KI–chloramine T, tetranitro-methane	Tyr	Tyr associated with antiviral action	

Invertase	yeast	citraconic anhydride, methyl acetimidate	Lys	salt linkages involved in stabilizing enzyme	264
Isocitrate dehydrogenase	porcine	1-[^{14}C]ethylethoxyformic anhydride	His	essential His in nucleotide binding-site	265
Isocitrate dehydrogenase	porcine	butane-2,3-dione	Arg	2 Arg per subunit modified	266
Kallikrein	human	Ala-Phe-Arg-CH_2Cl, Pro-Phe-Arg-CH_2Cl	Ser	syntheses described	267
Keratin	wool	dimethylsuberimidate	Lys	subunit study	268
2-Keto-3-deoxygluconate-6-P aldolase	*Pseudomonas putida*	bromopyruvate	Glu	modified tryptic peptide sequenced	269
Laccase	*Rhus vernicifera*	2-hydroxy-5-nitrobenzyl bromide	Trp	unique Trp	270

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Lac repressor	*E. coli*	fluorescein mercuric acetate, 2-bromoacetamido-4-nitrophenol	Cys	fluorescent reporter group	271
α-Lactalbumin	bovine, human	2-hydroxy-5-nitrobenzyl bromide	His-32 Trp	chemistry of reaction studied	272
Lactose synthase	bovine	Sepharose-glutathione-2(5-nitropyridyl)disulphide	Cys	reactive thiol not at α-lact-albumin binding-site	273
Lactate dehydrogenase	porcine	*N*-(4-azidocarbonyl-3-hydroxy phenyl)maleimide	Cys-165, Lys-179	Cys-165 and Lys-179 connected *via* a hydrophobic channel	274
Lactate oxidase	*Mycobacterium smegmatis*	fluorodinitrobenzene	His,Lys	Lys protected by competitive inhibitors	275
β-Lactoglobulin	bovine	*N*-dansylaziridine	Cys	sulphydryl is electron donor?	276
Leghemoglobin *a*	soybean	mesohememonosulphuric anhydride	Lys	Lys-6,19 and 57 react	277
Ligandin	rat	succinic anhydride, dimethyl suberimidate	Lys	ligandin is a heterodimer	278
		iodoacetamide	Cys		
Lipoamide dehydrogenase	porcine	8-Cl-FAD	Cys	model reactions suggest Cys modified	279
Lipoprotein B	human	*N*-(1-oxyl-2,2,5,5-tetramethyl pyrrolidin-3-yl)maleimide	Cys	weak and tight binding-sites observed	280
Low density lipoprotein	human	^{125}I followed by succinylated [*U*-^{14}C]sucrose		new approach to assessing lysosomal degradation	281
Low density lipoprotein	human	various reagents	Lys, Cys	Lys not Cys essential for receptor binding	282
Luciferase	*Beneckea harveyi*	*N*-(1-pyrene)maleimide, *N*-*p*-(2-benzoxazolyl)phenyl maleimide	Cys	fluorescence studies indicate elongated molecule	283
Lutropin	ovine	4-fluoro-3-nitrophenylazide	Lys-β20	Lys-20 in interface of α and β	284
Lutropin	porcine, ovine	methylacetimidate	Lys	84 % loss of activity after 2 Lys modified	285
Lysosyme	chicken egg-white	*O*-methylisourea	Arg	^{13}C-n.m.r. study of carboxyls	286
Lysosyme	chicken egg-white	*N*-bromosuccinimide, iodine	Trp Tyr-23	n.m.r. studies	287,288
		4-chloro-7-nitro-benzofurazan			
Lysosyme	chicken egg-white	iodo-1-[^{14}C]acetic acid	Cys-6,127	disulphide equilibration study	289
Lysosyme	chicken egg-white	dithioerythritol	cystine	thermodynamic study	290
Malate dehydrogenase	*Mycobacterium*	*p*-chloromercuribenzoate	Cys	1 Cys per mole	291
Malate dehydrogenase	porcine	ethoxyformic anhydride	His	2 reactive His per dimer	292

L-Malate: NAD^+ oxido reductase	porcine	iodine, tetranitromethane	Tyr	2 out of 4 Tyr essential	293
Malic enzyme	pigeon	DTNB, *N*-ethylmaleimide, bromopyruvate	Cys	half-of-the-sites behaviour of affinity label	294
Malic enzyme	porcine	[^{3}H]-4,4′-bis(dimethylamino) diphenylcarbinol	Cys	essential Cys peptide isolated	295
α-Mannosidase	*Phaseolus vulgaris*	various reagents	Ser,Cys, Arg,Lys, Asp,Glu	essential carboxyl groups	296
α-Mannosidase	*Phaseolus vulgaris*	*N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide	Trp	essential Trp	297

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Membrane proteins	bovine adrenal	diazobenzenesulphonate		study of steroid hydroxylase complexes	298
Membrane proteins	bovine corpus luteum	dinitrofluorobenzene, acetic anhydride	Lys	study of gonadotropin receptors	299
		tetranitromethane	Tyr		
Membrane proteins	bovine heart	diazobenzene[^{35}S]sulphonate		labelled proteins identified	300
Membrane proteins	cat myelin	dinitrodifluorobenzene	Lys	model for protein dimer proposed	301
		NN'-phenylenedimaleimides	Cys		
Membrane proteins	*E. coli*	1,2-[^{14}C]glycyl-4-azido-2-nitro-L-Phe–photoirradiation		probe of dipeptide transport in intact cells	302
Membrane proteins	*E. coli*	diazinedicarboxylic acid bis (*NN*-dimethylamide), *N*-ethyl maleimide	Cys	study of transport systems	303
Membrane proteins	*E. coli*	*N*-(1-oxyl-2,2,5,5-tetramethyl pyrrolidin-3-yl)maleimide	Cys	specific labelling	304
Membrane proteins	*E. coli*	dansyl cadaverine–transglutaminase	Gln	nitrate reductase buried	305
Membrane proteins	fly mitochondria	*N*-[^{3}H]ethylmaleimide	Cys	inhibits phosphate transport	306
Membrane proteins	*E. coli*	$NaB^{3}H_4$, performic acid		allyl-lysine present in 2 proteins	307
Membrane proteins	hamster, guinea-pig	8-azido-ATP–photoirradiation		binding-site identified	308
Membrane proteins	hamster fibroblast	DTT	cystine	removes LETS protein	309
Membrane proteins	human erythrocytes	photoirradiation–protoporphyrin		His probably involved in cross-linking	310,311
Membrane proteins	human erythrocytes	sodium periodate		specific cross-linking of spectrin	312
Membrane proteins	human erythrocytes	maleimide and iodoacetamide spin labels	Cys	reappraisal of e.s.r. spectra	313
Membrane proteins	human erythrocytes	various sulphydryl reagents	Cys	selective alteration of deformability	314
Membrane proteins	human erythrocytes	photoirradiation–bilirubin		evidence for covalent cross-linking	315
		N-ethylmaleimide	Cys	which does not involve Cys	
Membrane proteins	human erythrocytes	hydrogen peroxide		oxidative stress in β-thalassemia results in cross-linking	316
Membrane proteins	human erythrocytres	3,4,5,6-tetrahydrophthalic anhydride	Lys	reversible modification	317
Membrane proteins	human erythrocytes	Cu^{2+}	Cys	oxidation	318

Membrane proteins	human erythrocytes	fluorescein isothiocyanate–photoirradiation	Lys,Cys	cross-linking	319
Membrane proteins	human erythrocytes	diazonium salt of oligodeoxythymidylic acid 5′-*p*-aminophenylphosphate		new reagent for surface labelling	320
Membrane proteins	human erythrocytes	[^{3}H]-*NNN*-trimethylamino-β-alanyl-*N*-hydroxysuccinimido ester	Lys	new cationic impermeable reagent	321
Membrane proteins	human erythrocytes	pyridoxal phosphate or galactose oxidase–NaB^3H_4,1-(*p*-benzenediazonium)EDTA–$^{111}In^{3+}$	Lys	three reagents compared	322

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Membrane proteins	human erythrocytes	4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid	Lys	Cl^- transport site identified	323,324
Membrane proteins	human lymphocytes	dansyl chloride	Lys	mitogen susceptibility retained	325
Membrane proteins	human placenta	*N*-bromoacetyl-*p*-azido-L-Phe methyl ester–photoirradiation	Cys	bifunctional reagent	326
Membrane proteins	human platelets	5′-*p*-fluorosulphonylbenzoyl-[^{3}H] adenosine		identification of adenine nucleotide binding proteins	327
Membrane proteins	lettuce	dimethyladipimidate	Lys	25 000 and 23 000 dalton polypeptides cross-linked	328
Membrane proteins	mouse synaptosomes	dimethyl-malonimidate, -adipimidate and -suberimidate	Lys	plasma membrane proteins mobile, synaptic junction proteins	329,330
		o-phenanthroline–Cu^{2+}	Cys	immobilized	
Membrane proteins	pigeon erythrocytes	cholera toxin–[^{32}P]NAD^+		ADP-ribosylation of GTP-binding protein	331
Membrane proteins	pigeon heart	[^{3}H]-2-nitro-4-azidocarbonyl cyanide phenylhydrazone–photo irradiation		80—85 % bound to a single peptide	332
Membrane proteins	porcine heart	DTNB	Cys	asymmetrical distribution of thiols involved in ATP–$^{32}P_i$ exchange	333
Membrane proteins	rabbit muscle	dithiobis (succinimidyl propionate)	Lys	effect of deoxycholate studied	334
Membrane proteins	rabbit muscle	dansyl chloride	Lys	conformational changes monitored	335
Membrane proteins	rabbit muscle	DTNB	Cys	effect of cations and nucleotides studied	336
Membrane proteins	rabbit erythrocytes	^{125}I–lactoperoxidase	Tyr	Con A receptor is a transmembrane protein	337
		4,4′-dithiobisphenylazide		components 6 and 7 cross-linked	
Membrane proteins	rat adipocytes	*N*-hydroxysuccinimide ester of 4-azidobenzoic acid–photoirradiation		labelling of insulin receptor?	338
Membrane proteins	rat erythrocytes	protein methylase II–S-adenosyl-L-[*methyl*-^{14}C]methionine	carboxyl	bands 3,4,4.5 selectively esterified	339
Membrane proteins	rat liver	diazobenzene sulphonate		investigation of transverse topology	340,341
Membrane proteins	rat liver	[^{125}I]insulin–glutaraldehyde–$NaBH_4$	Lys	affinity cross-linking	342

Membrane proteins	rat liver	*N*-ethylmaleimide	Cys	Cys in triethyl tin site	343
Membrane proteins	rat liver	fluorescamine	Lys	energization of membrane exposes Lys	344
Membrane proteins	spinach chloroplasts	[^{3}H]acetic anhydride iodo[^{3}H]acetic acid	Lys Cys	study of electron transport	345
Membrane proteins	squid axon	glyoxal, phenylglyoxal, butane-2,3-dione	Arg	sodium channel inactivation removed	346
Membrane proteins	*Torpedo marmorata*	4-(*N*-maleimido)phenyl[^{3}H]trimethyl ammonium	Cys	affinity label	347
Meromyosin	rabbit muscle	iodo[^{14}C]acetamide, [^{14}C]-*N*-ethylmaleimide	Cys	2 essential thiols in 20 000 dalton segment	348

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Meromyosin	rabbit muscle	*N*-(1-oxyl-2,2,6,6-tetramethyl piperidin-4-yl)iodoacetamide, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid	Cys-1	ATPase activity affected	349
Meromyosin	rabbit muscle	(*N*-iodoacetyl-*N'*-1-sulpho-5-naphthyl)ethylenediamine	Cys	binding to actin studied	350
Microtubule proteins	porcine	*N*-ethyl[^{14}C]maleimide, 2-amino-6-(*S*-dinitrophenyl) thiopurineriboside triphosphate	Cys	2 Cys at GTP binding-site modified	351
Monoamine oxidase		*N*-ethyl[1,4-^{14}C]maleimide	Cys	unique Cys	352
arom Multienzyme complex	*Neurospora crassa*	iodo[^{14}C]acetic acid	Cys,Met	subunit study	353
Myelin basic protein	bovine	[^{13}C]methyl iodide	Met	^{13}C-n.m.r. study	354
Myoglobin	sperm whale	methyl acetimidate	Lys	modified protein characterized	355
Myoglobin	sperm whale	4-isothiocyanate-2,2,6,6-tetramethylpiperidino-oxyl	α-NH_2	label for probing helix A	356
Myosin	rabbit muscle	6-thio[^{14}C]cyanato-9-β-D-ribofuranosylpurine	Cys	sulphydryls not essential for ATPase activity	357
Myosin	invertebrate	various spin labels	Cys	study of calcium switch	358
Myosin		dimethylsuberimidate, methyl acetimidate	Lys	effect of pH on cross-bridging studied	359
Myosin	rabbit muscle	*N*-ethyl[1-^{14}C]maleimide, iodo-[1-^{14}C]acetamide	Cys	2 essential thiols in heavy chain	360
Neuraminidase	*Arthrobacter*	various reagents		essential Trp	361
Neurophysins	bovine	dimethylsuberimidate	Lys	covalent dimers do not bind hormones	362
		tetranitromethane	Tyr-49	not involved in high-affinity binding	
Neocarzinostatin	*Streptomyces carzino staticus*	performic acid	cystine	prevents cleavage of DNA	363
Nitrate reductase	*Neurospora crassa*	phenylglyoxal	Arg	Arc, Cys in binding site of NADPH or FAD?	364
		p-hydroxymercuribenzoate	Cys		
5'-Nucleotide phosphodiesterase	calf	3',5'-[^{3}H]cAMP		covalent intermediate isolated	365
Nucleoside diphosphatase	rat	pyridoxal 5'-phosphate–$NaBH_4$	Lys	N^6-pyridoxyl-lysine isolated	366

Nuclear envelope proteins	chicken erythrocytes	*o*-phenanthroline–Cu^{2+}	Cys	homogeneous oxidation products	367
L-Ornithine: 2-oxoacid aminotransferase	rat	4-aminohex-5-ynoic acid, 5-amino-cyclohexa-1,3-dienylcarboxylic acid		suicide substrates	368
Ovalbumin		various anhydrides	Lys	immunological activity study	369
Papain	papaya latex	$TosLysCH_2Cl$	Ser	cryoenzymatic studies of	370
		methyl methanethiolsulphonate	Cys-25	mechanism	
Papain	papaya latex	substituted benzoylimidazoles	Cys	study of catalysis	371
Papain	papaya latex	substituted imidazoles	Cys	resonance Raman study	372
Papain	papaya latex	various sulphydryl reagents	Cys	evidence for multiple forms	373
Papain	papaya latex	2,2′-dipyridyldisulphide, n-propyl 2-pyridyl disulphide	Cys	reactivity probes	374
Parathyroid hormone	human	cyclohexane-1,2-dione	Arg-20, 25	activity reduced to 16 %	375

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Penicillinase	*Staphylococcus aureus*	tetranitromethane	Tyr-72	preferential nitration	376
Phenol-sulphotransferase	rat	dialdehyde of ADP	Lys	protected by 3′-phosphoadenosine-5′-phosphosulphate	377
Phenylalanyl-tRNA synthetase	yeast	ethoxyformic anhydride, photoirradiation–Rose Bengal	His	4 His involved in acylation	378
Phosphate translocator	spinach	trinitrobenzenesulphonate	Lys	Lys at substrate binding-site	379
Phosphatidylserine decarboxylase	*E. coli*	NaB^3H_4		essential bound pyruvate	380
Phosphoenolpyruvate carboxykinase	rat	various sulphydryl reagents	Cys	*vicinal* dithiol has essential Cys	381
Phosphofructokinase	ovine	8-[*m*-(*m*-fluorosulphonylbenzamido)benzylthio]adenine		affinity label for Amp, ADP binding-site	382
Phosphofrucktokinase	ovine	5′-*p*-fluorosulphonylbenzoyladenosine		affinity label for AMP, ADP	383
Phosphofructokinase	rabbit	5′-*p*-fluorosulphonylbenzoyladenosine		affinity label	384
6-Phosphogluconate dehydrogenase	*Candida utilis*	DTNB	Cys	thiols at active site close in space	385
6-Phosphogluconate dehydrogenase	*Candida utilis*	dansyl chloride	Cys,Tyr	pH-dependent reactivities	386
6-Phosphogluconate dehydrogenase	*Candida utilis*	various reagents		Arg,His,Cys,Lys modified	387
6-Phosphogluconate dehydrogenase	*Candida utilis*	3-chloroacetylpyridine-ADP^+ and -AD^+	Cys	first is affinity label, second is a second order reagent	388
D-3-Phosphoglycerate dehydrogenase	chicken	*p*-hydroxymercuribenzoate	Cys	12 free thiols per subunit	389
Phosphoglycerate kinase	yeast	2-chloromercuri-4-nitrophenol, DTNB	Cys	study of anion binding	390
Phosphoglycerate kinase	yeast	2-nitro-5-thiocyanobenzoic acid	Cys	Cys near position 100	391
Phosphoglycerate kinase	yeast	phenylglyoxal	Arg	2 essential Arg	392
Phosphoglycolate phosphatase	tobacco	*N*-ethylmaleimide	Cys	slow inactivation	393
Phospholipase A_2	bee venom	imidazolide derivatives of various fatty acids	Tyr?	oleoyl imidazolide is potent activator	394
Phospholipase A_2	bovine	^{125}I	Tyr-69	labelled peptide isolated	395
Phosphoribosylpyrophosphate synthetase	*Salmonella typhimurium*	pyridoxal 5′-phosphate–$NaBH_4$, trinitrobenzenesulphonate	Lys	first reagent specific	396

Phosphorylase b	rabbit	glycogen–cyanogen bromide		study of bound enzyme	397
Phosphorylase b	rabbit	potassium ferrate	Tyr-75	modified tryptic peptide sequenced	398
Phosphorylase b	rabbit	*N*-(1-oxyl-2,2,5,5-tetramethyl pyrrolidin-3-yl)iodoacetamide	Cys	properties of monomer studied	399
Porphobilinogen deaminase	wheat germ	butane-2,3-dione	Arg	inhibition of enzyme	400
Progesterone receptor	hen oviduct	pyridoxal 5′-phosphate–$NaBH_4$	Lys	critical Lys at nucleotide binding-site	401
Proline reductase	*Clostridium sticklandii*	NaB^3H_4		study of covalently bound pyruvate	402
Prostaglandin synthetase	bovine	[*acetyl*-^{3}H]aspirin	α-NH_2	*N*-acetylserine identified	403

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Prostatic acid phosphatase	human	butane-2,3-dione, cyclohexane-1,2-dione	Arg	essential Arg	404
Prostatic acid phosphatase	human	ethoxyformic anhydride, *p*-nitrophenyl ^{32}P phosphate	His	essential His	405,411
S-100 Protein	bovine brain	formaldehyde–NaB^3H_4	Lys	preparation of hot active protein	406
Protein kinase	*Bombyx mori*	*N*-ethylmaleimide, *p*-chloromercuribenzoic acid	Cys	essential Cys	407
Protein kinase	bovine heart	DTNB	Cys	study of subunit interactions	408
Protein kinase	bovine lung	butane-2,3-dione	Arg	Arg plays role in inhibiting activity?	409
Protein kinase	calf artery	8-azidoinosine 3′:5′-[^{32}P]monophosphate–photoirradiation		photoaffinity label for cGMP binding-site	410
Proteoglycan	porcine	iodoacetic acid	Cys	inhibition of multimerization	412
Purine nucleoside phosphorylase	calf, human	butane-2,3-dione, phenylglyoxal	Arg	1 essential Arg	413
Purple membrane	*Halobacterium halobium*	dimethyl-3,3′-dithiobispropionimidate, dimethylsuberimidate, tartryldiazide	Lys	selective formation of trimers	414
Purple membrane	*Halobacterium halobium*	biotin *N*-hydroxysuccinimide ester	Lys	extracellular surface labelling	415
Pyridine dinucleotide transhydrogenase	bovine	various sulphydryl reagents	Cys	2 classes of sulphydryls	416
Pyridoxamine-pyruvate transaminase	*Pseudomonas*	pyridoxal 5′-phosphate–$NaBH_4$	Lys	sequence of binding-site established	417
Pyruvate dehydrogenase	bovine	pyruvate dehydrogenase kinase–[γ-^{32}P]ATP	Ser	phosphopeptides sequenced	418
Pyruvate kinase	rabbit	[*Me*-^{3}H]methylmethanethiosulphonate	Cys	all thiols equally accessible	419
Pyruvate kinase	rabbit	iodoacetamide	Cys	inactivates decarboxylase	420
		trinitrobenzenesulphonate	Lys	inactivates kinase	
		dithionitrobenzoate	Cys	inactivates kinase *and* decarboxylase	
Pyruvate, phosphate dikinase	*Bacteroides symbiosus*	ethoxyformic anhydride	His	essential His	421,422
		bromo[3-$^3H_2$]pyruvate, DTNB	Cys	*S*-carboxyhydroxyethylcysteine identified	
Quinate dehydrogenase	*Neurospora crassa*	various sulphydryl reagents	Cys	essential Cys not in active site	423

Receptor protein (cAMP)	*E. coli*	DTNB	Cys	2 accessible Cys	424
Rhodanese	bovine	iodoacetate, phenylglyoxal	Cys	novel reaction of phenylglyoxal	425
Rhodopsin	bovine	transgluaminase–dansyl cadaverine or putrescine	Gln	specific labelling	426
Rhodopsin	bovine	*N*-[^{3}H]ethylmaleimide, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)maleimide	Cys	distribution of sulphydryls determined	427
Rhodopsin	bovine	^{125}I *N*-(*p*-hydroxyphenylethyl)-maleimide	Tyr Cys	polypeptide folding in membrane discussed	428
Rhodopsin	bovine	maleimido spin label	Cys	study of protein–lipid interaction	429

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Rhodopsin	bovine	DTNB	Cys	reactivity as structural probe	430
Ribonuclease	bovine	bromo[$^{13}C_1$]acetate	His-119	n.m.r. study	431
Ribonuclease A	bovine	phthalyl-4-isothiocyanate	α-NH_2	binding-site for lanthanides	432
Ribonuclease A	bovine	1. *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl)bromoacetamide 2. performic acid, iodoacetate	1. His 2. Cys	spin label used to assess conformational change on chemical modification	433
Ribonuclease	bovine	potassium cyanate HCl	Lys Gln,Asn	nature of allosteric interactions studied	434
Ribonuclease A	bovine	diazonium salt of 5′-(4-aminophenylphosphoryl)-uridine 2′(3′)-phosphate	Tyr	specific modification of Tyr-73	435
Ribonuclease A	bovine	methanol–HCl	Glu-49, Asp-53	dimethyl ester isolated	436
Ribonuclease T_1	*Aspergillus oryzae*	photoirradiation–Methylene Blue	His	photoxidized His-27 identified	437
Ribosomal protein L12	*E. coli*	hydrogen peroxide	Met	decreases activity	438
Ribosomal protein S1	*E. coli*	formaldehyde–NaB^3H_4	Lys	6 out of 30 Lys modified	439
Ribosomal protein S4	*E. coli*	2-nitro-5-thiocyanobenzoic acid	Cys-31	removal of residues 1—30	440
Ribosomes	*Bacillus stearothermophilus*	various reagents	His,Cys Trp,Tyr	study of peptidyltransferase site	441
Ribosomes	*E. coli*	*N*-bromosuccinimide	Cys	stimulation of polymerization	442
Ribosomes	*E. coli*	^{125}I	Tyr	conformational study	443
Ribosomes	*E. coli*	succinic, maleic, and acetic anhydrides	Lys	inactivation of polyPhe synthesis	444
Ribosomes	*E. coli*	[^{14}C]spermine–1,5-difluoro-2,4-dinitrobenzene	Lys	labels protein associated with rRNA	445,446
Ribosomes	*E. coli*	methyl 4-mercaptobutyrimidate	Lys,Cys	cross-linked protein pairs	447,448
Ribosomes	*E. coli*	2-nitro-5-thiocyanobenzoic acid	Cys	functional cleaved fragment	449
Ribosomes	*E. coli*	*N*-(1-oxyl-2,2,6,6-tetramethyl-piperidin-4-yl)-maleimide or -iodoacetamide	Cys	study of ligand binding	450
Ribosomes	*E. coli*	ethoxyformic anhydride	His	His involved in peptidyl transferase activity	451

Ribosomes	*E. coli*	cyclohexane-1,2-dione	Arg-73	*N*-terminal sequence of L7/L12 is responsible for its dimerization	452
		dimethylsuberimidate	Lys		
		hydrogen peroxide	Met		
Ribosomes	*E. coli*	diepoxybutane	Cys,Lys	reacts with both RNA and protein	453
Ribosomes	*E. coli*	various reagents		Lys plays role in mRNA binding?	454
Ribosomes	*E. coli*	tetranitromethane	Tyr	S1, S11, S21 modified	455
Ribosomes	*E. coli*	photoirradiation		sites of cross-linking identified	456,457
Ribosomes	*E. coli*	photoirradiation–[^{3}H]poly(U)		specific reaction	458

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ribosomes	*E. coli*	photoirradiation–Methylene Blue		S6 protein first affected	459
Ribosomes	*E. coli*	photoirradiation–Methylene Blue–oxygen		S3 and S5 newly identified as cross-linked to RNA	460
Ribosomes	*E. coli*	[^{3}H]-P^1-(3-azido-6-hydroxymethyl-phenyl)-P^3-(5′-guanosyl)tri-phosphate–photoirradiation		labelling of L11	461
Ribosomes	*E. coli*	N^6-(2-nitro-4-azidophenyl)-L-lysinylpuromycin aminonucleoside–photoirradiation		photoaffinity label for peptidyl transferase site	462
Ribosomes	*E. coli*	puromycin–photoirradiation		affinity labelling	463
Ribosomes	*E. coli*	(1*R*,2*R*)-1-(4-azidophenyl)-2-dichloroacetamidopropane-1,3-diol–photoirradiation		labelling is non-specific	464
Ribosomes	*E. coli*	*p*-azidobenzoyl-[^{3}H]-glycyl-hydrazide derivative of oligo-adenylic acids–photoirradiation		50*S* subunit not labelled	465
Ribosomes	*E. coli*	5′-{4-bromo[2-^{14}C]acetamido (phenyl)phospho}adenylyl-(3′,5′)uridylyl-(3′,5′)guanosine	Cys	linked to Cys-10 of S18	466
Ribosomes	*E. coli*	6-dimethylamino-9-[3-(*p*-azido-L-*β*-phenylalanylamino)-3-deoxy-*β*-ribofuranosyl]purine–photoirradiation		affinity label for ribosomal A site	467,468
Ribosomes	HeLa cells	1. sodium periodate 2. $NaBH_4$	Lys	S3a linked to 18*S* RNA; L3 to 28*S* RNA; L7′, L23′ to 5.8*S* RNA	469
Ribosomes	rat	[^{14}C]formaldehyde–NaB^3H_4	Lys	optimal labelling studied	470
Ribosomes	rat	photoirradiation		cross-linking to 18S RNA	471
Ribulose bisphosphate carboxylase	barley, *P. oxalaticus*	butane-2,3-dione	Arg	pseudo-first order inactivation	472
Ribulose bisphosphate carboxylase	*pea*	tetranitromethane	Tyr		
		dimethyl suberimidate, dimethyl adipimidate, methyl 4-mercaptobutyrimidate	Lys	small subunits arranged at least in pairs	473

Ribulose bisphosphate carboxylase	*Rhodospirillum rubrum*	pyridoxal 5′-phosphate–NaB^3H_4	Lys	essential Lys	474
Ribulose bisphosphate carboxylase	*Rhodospirillum rubrum*	[^{14}C]phenylglyoxal	Arg	2—3 Arg modified inactivates	475
Ribulose bisphosphate carboxylase/oxygenase	spinach	*N*-bromoacetylethanolamine phosphate	Lys,Cys	affinity reagent	476
Ribulose bisphosphate carboxylase/oxygenase	spinach	pyridoxal 5′-phosphate–NaB^3H_4 iodoacetamide, *p*-chloro-mercuribenzoic acid	Lys Cys	all Lys in large subunit inhibition	477
Ribulose bisphosphate carboxylase/oxygenase	spinach	3-bromo-1,4-dihydroxy butan-2-one 1,4-bisphosphate	Lys	labelled tryptic peptides sequenced	478
Ribulose bisphosphate carboxylase	tobacco	butane-2,3-dione	Arg	essential Arg	479
Ribulose bisphosphate carboxylase	tobacco	potassium[^{14}C]cyanate	Lys	essential Lys	480

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ricin	*Ricinus communis*	2-mercaptoethanol–iodoacetamide	cystine	essential disulphide	481
RNA polymerase	*Bacillus subtilis*	3-(2-bromoacetamidoethyl) and 3-(2-acetamidoethyl)-thio-rifamycin		affinity labels	482
RNA polymerase	*E. coli*	photoirradiation		complex with DNA studied	483
RNA polymerase	*E. coli*	photoirradiation–T7 DNA		β'subunit not cross-linked	484
RNA polymerase	*E. coli*	periodate oxidized nucleoside triphosphates–$NaBH_4$	Lys	affinity labels	485
Saccharopine dehydro-genase	yeast	iodoacetate, *p*-chloromercuri-benzoate	Cys	protection by co-enzyme	486
Sialyltransferase	human	iodoacetate, *p*-chloromercuri-benzoyl sulphonate	Cys	authors claim Met reaction	487
Somatotropin	human	iodo 1-[^{14}C]acetamide	Cys	study of dissociation	488
Spinin	marine bacterium	dimethylsuberimidate, glutaraldehyde	Lys	sedimentation study	489
Spectrin	bovine	nickel sulphate–sodium glyoxylate	α-NH_2	estimation of amino terminus	490
Stearylcoenzyme A desaturase	rat	various reagents	Arg,Tyr	Arg in CoA binding-site? Tyr involved in iron chelation?	491
Succinylcoenzyme A synthetase	porcine, *E. coli*	oxidized CoA disulphide	Cys	affinity label	492
Sulphotransferase	rat	iodoacetate	Cys	essential Cys?	493
Superoxide dismutase	*S. faecalis, E. coli*	[^{14}C]streptomycin		3.2 molecules bound	494
Superoxide dismutase	human	various sulphydryl reagents	Cys	2 reactive thiols	495
Thermolysin		*N*-[1-^{14}C]chloroacetyl-DL-*N*-hydroxy-Leu-methyl ester, 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile	Glu-143	affinity labels for metal	496
Thermolysin	*Bacillus thermor-proteolyticus*	mixed anhydrides of tetradecanoic acid analogues	Lys	6—7 acyl groups per molecule	497
Thrombin	*bovine*	cylcohexane-1,2-dione	Arg	decreases heparin sensitivity	498
Thrombin	*human*	cylcohexane-1,2-dione di-isopropylfluorophosphate	Arg Ser	interaction with heparin studied	499
Thymidylate synthetase	*Lactobacillus casei*	butane-2,3-dione	Arg	essential Arg	500

Thymidylate synthetase	*Lactobacillus casei*	methyl methanethiolsulphonate, DTNB	Cys	general base activation of essential Cys	501,502
Thymidylate synthetase	*Lactobacillus casei*	5-nitro-2′-deoxyuridine 5′-monophosphate		irreversible inhibitor	503
Toxin I	*Androctonus australis*	iodo[^{14}C]acetate	Lys-56	labelled chymotryptic peptide isolated	504
26*S*-Transcarboxylase	*Propionibacterium shermanii*	*p*-azido[^{14}C]benzoyl CoA–photoirradiation		12 CoA ester sites observed	505
Transcortin	human	DTNB, ethyleneimine	Cys	accessible Cys partially oxidized	506
Transferrins	chicken, human	phenylglyoxal, butane-2,3-dione, cyclohexane-1,2-dione	Arg	1 critical Arg per iron binding site	507

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Table 1 (*cont.*)

Protein	*Source*		*Residue*	*Comments*	*Ref.*
Trehalose-phosphate synthase	*Mycobacterium smegmatis*	ethoxyformic anhydride acetic anhydride cyclohexane-1,2-dione	His Lys Arg	His, Lys, Arg involved in binding of heparin	508
Tropomyosin	rabbit	DTNB, iodoacetamide	Cys	effects of disulphide bond on structure studied	509
Tropomyosin	rabbit	*N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine	Cys	study of interaction with troponin and actin	510
Tropomyosin	rabbit	*N*-(1-pyrene)maleimide	Cys-190	probe for proximal thiols	511
Troponin C	rabbit, dog	dansylaziridine	Met-25	fluorescent probe for Ca^{2+} binding	512,513
Troponin C	rabbit	[^{3}H]ethanolamine–Ca^{2+}	carboxyl	Ca^{2+} binding-sites identified	514
Trypsin	bovine	DTT, cysteine, mercaptoethanol	Cys	inhibition	515
Trypsin	bovine	*m*- and *p*-azidobenzamidine–photoirradiation		photoaffinity analogues of Lys and Arg	516
Trypsin inhibitor	bovine	CNBr	Met-52	converted to Ser	517
Tryptophan synthase	*E. coli*	nitrothiocyanobenzoic acid	Cys	identification of essential Cys	518
Tryptophan synthase	*E. coli*	bromo[^{14}C]acetylpyridoxamine	Cys	new affinity-labels	519
Tryptophanyl-tRNA synthetase	bovine	ethoxyformic anhydride	His	essential His	520
Tubulin	human	sulphydryl reagents	Cys	polymerization affected	521
Tyrosinase	*Neurospora*	2-mercaptoethanol	Cys	e.s.r. study	522
Tyrosine aminotransferase	guinea-pig	sulphydryl reagents	Cys	study of inactivation	523
Tyrosyl-tRNA synthetase	*Bacillus stearothermophilus*	[^{3}H] and [^{14}C]acetic anhydride	Lys	competitive labelling	524
UDP galactose: lipopolysaccharide α,3-galactosyl transferase	*Salmonella typhimurium*	pyridoxal 5'-phosphate–NaB^3H_4	Lys	fluorescence study	525
UDP glucose dehydrogenase	bovine	6,6'-dithiodinicotinamide	Cys	half-sites oxidation	526
Ubiquinone cytochrome *c* reductase	bovine	disuccinimidyl tartarate, *NN'*-bis(3-succinimidyloxycarbonylpropyl)tartaramide	Lys	novel cleavable cross-linkers	527
β-D-Xylosidase	*Bacillus pumilus*	sulphydryl reagents	Cys	essential Cys	528

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1 Re-investigation of Known Reagents and Reactions

Phenylmercuric Acetate.—This reagent reversibly 'de-greens' butyryl-coenzyme A dehydrogenase by abolishing the absorption band at 710 nm. The reaction has been investigated in detail[529] and the authors conclude that the bleaching is not a result of modification of a protein thiol as was previously believed, but is probably due to the chemical modification of the tightly bound acyl-coenzyme A molecule.

Glyoxal.—The reversible modification of arginine with glyoxal has been assessed[530] for use in polypeptide semi-synthesis. Conditions are given for the regeneration of free arginine with *o*-phenylenediamine and it is concluded that glyoxal and cyclohexanedione could be complementary for reversible protein modification.

Methyl Acetimidate.—The effects of pH, reagent concentration, temperature, and reaction time on the reaction of methyl acetimidate with sperm whale myoglobin have been reported.[531] Reaction with a 6-fold excess of acetimidate relative to each amino-group at pH 9.8, 15 °C, for 30 min yielded six acetamidomyoglobin derivatives which were separated and purified.

2,3-Dioxo-5-indoline Sulphonic Acid.—This reagent is claimed[532] to modify Trp-123 of lysosyme. However, in a reinvestigation of the reaction[533] it has been demonstrated that no covalent adduct is produced.

2-Hydroxy-5-nitrobenzyl Bromide.—This tryptophan-modifying reagent has been reported to react with His-32 of α-lactalbumin. This result has been confirmed in the new study[534] and the modified histidine has been characterized. The reaction requires the native conformation of the protein and is restricted to position 1 of the imidazole.

β-Elimination of β-Halo Substrates by D-Amino-acid Transaminase.—Evidence is presented[535] for the involvement of an aminoacrylate–Schiff base intermediate in the β-elimination of β-chloro-D-alanine which leads to inactivation of the enzyme.

Halogenation.—A comparison has been made of the radioiodination of guinea-pig serum lipoproteins using ICl and chloramine-T.[536] It was concluded that the products of each method of iodination are equally acceptable provided that optimum conditions for the chloramine-T reaction are carefully established. The sites of direct and indirect halogenation of albumin have been established[537] by pronase digestion and amino-acid analysis. For optimum labelling the authors recommend the use of radioiodinated *N*-succinimidyl-3-

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(4-hydroxyphenyl)-propionate provided the protein does not contain essential lysine residues.

Sulphonyl Fluorides.—The selectivity and reactivity of a variety of sulphonyl fluorides towards several closely related serine proteases have been reported.[538]

Cupric–*o*-Phenanthroline Complex.—The quaternary structure of the Na^+, K^+–ATPase has been probed using this complex for cross-linking the enzyme subunits. In a re-investigation of the reaction it has been demonstrated[539] that the same products (α,α dimer, α,β dimer, and higher mol. wt. aggregates) are also obtained when the enzyme is exposed to Cu^{2+} alone. In addition the α,β dimer is *not* covalently cross-linked since it is readily dissociated in the presence of EDTA. The findings suggest that other results of 'cross-linking' with the Cu^{2+}–*o*-phenanthroline complex should be treated with caution. Since the α,α dimer appears to be covalently cross-linked, the results do not negate Cu^{2+}-induced S–S bridging and indeed evidence for direct oxidation of human erythrocyte membrane thiols by copper has been reported.[540]

3-Bromo-1,1,1-trifluoropropanone.—Methods of quantitation of the extent of sulphydryl conversion to trifluoroacetonyl derivatives have been described.[541] The derivatives were first stabilized by borohydride reduction and then quantitated by amino-acid analysis.

2 Synthetic Procedures for Radiolabelled Reagents

The discovery that 8-azido ATP could be used as a photoaffinity label opened up a whole new area of endeavour in photoaffinity labelling and comparable analogs of cAMP, NAD^+, FAD, co-enzyme A and GTP have now been synthesized and used in a variety of labelling studies. It is essential for such work to have appropriately labelled radioactive analogues and a step in this direction has been made with the report[542] of the microsynthesis of 8-[2-^{3}H]azido adenosine nucleotides of high specific activity in overall radiochemical yields of 30—40 %.

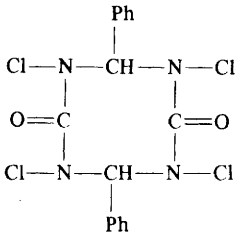
p-Fluorosulphonylbenzoyl-5′-adenosine is an affinity label for enzymes that bind adenine nucleotides. A procedure has been reported for the synthesis of this reagent with ^{14}C in the benzoyl moiety.[543] An overall yield of 25—30 % of radiolabelled product was obtained from 1 mmol of *p*-amino-[^{14}C]benzoic acid after a four step synthetic route.

A simplified route to [^{14}C]dimethyl suberimidate has been described.[544] The method involves the synthesis of [^{14}C]suberonitrile by phase transfer catalysis using an 18-crown-6-ether. The reagent is produced with a high specific activity and this should facilitate the detection of cross-linked species from limited amounts of protein and the interpretation of complex maps.

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[541] W.E. Brown and K.B. Seamon, *Analyt. Biochem.*, 1978, **87**, 211.
[542] K.-S. Boos, R. Bridenbaugh, R. Ronald, and R.G. Yount, *F.E.B.S. Letters*, 1978, **91**, 285.
[543] F.S. Esch and W.S. Allison, *Analyt. Biochem.*, 1978, **84**, 642.
[544] L.C. Packman and D.J.H. Smith, *F.E.B.S. Letters*, 1978, **91**, 178.

3 New Reagents and Techniques

The well established methods of cell-surface iodination using lactoperoxidase or chloramine-T suffer from a number of disadvantages not the least of which is their potential for membrane damage. To overcome these problems other approaches to surface labelling have been explored. An interesting 'solid-phase' method has been reported[545] in which films of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (1) react with aqueous mixtures of I^- and proteins or membranes to yield iodinated species. The stability of the reagent and its sparing solubility in water account for the minimal damage to proteins and living cells. The method has been successfully applied to the labelling of erythrocytes and the envelope proteins of two paramyxoviruses[546] and these workers[546] fully endorse the conclusions of Fraker and Speck[545] concerning the effectiveness, simplicity of use, and versatility of chloroglycoluril.



(1)

The fluorescent reporter-group, fluorescein isothiocyanate, has been radio-iodinated with ^{125}I in a lactoperoxidase-catalysed reaction[547] and used as a reagent for labelling proteins and cells to a high specific radioactivity. The authors suggest that this label would be useful for studies in which it is not convenient to measure fluorescence intensity.

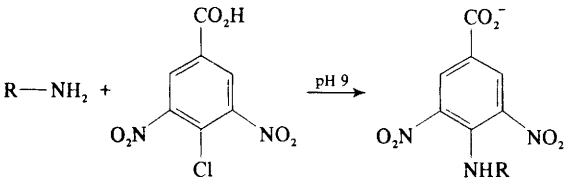
5-[^{125}I]Iodonaphthyl azide has been synthesized and used to photolabel rabbit-muscle sarcoplasmic reticulum.[548] In earlier studies [^{3}H]-1-azidonaphthalene and 4-[^{125}I]iodobenzene 1-azide have been used but these reagents have a low specific activity, do not store well, and have an absorption maximum close to that of membranes. The new reagent overcomes these problems. To complement the anionic cell-surface reagents which are available, the cationic species [^{3}H]-labelled *NNN*-trimethylamino-β-alanyl-*N*-hydroxysuccinimido ester has been synthesized and shown to react under mild physiological conditions with human ghosts and intact erythrocytes.[549] This reagent could be useful for the labelling of nerve cells where cationic ligands are thought to play an important role in triggering the excitable membranes.

The coupling of reactive functional groups including mercury and diazonium ions to dextran has been investigated[550] with the aim of preparing

[545] P.J. Fraker and J.C. Speck, *Biochem. Biophys. Res. Comm.*, 1978, **80**, 849.
[546] M.A.K. Markwell and C.F. Fox, *Biochemistry*, 1978, **17**, 4807.
[547] C.A. Gabel and B.M. Shapiro, *Analyt. Biochem.*, 1978, **86**, 396.
[548] T. Bercovici and C. Gitler, *Biochemistry*, 1978, **17**, 1484.
[549] N. Zisapel and U.Z. Littauer, *Biochim. Biophys. Acta*, 1978, **512**, 156.
[550] J. Pitha, *European J. Biochem.*, 1978, **82**, 285.

true surface-specific reagents. A preliminary exploration of the potential of phenylisothiocyanate as a specific hydrophobic membrane label has been reported.[551] The reagent overcomes some of the disadvantages of hydrophobic azides associated with their low degree of selectivity.

The EDTA derivative 1-(*p*-benzenediazonium)ethylenediamine tetra-acetic acid has been used together with $^{111}In^{3+}$ as an impermeant metal chelating probe of erythrocyte and platelet surfaces.[552] A variety of metals can be used in place of $^{111}In^{3+}$, thus permitting the chelation of various reporter groups to cell membranes.

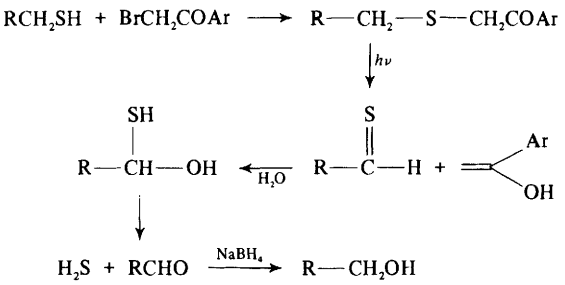


Scheme 1

A general strategy for the isolation and characterization of cell-surface components has been proposed.[553] The method involves the covalent attachment of the diazonium salt of oligodeoxythymidylic acid 5′-*p*-aminophenylphosphate to the surface of the intact cell, the enzymatic extension of the oligomer, and then hybridization to columns of polyriboadenylic acid-agarose. An important aspect of the procedure is its applicability to SDS solutions. A new protein arylating reagent, 4-chloro-3,5-dinitrobenzoate, which reacts with ε-NH_2 to give negatively charged derivatives (Scheme 1) has been used to probe the binding domains of cytochrome *c*.[554] The formation of a coloured derivative facilitates peptide mapping and the charge reversal enabled the cation exchange separation of a set of singly modified cytochromes which were well suited to the study of electron-transfer activity. A series of bromoacetamido-substituted acids were synthesized and evaluated as active-site-directed inactivators of liver-alcohol dehydrogenase.[555] 4-(*p*-Bromoacetamidophenyl)butyric acid and its amide were shown to be ambivalent inactivators whose reaction specificity was determined by the co-enzyme. A general procedure for the synthesis of chloromethyl ketone derivatives of fatty acids has been described.[556] The reagents were used as specific inhibitors of acetoacetyl–co-enzyme A thiolase, cholesterol biosynthesis, and fatty acid synthesis.

The novel conversion of the active-site cysteine of papain into serine has been achieved photochemically after reaction with 2-bromo-2′,4′-dimethoxy-

[551] H. Sigrist and P. Zahler, *F.E.B.S. Letters*, 1978, **95**, 116.
[552] K.J. Hwang and A.W. Wase, *Biochim. Biophys. Acta*, 1978, **512**, 54.
[553] L.P. Wennogle and H.C. Berg, *J. Mol. Biol.*, 1978, **123**, 471.
[554] D.L. Brautigan, S. Ferguson-Miller, and E. Margoliash, *J. Biol. Chem.*, 1978, **253**, 130; D.L. Brautigan, S. Ferguson-Miller, G.E. Tarr, and E. Margoliash, *ibid.*, **253**, 140; S. Ferguson-Miller, D.L. Brautigan, and E. Margoliash, *ibid.*, **253**, 149.
[555] W.-S. Chen and B.V. Plapp, *Biochemistry*, 1978, **17**, 4916.
[556] D.P. Bloxham, R.A. Chalkley, S.J. Coghlin, and W. Salam, *Biochem. J.*, 1978, **175**, 999.



Scheme 2

acetophenone.[557] The intermediate aldehyde was reduced by borohydride to yield serine (Scheme 2).

The synthesis of four new fluorescent sulphydryl reagents related to *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulphonic acid has been reported.[558] The new derivatives extend the range of compounds available for fluorescence energy transfer experiments and allow the selection of appropriate energy donors. The substituted maleimide, *N*-(4-trifluoromethylphenyl)maleimide is readily prepared[559] and can be used as a highly specific sulphydryl reagent for ^{19}F-n.m.r. studies. This reagent is a potential cross-linking reporter group. An enzymatic approach to the labelling of tryptophan in peptides and proteins to a high specific activity has been proposed.[560] The method utilizes the recently discovered bacterial enzyme indolyl-3-alkane α-hydroxylase which catalyses the formation of 3-alkylidene indoline which can be reduced catalytically with carrier-free tritium. The radiolabelling of proteins by reductive alkylation is considerably improved by the replacement of borohydride by cyanoborohydride which allows more flexibility in the choice of reaction conditions.[561]

A step towards the better understanding of the mechanism of tRNA synthetase catalysed reactions has been made with the isolation of a covalent derivative of tryptophan and tryptophanyl tRNA synthetase from bovine pancreas.[562] Evidence for the covalent derivative being a mixed anhydride was obtained from reaction with [^{14}C]-labelled methoxylamine followed by tryptic mapping of the radiolabelled protein.

4 Chemical Cross-linking

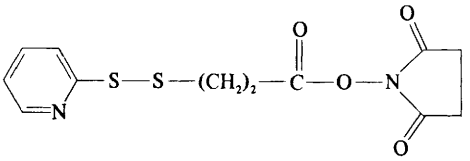
A number of approaches to the preparation of protein conjugates *via* intermolecular disulphide bond formation have been investigated. The practical uses of such procedures are varied and include important applications to immunoassays, hormone-receptor studies, and immunohistochemistry.

[557] P.I. Clark and G. Lowe. *European J. Biochem.*, 1978, **84**, 293.
[558] A. Steinemann, J. Bietenhader, and M. Dockter, *Analyt. Biochem.*, 1978, **86**, 303.
[559] T.T. Sakai and J.L. Dallas, *F.E.B.S. Letters*, 1978, **93**, 43.
[560] A. Maelicke, D. Krüger, J. Roberts, and H. Rosenfeld, *F.E.B.S. Letters*, 1978, **85**, 296.
[561] D. Dottavio-Martin and J.M. Ravel, *Analyt. Biochem.*, 1978, **87**, 562.
[562] G.K. Kovaleva, S.G. Moroz, O.O. Favorova, and L.L. Kisselev, *F.E.B.S. Letters*, 1978, **95**, 81.

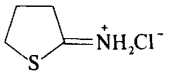
Protein thiolation and reversible protein–protein conjugation has been achieved with the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridyldithio)-propionate (2) which reacts with lysine and cysteine.[563] Reaction occurs rapidly under mild conditions and intermolecular cross-linking can be effected without competing intramolecular processes. Conjugates of unlike proteins have been obtained by the reaction of thiol groups introduced into a protein with dithiopyridyl derivatives of a second protein.[564] The disadvantages of heterogeneity of S–S linkage and non-uniform thiol incorporation are balanced by the ease of preparation, good yields, and retention of biological activity.

The bifunctional reagent, 2-iminothiolane (3), previously believed to be methyl 4-mercaptobutyrimidate (4), has been fully characterized[565] and used to cross-link *E. coli* ribosomal proteins.[566] The latter experiments establish the reliability of disulphide cross-linking and show that random cross-links are not formed during extraction and analysis of the proteins. The novel periodate cleavable cross-linkers, disuccinimidyl tartarate and *NN'*-bis(3-succinimidyl-oxycarbonylpropyl) tartaramide have been synthesized[567] and used in a study of ubiquinone–cytochrome *c* reductase. With a linking distance of 6 Å and 18 Å respectively the reagents can modify lysines at widely differing positions in the protein complex.

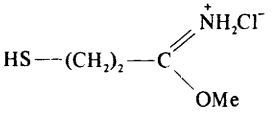
p-NN'-Phenylenebismaleimide has been shown[568] to be a suitable reagent for the insertion of a single cross-link between neighbouring actin subunits. An



(2)



(3)

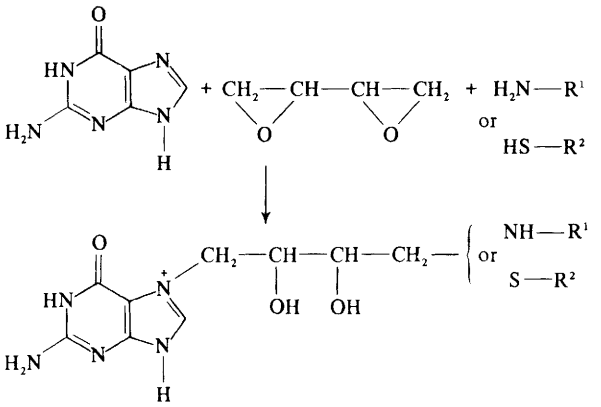


(4)

[563] J. Carlsson, H. Drevin, and R. Axén, *Biochem. J.*, 1978, **173**, 723.
[564] T.P. King, Y. Li, and L. Kochoumian, *Biochemistry*, 1978, **17**, 1499.
[565] R. Jue, J.M. Lambert, L.R. Pierce, and R.R. Traut, *Biochemistry*, 1978, **17**, 5399.
[566] R. Jue, J.M. Lambert, L.R. Pierce, and R.R. Traut, *Biochemistry*, 1978, **17**, 5406.
[567] R.J. Smith, R.A. Capaldi, D. Muchmore, and F. Dahlquist, *Biochemistry*, 1978, **17**, 3719.
[568] P. Knight and G. Offer, *Biochem. J.*, 1978, **175**, 1023.

approach to the probing of very close contacts has resulted from the observation[569] that the *mono*-functional compound methyl acetimidate yields cross-linked products from rat-liver nucleosomes. The formation of oligomers over such extremely short distances and the observation of an H3 homodimer suggest a very close proximity of half-nucleosomes.

Cyanoborohydride reductive cross-linking of RNA to proteins has been successfully applied[570] to the study of eukaryotic initiation factors. Protein synthesis initiation factors prepared from rabbit reticulocyte and mouse ascites ribosomes were tested for their ability to cross-link to the 5′ cap of mRNA. One 24 000 dalton polypeptide was shown to interact specifically with the cap. A method which is claimed to be superior to reductive alkylation for the cross-linking of proteins to nucleic acids has been investigated.[571] Reaction of ribosomes with diepoxybutane yields a rich crop of complexes between guanine and cysteine or lysine (Scheme 3). The cross-links are cleavable under mild conditions.



Scheme 3

Although it is well known that bis(2-chloroethyl)methylamine (nitrogen mustard) produces DNA–protein associations there is very little direct evidence available on the chemistry of the protein–DNA cross-links. An approach to the problem has now been made[572] with the caesium chloride purification of cross-linked species and the demonstration that the cross-links are cleaved in dilute acid but can be stabilized by alkali pre-treatment. It was concluded that the proteins are bound to alkylated purines.

5 Photocross-linking

The specificity of the photocross-linking reaction between poly-4-thiouridylic

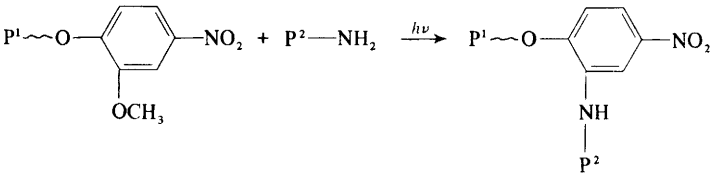
[569] W. Wyns, I. Lasters, and R. Hamers, *Nucleic Acids Res.*, 1978, **5**, 2345.
[570] N. Sonenberg, M.A. Morgan, W.C. Merrick, and A.J. Shatkin, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4843.
[571] H.G. Bäumert, S.-E. Sköld, and C.G. Kurland, *European J. Biochem.*, 1978, **89**, 353.
[572] C.B. Thomas, K.W. Kohn, and W.M. Bonner, *Biochemistry*, 1978, **17**, 3954.

acid and protein S1 in the *E. coli* ribosome has been investigated.[573] It was demonstrated that cross-linking is strongly stimulated by purified $tRNA^{Phe}$ but not by four non-cognate tRNAs. A detailed analysis has been made of the products obtained after u.v. irradiation of *E. coli* 30*S* ribosomal subunits.[574] The oligopeptide and the oligonucleotide at the cross-linking point have been identified by direct analysis of (i) the peptide remaining attached to the oligonucleotide after total digestion of RNA with ribonucleases A and T_1 followed by digestion with trypsin and (ii) the nucleotide remaining attached to cross-linked protein after ribonuclease T_1 digestion. The cross-link was located in the peptide spanning residues 113—117 of protein S7. The reported methodology is designed with a view to being generally applicable. The first application of photocross-linking to mammalian ribosomes has been reported.[575] The results have been tentatively correlated with chemical studies. The subunits of RNA polymerase which are in close contact with the T7 phage DNA template have been identified by u.v. irradiation at 254 nm.[576] In non-specific complexes, subunits σ, β and β' were cross-linked to the DNA; in contrast, only σ and β were cross-linked in specific binary complexes and it is possible that the σ subunit participates in promoter recognition even though isolated σ does not bind to DNA. Control experiments showed that binding and initiation were not affected by irradiation. The photochemical cross-linking of histones to DNA in nucleosomes was effected by irradiation in the presence of a photosensitizer.[577] The results indicated that the four core histones are not positioned equivalently with respect to DNA. The proto-porphyrin-sensitized modification of proteins in human erythrocyte membranes has been studied.[578] Results from the study of model systems suggest that cross-links could be formed by reactions of amino groups with oxidized histidine residues. Cross-linking *in situ* of ribosomal RNA and proteins using visible light and Methylene Blue as sensitizer yields covalent complexes containing proteins S3, S4, S5, S7, L2, and L3.[579] S3 and S5 had not previously been identified as RNA-binding proteins.

4-Nitrophenyl ethers have been proposed as new high-yield photoreagents for protein cross-linking and affinity labelling.[580] These reagents react quantitatively with amines at pH 8 upon irradiation with 366nm light (Scheme 4). The method was used for the formation of γ–γ cross-linked human fetal haemoglobin in 80 % yield.

The use of fluorescein isothiocyanate (FITC) as a reporter group for membrane proteins is well established and recently it has been used to measure

[573] P. Margaritella and E. Kuechler, *F.E.B.S. Letters*, 1978, **88**, 131.
[574] C. Zwieb and R. Brimacombe, *Nucleic Acids Res.*, 1978, **5**, 1189; K. Möller, C. Zwieb, and R. Brimacombe, *J. Mol. Biol.*, 1978, **126**, 489.
[575] A.-M. Reboud, M. Buisson, M.-J. Marion, and J.-P. Reboud, *European J. Biochem.*, 1978, **90**, 421.
[576] Z. Hillel and C.-W. Wu, *Biochemistry*, 1978, **17**, 2954.
[577] J. Sperling and R. Sperling, *Nucleic Acids Res.*, 1978, **5**, 2755.
[578] T.M.A.R. Dubbelman, A.F.P.M. De Goeij, and J. Van Steveninck, *Biochim. Biophys. Acta*, 1978, **511**, 141; T.M.A.R. Dubbelman, A.F.P.M. De Goeij, and J. Van Steveninck, *Photochem. and Photobiol.*, 1978, **28**, 197.
[579] D.E. Zook and S.R. Fahnestock, *Biochim. Biophys. Acta*, 1978, **517**, 400.
[580] P.C. Jelenc, C.R. Cantor, and S.R. Simon, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3564.



Scheme 4

the diffusion rate of proteins in membranes. Such experiments assume that the membrane is not affected by the photoirradiation required for fluorescence measurements. Recent work[581] casts doubt on the validity of this assumption. It has been demonstrated that high molecular weight aggregates are produced after 488 nm irradiation of FITC-labelled membranes.

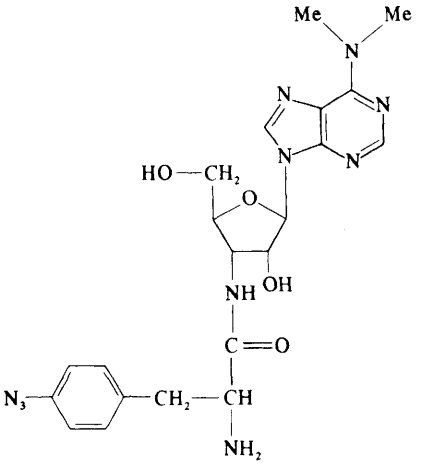
6 Competitive Labelling

The technique of competitive labelling has been used to probe the cytochrome *c* oxidase binding site on cytochrome *c*[582] by examining the reactivity of lysine residues in both free and oxidase bound cytochrome *c*. When cytochrome *c* was bound to the oxidase, lysines-1,7,8,13,72,73,86,87,88 became less reactive relative to the remaining eleven lysine residues by factors of 1.5 to 5. Based upon this information, a model for the binding site has been proposed. In similar experiments it has been found that the same groups of residues are less reactive in a cytochrome *c*–cytochrome bc_1 complex.[583] These results are consistent with those reported for the mapping by differential reductive methylation (not competitive labelling) of the cytochrome *c*–peroxidase complex.[584] A true competitive-labelling experiment using reductive methylation has been applied to elongation factor EF-Tu.[585] The complexes are first labelled with trace amounts of formaldehyde and tritiated borohydride, then methylated fully with cold reagents, and finally mixed with standard amounts of fully [^{14}C]methyl-labelled protein. Determination of ^{3}H:^{14}C ratios revealed a few remarkable changes in the reactivity of lysine in EF-Tu–GTP–Phe-tRNA as compared with EF-Tu–GTP. Advantages of reductive methylation compared with acetylation for competitive labelling include higher specific activities of reagents at lower cost, the retention of charge and hence facilitation of mapping and the stability to acid hydrolysis. Results of competitive labelling with acetic anhydride of the tyrosyl-tRNA synthetase–$tRNA^{Tyr}$ complex have been interpreted as supporting a single binding-site spanning both subunits.[586] This conclusion has been challenged[587] and the experimental data have been re-evaluated by a more rigorous procedure. The authors[587] claim that the results are in agreement with an antico-operative binding scheme.

[581] J.R. Lepock, J.E. Thompson, J. Kruuv, and D.F.H. Wallach, *Biochem. Biophys. Res. Comm.*, 1978, **85**, 344.
[582] R. Reider and H.R. Bosshard, *J. Biol. Chem.*, 1978, **253**, 6045.
[583] R. Reider and H.R. Bosshard, *F.E.B.S. Letters*, 1978, **92**, 223.
[584] G. Pettigrew, *F.E.B.S. Letters*, 1978, **86**, 14.
[585] B. Kraal and B.S. Hartley, *J. Mol. Biol.*, 1978, **124**, 551.
[586] H.R. Bosshard, G.L.E. Koch, and B.S. Hartley, *J. Mol. Biol.*, 1978, **119**, 377.
[587] A.M. Pingoud, F. Peters, and G. Maass, *J. Mol. Biol.*, 1978, **125**, 397.

7 Affinity Labelling

The technique of affinity labelling continues to be an important tool in structural and functional studies of biological macrostructure. Excellent reviews are now available which give a critical summary of affinity labelling of *E. coli* ribosomes,[588] estrogen photoaffinity reagents,[589] and the use of puromycin analogues and related compounds to probe the active centre of peptidyl transferase on *E. coli* ribosomes.[590] Native puromycin has been shown to be a photoaffinity label for ribosomes,[591] and the photochemical reactions leading to incorporation have been studied using the photolabile derivative 6-dimethylamino-9[3′-deoxy-3′-(*p*-azido-L-phenylalanylamino)*β*-D-ribofuranosyl]purine (5).[592] It has been pointed out[590] that such affinity-labelling experiments may not yield sufficiently good data to delineate binding



(5)

sites at high resolution and experiments with (5) have shown this to be true. Whereas Nicholson and Cooperman[592] find predominant labelling of S18, L11, L2, L3, L17, L18, L27, S3, S4, and S5 another group of workers[593] using the same reagent conclude that primary labelling of L6, L13, L18, L22, L25, S4, and S15 occurs. 5′-*O*-(*N*-Bromoacetyl-*p*-aminophenylphosphoryl-3′-*N*-L-phenylalanyl puromycin aminonucleoside is an affinity label for puromycin which retains the free *α*-amino group of the amino-acid moiety.[594] This reagent suffers from the disadvantage that the reactive group is at a position removed

[588] B.S. Cooperman, in 'Bioorganic Chemistry', ed. E.E. van Tamelen, Academic Press N.Y., 1978, p. 81.
[589] J.A. Katzenellenbogen, H.J. Johnson, H.N. Myers, K.E. Carlson, and R.J. Kempton, in ref. 588, p. 207.
[590] R.H. Symons, R.J. Harris, P. Greenwell, D.J. Eckerman, and E.F. Vanin, in ref. 588, p. 409.
[591] E.N. Jaynes, P.G. Grant, G. Giangrande, R. Wieder, and B.S. Cooperman, *Biochemistry*, 1978, **17**, 561.
[592] A.W. Nicholson and B.S. Cooperman, *F.E.B.S. Letters*, 1978, **90**, 203.
[593] F. Krassnigg, V.A. Erdmann, and H. Fasold, *European J. Biochem.*, 1978, **87**, 439.
[594] D.J. Eckermann and R.H. Symons, *European J. Biochem.*, 1978, **82**, 225.

from the amino-acid binding-site of peptidyl transferase and some non-specific labelling results. Such problems are not encountered using the photoaffinity label N^6(2-nitro-4-azidophenyl)-L-lysinyl puromycin aminonucleoside (NAP-Lys-Pan).[595] This reagent has the photoreactive group in the aminoacyl moiety and therefore is expected to label proteins within the amino-acid binding-site. After covalent linkage of the affinity label, poly(uridylic acid) was employed to direct Ac[^{14}C]Phe-tRNA to the P sites of the ribosomes. Transpeptidation of Ac[^{14}C]Phe to the bound NAP-Lys-Pan resulted in radio-labelling of the A-site. After dissociation and digestion of RNA the label was shown to be incorporated into a protein fraction of the 50*S* subunit. The authors compared their work to the results of affinity labelling with puromycin[591] in which no AcPhe transfer to puromycin could be detected and suggest that this may indicate puromycin does not bind at a functional site.

The synthesis of a series of potential photoaffinity labels for the ribosomal peptidyl transferase has been reported.[596] The reagents include 6-azidopurine ribonucleoside 5′-triphosphate which was designed to be incorporated enzymatically into the 3′-terminus of tRNA to yield a photoactive tRNA. Preliminary experiments with 6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside have so far failed to demonstrate that peptidyl transferase catalysed transfer of AcPhe to the covalently bound reagent has taken place. The mRNA binding region of *E. coli* ribosomes has been investigated using photolabile derivatives of oligoadenylic acids as mRNA analogues.[597] The derivatized oligonucleotides, which were shown to be functional messengers, labelled S3 and S5 with high specificity. Using a bromo 2-[^{14}C]-acetamidophenyl derivative of the initiation codon A-U-G, exclusive labelling of protein S18 has been observed.[598] It was concluded that S18 is at, or near, the aminoacyl-tRNA binding site on the ribosome. Peptide and amino-acid analyses of the modified protein identified Cys-10 as the derivatized residue.

The photoreactive GTP analogue P^1-(3-azido-6-hydroxy[^{3}H]methylphenyl-P^3-(5′-guanosyl)triphosphate has been prepared with high specific activity by tritiated borohydride reduction of P^1-(3-azido-6-formylphenyl)-P^3-(5′-guanosyl) triphosphate and used to label ribosomes in an elongation factor G-dependent process:[599] L11 was the main protein labelled.

Photoaffinity probes for various receptors have been reported. These include the glucagon receptor,[600] receptors involved in dipeptide transport,[601] the acetylcholine receptor,[602] and insulin and epidermal growth-factor receptors.[603] Cyclic nucleotide binding proteins have been detected by photoaffinity

[595] R. Vince, J. Brownell, and K.-L.L. Fong, *Biochemistry*, 1978, **17**, 5489.
[596] K. Quiggle, M.L. Wejrowski, and S. Chládek, *Biochemistry*, 1978, **17**, 94.
[597] H. Towbin and D. Elson, *Nucleic Acids Res.*, 1978, **5**, 3389.
[598] M. Yaguchi, E. Lanka, B. Dworniczak, H.-H. Kiltz, and O. Pongs, *European J. Biochem.*, 1978, **92**, 243.
[599] J.A. Maassen and W. Möller, *J. Biol. Chem.*, 1978, **253**, 2777.
[600] M.D. Bregman, S. Cheng, and D. Levy, *Biochim. Biophys. Acta*, 1978, **539**, 489.
[601] J.V. Staros and J.R. Knowles, *Biochemistry*, 1978, **17**, 3321.
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labelling in both the nucleus and cytoplasm of bovine liver.[604] The results suggest that the major, specific cAMP-binding proteins from both sources are the type I and type II regulatory subunits of cAMP-dependent protein kinase. Azidophenantridinium compounds have been proposed as photoaffinity labels for cholinergic proteins.[605] The label is very light sensitive and hence can be covalently attached at doses of light which do not alter or destroy the protein.

Haloacetyl-*N*-hydroxy derivatives of amino-acids have been used irreversibly to inhibit thermolysin[606] and such compounds should be useful in the study of other metalloproteases. Trypsin-like proteases can be probed with the lysine and arginine analogues, *m* and *p*-azidobenzamidines.[607] New affinity labels for acetyl CoA,[608] guanosine nucleotides,[609] and steroid binding-sites [610–613] have been reported.

E.coli Met, Ile, Trp, and Phe-tRNA synthetases and *B. stearothermophilus* Met and Tyr-tRNA synthetases are irreversibly inactivated by reaction of their active ATP sites with the 2′,3′-dialdehyde derivative of ATP obtained by periodate oxidation.[614] The results support the presence at the active site of a common lysine in all the synthetases.

Two reports on the use of fluorescent photoaffinity-labelling as an alternative to radioactive reagents have appeared.[615,616] The method combines the merits of directed photoaffinity-labelling with the probing power of fluorescent ligands.

Finally, a cautionary paper[617] describes the rapid reduction of aryl azides by dithiothreitol and it is recommended that this reducing agent not be used in aryl azide photoaffinity-labelling of biological systems that require thiols for activity.

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

1 Introduction

The year 1978 has seen many interesting advances in *X*-ray crystallographic studies of amino-acids, peptides, and proteins.

In the methods used during structure analysis three areas have shown considerable achievements in 1978. The use of linear position-sensitive detectors has allowed the construction of diffractometers capable of collecting data at very high rates, new algorithms for the refinement of structures have allowed hours of work to be performed in minutes, and the use of computer graphics systems to interpret electron-density maps and display molecular structures has continued to increase.

The protein structures determined at high resolution are considered in Sections 3 to 11. Among the oxygen-carrying and electron transport proteins the structure of haemerythrin, erythrocruorin, azurin, and plastocyanin are particularly interesting. In the glycolytic pathway the functioning and control properties of phosphorylase are slowly being elucidated.

The two outstanding protein-structure determinations reported during 1978 were of tobacco mosaic virus disks and of tomato bushy stunt virus particles. The size of these structures and the consequent large amounts of data that had to be recorded must surely set the limits for the current 'state of the art'.

Other structures of interest are those of tyrosyl-tRNA synthetase and the refined structures of tRNA itself. The structures of another species of dihydrofolate reductase, the flavoenzyme glutathione reductase, phospholipase A2, and the complex enzyme aspartate carbamoyl transferase have all been reported in 1978.

The analysis of protein structures has also continued apace and many of the principles of protein structure gleaned from these analyses are finding application in the methods of structure prediction. Several improvements to the structure prediction method have been reported in 1978 but as yet there seems to be no certain way of predicting the folding of a protein from just its amino-acid sequence.

X-ray diffraction studies on muscle, membranes and membrane proteins, ribosomes, and some small molecules of biological interest are reviewed here together with the work on fibrous proteins and synthetic polypeptides.

The crystal structures of amino-acids and small peptides, some of them biologically active, can give useful conformational information on these amino-acids in proteins. Structures reported in 1978 and some from 1977 are tabulated. Table 1 lists amino-acid and peptide structures while Table 2 covers metal-ion complexes of amino-acids and peptides. Much of the data for these tables were obtained by searching the Cambridge Crystallographic Data File (see Section 2 – Structure Storage) and use of this system is gratefully acknowledged.

Preliminary crystallization reports for proteins are detailed in Table 3 and low resolution structure determinations are listed in Table 4. Table 5 covers the results obtained from low-angle *X*-ray scattering measurements from biological molecules.

2 Equipment and Methods

Equipment and Crystal Growth.—The design and construction of a flat-cone diffractometer utilizing a linear position-sensitive detector has been described.[1] The instrument has been used with neutron radiation and, due to the two-dimensional position-sensitive detector, is able to acquire data at very fast rates. A concise review of such detectors has been published.[2] A diffractometer using a multiwire area detector has been described for use with *X*-ray diffraction studies on crystals with large unit cells.[3] The diffractometer has been used to collect diffraction intensities at the rate of 120 000 reflections per month and the necessary system hardware and software is outlined. A sensitive and efficient image intensifier–TV *X*-ray detector has been described.[4] All the major components are available commercially and the system is well suited to measuring weak or changing diffraction patterns. Since there is no limit on the counting rate the detector is very well suited for studies using the high *X*-ray fluxes of synchrotron sources. The optimization of the measurement of diffraction intensities has been discussed and a third, so-called absolute optimum, method described.[5]

Two reviews on the use of synchrotron *X*-ray radiation for research into biological structure have been published.[6,7] A grazing incidence monochromator for synchrotron radiation has been described[8] and an analysis of the counting losses of detectors measuring *X*-rays from storage rings has been presented.[9] High-flux micro-resonators for *X*- and γ-ray lasers have been discussed.[10]

A simple method for mounting crystals on the end of glass fibres has been described[11] as has a crystal-cooling device.[12] The cooling device is designed to provide good temperature stability while avoiding ice formation on the crystal, and is to be made available commercially.

Kam and co-workers[13] have reported theoretical and experimental work aimed at a systematic approach to the crystallization of proteins. Light scattering methods were used to determine if solutions would yield crystals or amorphous precipitate, and once crystallization had begun the protein concentration near the crystal surface was monitored. A self-limiting phenomenon causes crystals to stop growing after a certain size has been reached, but when these crystals are cleaved growth occurs at the surface until the original size is approximately restored. The chemistry of the chloroplatinite ion and its reactions with protein crystals has been discussed by Petsko and co-workers.[14] The

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Table 1 *Amino-acid Structures*

Compound	*Space group*	*Cell dimensions*						*Z*	*R*	*Ref.*
		a/Å	*b*/Å	*c*/Å	*α*/°	*β*/°	*γ*/°			
β-Ala-ciliatine monohydrate	$P2_1/c$	12.007	9.965	8.643	90	112.85	90	4	0.048	*a*
3-[(5*S*)-5-carboxy-2-oxotetrahydrofur-5-yl]-(2*S*)-Ala[b]	$P2_12_12_1$	6.411	22.889	6.270	90	90	90	4	0.059	*c*
5-*S*-Cysteinyluracil monohydrate	$P2_1$	14.59	6.82	5.05	90	95.10	90	2	0.101	*d*
L-pyroGlu	$P2_12_12_1$	9.018	13.495	14.662	90	90	90	12	0.049	*e*
5-Bromocytosine-phthaloyl-DL-Glu hemihydrate	$P\bar{1}$	10.578	19.640	9.890	88.13	107.86	104.26	4	0.110	*f*
N-Carboxy-*γ*-benzyl-L-Glu	$P2_12_12_1$	7.766	27.470	5.948	90	90	90	4	0.057	*g*
Glycyltaurine	$P2_1$	9.784	7.296	5.065	90	85.60	90	2	0.059	*h*
Cyclo-bis(L-*N*-methylisoleucyl-D-*α*-hydroxyisovaleryl)	$P2_12_12_1$	9.536	11.960	23.121	90	90	90	4	0.044	*i*
N-Carboxy-L-Leu anhydride	$P2_12_12_1$	6.527	23.920	5.527	90	90	90	4	0.049	*j*
Benzyloxycarbonyl-L-*α*-hydroxy-isovaleryl-D-*N*-methyl-Leu t-butyl ester	$P2_12_12_1$	22.306	10.969	10.462	90	90	90	4	0.038	*k*
DL-*p*-Introphenylalanine hydrochloride	$P2_1ab$	28.753	14.492	5.313	90	90	90	8	0.050	*l*
trans-Carboxy-4-L-Pro	$P2_1$	6.735	8.544	5.645	90	99.77	90	2	0.055	*m*
L-Prolinatodiphenyl boron	$P2_1$	5.943	14.463	8.965	90	98.42	90	2	0.037	*n*
O-Diazoacetyl-L-Ser	$P2_1^o$	15.291	5.251	9.523	90	96.52	90	4	0.064	*q*
	$P2_1^p$	15.234	5.265	9.527	90	96.33	90	4	0.039	*q*
1-Methyl-3-carbamido pyridinium *N*-acetyl-L-Trp monohydrate	$P2_12_12_1$	9.639	26.314	7.952	90	90	90	4	0.047	*r*
(2*S*,3*S*)-4-hydroxy Val lactone hydrochloride	$C2/c$	16.536	5.656	15.68	90	105.2	90	8	0.041	*s*
D-*N*-Methyl Val-D-*α*-hydroxy-valeric acid hydrobromide	$P2_12_12_1$	7.919	23.185	8.433	90	90	90	4	0.037	*t*
4-[(*N*-Benzyloxycarbonyl Val) oxyimino]-3-methyl-1-phenyl-2-pyrazolin-5-one	$P2_12_12_1$	12.164	25.130	7.417	90	90	90	4	0.048	*u*

Iron(II) (porphyrinato) (imidazole) complex	$C2/c$[v]	18.871	19.425	18.434	90	91.48	90	4	0.086	*x*
	$C2/c$[w]	18.864	19.451	18.287	90	91.45	90	4	0.083	*x*
(*N*-β-Ala-methyl-L-His) nitrate	$P2_1$	16.597	30.305	5.505	90	90.04	90	8	*y*	z
L-Ala-L-Ser[aa]	$P2_12_12_1$	18.053	9.389	4.858	90	90	90	4	0.055	*bb*
t-Butyloxycarbonyl Gly-L-Pro	$P2_12_12_1$	5.743	23.849	10.440	90	90	90	4	0.005	*cc*
Benzyl-t-butyloxycarbonyl Gly-L-Pro	$P2_12_12_1$	15.381	19.112	6.638	90	90	90	4	0.037	*cc*
Benzyloxycarbonyl Gly-D,L-Pro	$Pbc2_1$	10.227	10.490	25.590	90	90	90	8	0.060	*dd*
L-His-L-Asp monohydrate	$P2_1$	5.177	37.214	13.672	90	90.00	90	8	0.113	*ee*
t-Butyloxycarbonyl-D-Met-L-Met-methyl ester	$P2_1$	14.648	5.139	14.475	90	103.83	90	2	0.076	*ff*
Phe-Met	$I4_1$	23.891	23.891	5.589	90	90	90	8	—	*gg*
t-Butyloxycarbonyl-L-Pro-sarcosine	$P2_12_12_1$	13.955	16.767	6.662	90	90	90	4	0.049	*hh*
L-Ser-Gly[aa]	$P2_12_12_1$	4.550	8.506	17.380	90	90	90	4	0.043	*ii*
Cyclo-(L-His-LAsp) trihydrate[ll]	$P2_12_12_1$	16.771	13.792	6.143	90	90	90	4	0.056	*kk*
Cyclo-disarcosyl lithium perchlorate	$P\bar{1}$	11.575	8.107	11.561	110.95	89.97	116.63	2	0.065	*ll*
Cyclo(-L-Ser-L-His) monohydrate	$P2_12_12_1$	8.162	23.191	5.756	90	90	90	4	0.036	*mm*
Tyr-Gly-Gly	$P2_12_12$ or $P222_1$	9.549	8.073	18.424	90	90	90	4	—	*gg*
L-Tyr-Gly-Gly monohydrate[nn]	$P2_12_12_1$	9.549	18.405	8.012	90	90	90	4	0.029	*oo*
Sarcosyl-Gly-Gly	$Pbca$	20.846	10.252	8.719	90	90	90	8	0.049	*pp*
9-Methyl-10-methylanthryl sarcosyl-Gly-Gly	$P2_1/c$	19.021	10.727	10.728	90	94.24	90	4	0.078	*pp*
Cyclo(-L-Leu-L-Tyr-δ-aminovaleryl-δ-aminovaleryl-)	$P2_12_12_1$	17.201	18.806	9.327	90	90	90	4	0.099	*qq*
[Leu^5]Enkephalin[rr]	$C2$	31.871	8.535	12.467	90	96.53	90	4	0.14	*ss*
N-Benzyloxycarbonyl-Gly-L-Pro-L-Leu-Gly-L-Pro dihydrate[tt]	$P2_1$	15.999	7.557	13.792	90	100.86	90	2	0.064	*uu*
Cyclo(Gly-Pro-Gly-D-Ala-Pro)[vv]	$P2_12_12_1$	10.254	21.320	8.565	90	90	90	4	0.072	*ww*
Cyclo(L-Ala-L-Ala-Gly-L-Ala-Gly-Gly)[xx]	$P2_12_12_1$	9.274	24.357	9.168	90	90	90	4	0.037	*yy*

Table 1 *(cont.)*

Compound	Space group	Cell dimensions a/Å	b/Å	c/Å	α/°	β/°	γ/°	Z	R	Ref.
Cyclo(L-Ala-L-Ala-Gly-Gly-L-Ala-Gly)[zz]	$P2_1$	12.312	4.959	15.876	90	99.07	90	2	0.045	*yy*
Cyclo-hexasarcosyl methanol solvate	$Pna2_1$	14.379	10.203	16.236	90	90	90	4	0.080	*aaa*
D,L,L,L,L,L-Enniatin B[bbb]	$P6_1$	13.972	13.972	35.452	90	90	120	6	0.133	*ccc*
D,L,L,L,L,L-Enniatin B	$P6_1$	13.972	13.972	35.452	90	90	120	6	0.078	*ddd*
L,D,L,L,D,L-Enniatin B	$P4_32_12$	18.732	18.732	25.894	90	90	90	8	0.120	*eee*
L,D,L,L,D,L-Enniatin B	$P4_12_12$	18.732	18.732	25.895	90	90	90	8	0.085	*fff*
β-Amanitin[ggg]	$P2_12_12_1$	14.004	14.943	30.794	90	90	90	4	0.110	*hhh*
β-Amanitin[ggg]	$P2_12_12_1$	14.004	14.943	30.794	90	90	90	4	0.100	*iii*
Cyclo(D-Val-L-Ori-L-Val-D-Pro)$_3$[jj]	$P\bar{1}$	16.139	16.312	18.270	106.70	86.95	106.70	2	0.164	*kkk*
Gramicidin S–urea complex[lll]	$P3_121$	25.8	25.8	21.49	90	90	120	6	0.188	*mmm*
Virginiamycin Factor S[nnn]	$P2_12_12_1$	20.770	10.441	22.435	90	90	90	4	0.090	*ooo*
Nosiheptide[ppp]	$I4_1$	36.05	36.05	11.44	90	90	90	8	0.180	*qqq*
Nosiheptide	$I4_1$	36.05	36.05	11.44	90	90	90	8	0.133	*rrr*
Majusculamide B monohydrate[sss]	$P2_12_12_1$	9.027	12.102	26.512	90	90	90	4	0.031	*ttt*

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Braumann, E. Rose, and K.S. Suslick, *J. Amer. Chem. Soc.*, 1978, **100,** 6769. (*y*) Cannot be solved in $P2_1$ or $P2_12_12_1$. (*z*) Y. Barrans and M. Cotrait *J. Appl. Cryst.*, 1978, **11,** 288. (*aa*) Backbone chain in an extended conformation. (*bb*) P.G. Jones, L. Falvello, and O. Kennard, *Acta Cryst.*, 1978, **B34,** 1939. (*cc*) R.E. Marsh, M.R. Narasimha-Murthy, and K. Venkatesan, *J. Amer. Chem. Soc.*, 1977, **99,** 1251. (*dd*) T. Kojima, T. Yamane, and T. Ashida, *Acta Cryst.*, 1978, **B34,** 2896. (*ee*) T.N. Bhat and M. Vijayan, *Acta Cryst.*, 1978, **B34,** 2556. (*ff*) A. Immirzi, P. Avena, M.R. Ciajolo, J.M. Becker, and F. Naider, *Acta Cryst.*, 1978, **B34,** 179. (*gg*) M. Cotrait and S. Geoffre, *J. Appl. Cryst.*, 1978, **11,** 289. (*hh*) H. Itoh, T. Yamane, and T. Ashida, *Acta Cryst.*, 1978, **B34,** 2640. (*ii*) P.G. Jones, L. Falvello, and O. Kennard, *Acta Cryst.*, 1978, **B34,** 2379. (*jj*) Molecule is zwitterionic. Asp^{-}–His^{+}. (*kk*) R. Ramani, K. Venkatesan, and R.E. Marsh, *J. Amer. Chem. Soc.*, 1978, **100,** 949. (*ll*) N. Takahashi, I. Tanaka, T. Yamane, T. Ashida, T. Sugihara, Y. Imanishi, and T. Higashimura, *Acta Cryst.*, 1977, **B33,** 2132. (*mm*) M. Cotrait and M. Ptak *Acta Cryst.*, 1978, **B34,** 528. (*nn*) *N*-terminal portion of the enkephalins. The molecule assumes a conformation similar to a left-handed α-helix. (*oo*) W.M. Carson and M.L. Hackert, *Acta Cryst.*, 1978, **B34,** 1275. (*pp*) J.P. Glusker, H.L. Carrell, H.M. Berman, B. Gallen, and R.M. Peck, *J. Amer. Chem. Soc.*, 1977, **99,** 595. (*qq*) I.L. Karle and J.L. Flippen-Anderson, *Acta Cryst.*, 1978, **B34,** 3237. (*rr*) β-Bend in the sequence Tyr-Gly-Gly-Phe with 2 hydrogen bonds. All side-chains are in exposed positions on the surface. The tyrosine side-chain occurs in 2 discrete positions. (*ss*) G.D. Smith and J.F. Griffin, *Science*, 1978, **199,** 1214. (*tt*) Synthetic peptide substrate for collagenase. Folded into a 3_{10} helix with Pro and Leu at the corners. (*uu*) S. Bando, N. Tanaka, T. Ashida, and M. Kakudo, *Acta Cryst.*, 1978, **B34,** 3447. (*vv*) All *trans* backbone with two transannular hydrogen-bonds giving one β(II) bend and one γ bend. (*ww*) I.L. Karle, *J. Amer. Chem. Soc.*, 1978, **100,** 1286. (*xx*) Contains two β-bends. (*yy*) M.B. Hossain and D. van der Helm, *J. Amer. Chem. Soc.*, 1978, **100,** 5191. (*zz*) Contains a unique combination of a β(I) and a β(II) bend. (*aaa*) P. Groth, *Acta Chem. Scand.*, 1977, **A31,** 232. (*bbb*) *Cyclo*(D-*N*-methyl Val-L-α-hydroxy-isovaleryl-L-*N*-methyl Val-L-α-hydroxy-isovaleryl-L-*N*-methyl Val-L-α-hydroxy-isovaleryl). (*ccc*) T.G. Shishova, V.I. Andrianov, V.I. Simonov, V.Z. Pletnev, A.V. Evstratov, V.T. Ivanov, and Y.A. Ovchinnikov, *Bioorg. Khim.*, 1977, **3,** 172. (*ddd*) T.G. Shishova and V.I. Simonov, *Kristallografiya*, 1977, **22,** 515. (*eee*) G.N. Tischenko, Z. Karimov, V.I. Andrianov, B.K. Vainstein, A.V. Evstratov, V.T. Ivanov and Y.A. Ovchinnikov. *Bioorg. Khim.*, 1977, **3,** 467. (*fff*) G.N. Tischenko and Z. Karimov, *Kristallografiya*, 1978, **23,** 729. (*ggg*) Bicyclic octapeptide toxin from *Amanita phalloides*. Octapeptide ring is L-shaped. (*hhh*) E.C. Kostansek, W.N. Lipscomb, R.R. Yocum, and W.E. Thiessen, *J. Amer. Chem. Soc.*, 1977, **99,** 1273. (*iii*) E.C. Kostansek, W.N. Lipscomb, R.R. Yocum, and W.E. Thiessen, *Biochemistry*, 1978, **17,** 3790. (*jjj*) Rubidium picrate complex of Prolinomycin. Conformation very similar to that seen for Valinomycin. (*kkk*) J.A. Hamilton, M.N. Sabesan, B.F. Gisin, and L.K. Steinrauf, *Biochem. Biophys. Res. Comm.*, 1978, **80,** 949. (*lll*) Approximate 2-fold symmetry in the backbone. Interesting hydrogen bonds from ornithine side-chain nitrogen to main-chain carbonyl. (*mmm*) S.E. Hull, R. Karlsson, P. Main, M.M. Woolfson, and E.J. Dodson, *Nature*, 1978, **275,** 206. (*nnn*) Depsipeptide component of Virginiamycin. Structure contains one *cis*-peptide and is stabilized by a transannular hydrogen-bond. (*ooo*) J.P. Declercq, G. Germain, M. van Meersche, S.E. Hull, and M.J. Irwin, *Acta Cryst.*, 1978, **B34,** 3644. (*ppp*) Highly modified cysteine antibiotic from *Streptomyces actuosus*. (*qqq*) T. Prangé, A. Ducruix, C. Pascard, and J. Lunel, *Nature*, 1977, **265,** 189. (*rrr*) C. Pascard, A. Ducruix, J. Lunel, and T. Prangé, *J. Amer. Chem. Soc.*, 1977, **99,** 6418. (*sss*) *N*-Methyl-Val-*NO*-dimethyl-Tyr. (*ttt*) F.-J. Marner, R.E. Moore, K. Hirotsu, and J. Clardy *J. Org. Chem.*, 1977, **42,** 2815.

Table 2 *Amino-acid–Metal Complexes. Structures*

Compound	Space group	Cell dimensions						Z	R	Ref.
		a/Å	b/Å	c/Å	α/°	β/°	γ/°			
(*N*-Methyl Ala)-bis(ethylene diamine) cobalt(III) perchlorate	$P2_1$	9.437	12.241	8.417	90	104.23	90	2	0.068	*a*
(–)-*mer*-Tris(L-Ala)cobalt(III) monohydrate[b]	$P2_12_12_1$	5.247	13.555	19.351	90	90	90	4	0.076	*c*
Tetra-aquo-bis(β-Ala)zinc(II) nitrate	$P2_1/n$	23.891	6.576	5.519	90	95.67	90	2	0.042	*d*
trans-Dinitro-(β-Ala)-(1,3-diamino-propane)cobalt(III)	$P2_1/a$	12.928	11.177	8.174	90	93.95	90	4	0.046	*e*
Tris(β-Ala)cobalt(III)tetrahydrate	$P2_1/n$	5.666	12.868	22.215	90	90.66	90	4	0.046	*f*
mer-$(+)_{589}$-*cis*(05) *trans*(N_5)-(L-Asp) (L-ornithinato) cobalt(III)	$P2_12_12_1$	11.770	17.137	7.588	90	90	90	4	0.059	*g*
Hydrogen bis(L-Cys) mercury(II) chloride	$C2$	24.181	5.093	12.006	90	118.83	90	4	0.041	*h*
Dichloro(L-Cys)mercury(II)	$P2_12_12_1$	14.699	8.017	7.025	90	90	90	4	0.043	*h*
Bis(*S*-methyl-L-Cys)cadmium(II)	$P2_1$	13.314	5.074	9.839	90	96.34	90	2	0.052	*i*
Bis(*S*-methyl-L-Cys)zinc(II)	$P2_1$	13.433	5.323	9.603	90	107.52	90	2	0.059	*i*
Sodium bis(L-Cys)chromium(III) dihydrate	$P3_121$	10.644	10.644	9.809	90	90	120	3	0.022	*j*
Cystinato-bis[bis(ethylenediamine)-cobalt(III)] perchlorate hexahydrate	$P2_12_12$	35.708	8.869	6.847	90	90	90	2	0.073	*k*
N-Salicylidene Gly-(thiourea)copper(II)	$C2/c$	18.599	14.275	13.617	90	138.64	90	8	0.101	*l*
Diaquo-dibromo-(Gly)-manganese(II)	$P2_1/c$	7.961	16.420	9.692	90	136.60	90	4	0.070	*m*
μ-3-Oxo-hexakis[(μ-Gly)-tri (aquo-iron (III)] perchlorate	$P2_1$	19.260	15.610	16.690	90	100.71	90	4	0.074	*n*
mer-cis(*N*)-*cis*-(*O*)-Bis(Gly) amino ethanolato cobalt(III) hydrate	$P2_1/n$	9.687	11.957	9.629	90	103.75	90	4	0.041	*o*
Bis(Gly)manganese(II) bromide dihydrate	$Pbca$	21.613	12.649	8.990	90	90	90	8	0.047	*p*
	$P2_1/c$	11.943	6.060	8.979	90	111.65	90	2	0.040	*p*
Disodium bis(Gly-Gly)nickel(II) octa-hydrate	$C2/c$	27.83	6.19	13.93	90	121.92	90	4	0.047	*q*
Disodium bis(Gly-Gly)nickel(II) nona-hydrate	$P\bar{1}$	21.33	5.77	8.79	99.6	89.5	90.1	2	0.073	*q*
(2,9-Dimethyl-1,10-phenanthroline)(Gly-Gly) copper(II) pentahydrate	$P2_1/c$	9.591	14.478	17.238	90	114.79	90	4	0.037	*r*
Diphenyl tin Gly-Gly	$P2_12_12_1$	9.860	11.787	13.962	90	90	90	4	0.033	*s*

Gly-L-Ala copper(II) monohydrate	$P2_12_12_1$	9.180	9.750	8.940	90	90	90	4	0.045	*t*
TriGly-calcium iodide monohydrate	$P2_1/c$	12.070	7.370	19.130	90	109.60	90	4	0.117	*u*
Disodium triglycylglycinato nickel(II) octahydrate	$P\bar{1}$	7.73	9.79	14.06	70.07	75.50	84.92	2	0.090	*v*
Disodium triglycylglycinato nickel(II) decahydrate	$P\bar{1}$	7.74	10.17	14.48	92.8	107.2	95.4	2	0.043	*v*
α,β-Didehydro-Gly-Gly-histaminato copper(II) dihydrate	$P\bar{1}$	7.315	10.110	9.609	71.59	86.66	110.74	2	0.038	*w*
(L-His)(D-penicillaminato)cobalt(III) monohydrate	$P2_1$	7.078	12.263	8.856	90	96.06	90	2	0.032	*x*
(L-His(D-penicillaminato)chromium(III) monohydrate	$P2_1$	7.235	12.150	8.929	90	96.00	90	2	0.035	*y*
(α-*N*-(*o*-hydroxybenzyl)-L-His)(L-Ala) cobalt(III) dihydrate	$P2_12_12_1$	13.188	18.140	7.842	90	90	90	4	0.059	*z*
Diaquo-(L-His)(D-His)copper(II) tetra hydrate	$P\bar{1}$	6.894	8.478	9.553	98.61	116.13	95.88	1	0.076	*aa*
D-His-L-His-cobalt(III) bromide	$P2_1/c$	9.834	15.104	10.772	90	107.16	90	4	0.057	*bb*
Aquo-bis(*cyclo*-L-His-L-His) dicopper(II) perchlorate hydrate	$P2_1$	22.97	15.89	10.00	90	100.10	90	4	0.061	*cc*
Bis(L-His) nickel(II) monohydrate	$C2$	29.406	8.268	6.314	90	90.01	90	4	0.029	*dd*
$(-)_{435}$-L-Ile-[*NN*^{1}-ethylene bis(α-methyl salicylideneaminato)]cobalt(III) sesquihydrate	$P2_12_12_1$	21.56	11.86	9.80	90	90	90	4	0.116	*ee*
$[PtCl_6]^{2-}$L-Lys[ff]	$P2_12_12_1$	6.868	10.111	22.49	90	90	90	4	0.040	*gg*
(DL-Met)methyl mercury (II)	$P2_1/c$	7.048	5.826	25.323	90	93.20	90	4	0.099	*hh*
trans-Bis(L-Met) copper(II)	$P2_1$	9.487	5.061	15.563	90	92.46	90	2	0.046	*ii*
L-$(+)_{546}$-L-Phe-bis(acetylacetonato) cobalt (III) chloroform solvate	$P3_1$	12.319	12.319	14.504	90	90	120	3	0.060	*jj*
(L-Phe)dimethyl thallium(II)	$P1$	11.278	9.354	6.231	105.53	78.75	103.66	2	0.042	*kk*
N-salicylidene-L-Phe-aqua copper(II) dimer	$C2$	22.053	12.715	16.476	90	89.98	90	12	0.090	*ll*
cyclo-Tetrakis(L-Pro-Gly)rubidium thiocyanate trihydrate	$P4_12_12$	13.787	13.787	38.974	90	90	90	8	0.092	*mm*
Diaqua-*cis*-bis(L-Thr) zinc(II)	$P2_1$	5.875	22.275	5.807	90	119.69	90	2	0.054	*nn*
Sodium bis(3-methylsalicylidene-Thr) cobalt(II) ethanol solvate hydrate	$P2_1$	8.518	23.017	7.708	90	104.87	90	2	0.046	*oo*

Table 2 *(Cont.)*

Compound	Space group	Cell dimensions a/Å	b/Å	c/Å	α/°	β/°	γ/°	Z	R	Ref.
(DL-Trp)dimethylthallium(III) monohydrate	$P2_1/c$	7.955	19.507	10.679	90	117.52	90	4	0.044	*pp*
Methyl(L-Tyr)mercury(II) monohydrate	$P2_1$	9.084	6.151	11.5675	90	110.52	90	2	0.044	*qq*
Diaqua-bis(L-Tyr)nickel(II)	$P\bar{1}$	4.975	5.996	21.004	94.23	92.26	106.15	1	0.074	*rr*
Diaqua-bis(L-Tyr)nickel(II) monohydrate	$P\bar{1}$	6.135	4.844	18.236	89.05	77.69	107.54	1	0.090	*ss*
Catena-μ-(*N*-salicylidene-L-Tyr-*OO*′)copper(II)	$P2_12_12_1$	12.310	5.838	20.357	90	90	90	4	0.052	*tt*
Bis (DL-Val)calcium chloride dihydrate	*Pcca*	24.161	10.018	7.742	90	90	90	4	0.077	*uu*

(*a*) J.N. Brown, R.J. Majeste, and L.M. Trefonas, *Cryst. Struct. Comm.*, 1977, **6**, 61. (*b*) Cobalt co-ordination is distorted octahedral. (*c*) R. Herak, B. Prelesiuk, and I. Krstanovic, *Acta cryst.*, 1978, **B34**, 91. (*d*) G. Germain and M. van Meersche, *Inorg. Chim. Acta*, 1978, **30**, 197. (*e*) R.M. Herak, M.B. Celap, and I. Krstanovic, *Acta cryst.*, 1977, **B33**, 3368. (*f*) H. Soling, *Acta Chem. Scand.*, 1978, **A32**, 361. (*g*) S. Yano, M. Watanabe, and S. Yoshikawa, *Inorg. Nuclear Chem. Letters*, 1978, **14**, 279. (*h*) N.J. Naylor and A.J. Carty, *J. Amer. Chem. Soc.*, 1977, **99**, 6143. (*i*) P. de Meester and D.J. Hodgson, *J. Amer. Chem. Soc.*, 1977, **99**, 6884. (*j*) P. de Meester, D.J. Hodgson, H.C. Freeman, and C.J. Moore, *Inorg. Chem.*, 1977, **16**, 1494. (*k*) W.G. Jackson, A.M. Sargeson, and P.A. Tucker, *J. C. S., Chem. Comm.*, 1977, 199. (*l*) F. Pavelcik and J. Kratsmar-Smogrovic, *Z. Naturforsch.*, 1977, **B32**, 601. (*m*) T. Glowiak and Z. Ciunik, *Bull. Acad. polon. Sci., Sér. Sci. chim.*, 1977, **25**, 277. (*n*) R.V. Thundathil, E.M. Holt, S.L. Holt, and K.J. Watson, *J. Amer. Chem. Soc.*, 1977, **99**, 1818. (*o*) H. Miyamae and Y. Saito, *Acta Cryst.*, 1978, **B34**, 937. (*p*) T. Glowiak and Z. Cuinik, *Acta Cryst.*, 1978, **B34**, 1980. (*q*) H.C. Freeman and J.M. Guss, *Acta Cryst.*, 1978, **B34**, 2457. (*r*) C.J. Simmons, M. Lundeen, and K. Seff, *Inorg. Chem.*, 1978, **17**, 1429. (*s*) F. Huber, H.J. Haupt, H. Preut, R. Barbieri, and M.T. Lo Giudice, *Z. anorg. Chem.*, 1977, **432**, 51. (*t*) H.C. Freeman, M.J. Healy, and M.L. Scudder, *J. Biol. Chem.*, 1977, **252**, 8840. (*u*) S. Natarajan and J.K.M. Rao, *Curr. Sci.*, 1977, **465**, 595. (*v*) H.C. Freeman, J.M. Guss, and R.L. Sinclair, *Acta Cryst.*, 1978, **B34**, 2459. (*w*) P. de Meester and D.J. Hodgson, *Inorg. Chem.*, 1978, **17**, 440. (*x*) P. de Meester and D.J. Hodgson, *J. Amer. Chem. Soc.*, 1977, **99**, 101. (*y*) P. de Meester and D.J. Hodgson. *J. C. S. Dalton*, 1977, 1604. (*z*) K.E. Voss, R.J. Angelici, and R.A. Jacobson *Inorg. Chem.*, 1978, **17**, 1922. (*aa*) N. Camerman, J.K. Fawcett, P.A. Kruck, B. Sarkar, and A. Camerman, *J. Amer. Chem. Soc.*, 1978, **100**, 2690. (*bb*) N. Thorup, *Acta Chem. Scand.*, 1977, **A31**, 203. (*cc*) Y. Kojima, K. Hirotsu, and K. Matsumoto, *Bull. Chem. Soc. Japan*, 1977, **50**, 3222. (*dd*) T. Sakurai, H. Iwasaki, T. Katano, and Y. Nakahashi, *Acta Cryst.*, 1978, **B34**, 660. (*ee*) Y. Kushi, R. Tamura, M. Kuromoto, T. Yoshikawa, H. Yoneda, and Y. Fujii, *J. C. S., Chem. Comm.*, 1978, 266. (*ff*) Platinum atom is octahedrally co-ordinated by chlorine atoms. Protonated lysines bind to the chlorine atoms. (*gg*) P. L'Haridon, J. Lang, R. Pastuszak, and J. Dobrowolski, *Acta Cryst.*, 1978, **B34**, 2436. (*hh*) Y.-S. Wong, A.J. Carty, and P.C. Chieh, *J. C. S., Dalton*, 1977, 1157. (*ii*) C.-C Ou, D.A. Powers, J.A. Thich, T.R. Felthouse, D.N. Hendrickson, J.A. Potenza, and H.J. Schugar, *Inorg. Chem.*, 1978, **17**, 34. (*jj*) R. Fink, D. Revus and D. van der Helm, *Acta Cryst.*, 1977, **B33**, 1660. (*kk*) K. Henrick, R.W. Matthews, and P.A. Tasker, *Acta Cryst.*, 1978, **B34**, 1347. (*ll*) R. Hamalainen, U. Turpeinen, M. Ahlgren, and M. Rantala, *Acta Chem. Scand.*, 1978, **A32**, 549. (*mm*) Y.H. Chiu, L.D. Brown, and W.N. Lipscomb, *J. Amer. Chem. Soc.*, 1977, **99**, 4799. (*nn*) R. Hamalainen, *Finn. Chem. Letters*, 1977, 113. (*oo*) Y.N. Belokon, V.M. Belikov, S.V. Vitt, T.F. Saveleva, V.M. Burbelo, V.I. Bakhmutov, G.G. Aleksandrov, and Y.T. Struchkov, *Tetrahedron*, 1977, **33**, 2551. (*pp*) K. Henrick, R.W. Matthews, and P.A. Tasker, *Acta Cryst.*, 1978, **B34**, 935. (*qq*) N.W. Alcock, P.A. Lampe, and P. Moore, *J. C. S.*, Dalton, 1978, 1324. (*rr*) R. Hamalainen, K. Lajunen, and J. Valkonen, *Finn. Chem. Letters.*, 1977, 108. (*ss*) R. Hamalainen, M. Ahlgren, U. Turpeinen, and T. Raikas, *Cryst. Struct. Comm.*, 1978, **7**, 379. (*tt*) R. Hamalainen, M. Ahlgren, U. Turpeinen, and M. Rantala, *Acta Chem. Scand.*, 1978, **A32**, 235. (*uu*) T. Glowiak and Z. Ciunik, *Bull. Acad. polon. Sér. Sci., Sci. Chim.*, 1978, **26**, 43.

Table 3

Protein	Source	Space group	Cell dimensions a/Å	b/Å	c/Å	β/°	Mol. wt. and number of subunits	Mol. wt. of asymmetric units	V_m Å^3 dalton	Precipitant	pH	Ref.
Maltodextrin phosphorylase	*E. coli*	$C222_1$	104.8	191.5	297	—	164 000(2)	164 000	4.54	ammonium sulphate	6.4	*a*
Malate dehydrogenase	pig-heart mitochondria	$P2_1$	71.7	147.5	67.3	108.13	74 000(2)	148 000	2.28	PEG[b] 6000	5.7–6.0	*c*
		$P2_1$	67.0	144.1	64.1	101.0	74 000(2)	148 000	2.05	PEG 6000	5.7–6.0	*c*
		$P6_2$ or $P6_4$	97.8	97.8	134.0	—	74 000(2)	74 000	2.43	ammonium sulphate	7.5	*c*
Alcohol dehydrogenase	horse liver	*C*2	177.8	61.2	56.5	104.0	87 000(2)	?	?	trisHCl	8.4	*e*
										TES[d]	7.0	*e*
Neuraminidase heads	influenza virus	*I*422	143	143	210	—	48 000(1)	?	?	water from NaCl soln.	?	*f*
Semliki forest virus	—	*F*23	735	735	735	—	—	—	—	—	—	*g*
β-Lactamase I	*Bacillus cereus* 569	C2	143.0	35.8	52.7	97.0	28 000(1)	28 000	2.4	ammonium sulphate	8.6	*h*
Uteroglobin	rabbit	$P2_1$	43.3	31.1	34.5	90.7	15 800(2)	15 800	1.80	ammonium sulphate	5–7	*i*
Phospholipase C	*Bacillus cereus*	$P4_1$ or $P4_3$	63.6	63.6	73.9	—	23 000(1)	23 000	3.25	ammonium sulphate	7.4	*j*
Ricin D	*Ricinus communis*	$P2_12_12_1$	79.0	114.7	72.8	—	62 000(2)	62 400	2.64	from water	?	*k*
Adenosine triphosphatase	rat-liver mitochondria	*R*32	148	—	368	—	384 000(2)	190 000	—	ammonium sulphate	7.45	*l*
Fructose-1,6-diphosphatase	rabbit liver	*I*222	75	81	132	—	144 000(4)	36 000	2.69	PEG 4000	7.6	*m*
L7/L12 ribosomal proteins[n]	*E. coli*	$P6_1$ or $P6_5$[o]	72.2	72.2	137.4	—	3750(1)	15 000	6.9	ammonium sulphate	5.4	*q*
		$P4_12_12$[p] or $P4_32_12$	54.2	54.2	41.6	—	7350(1)	7350	2.08			
Glutamine synthetase	*E. coli*	$P2_12_12$	145	135.9	175.9	—	600 000(12)	300 000	2.8	ammonium sulphate	5.9	*r*

Table 3. *(cont.)*

Protein	*Source*	*Space group*	*Cell dimensions* a/Å	b/Å	c/Å	β/°	*Mol. wt. and number of subunits*	*Mol. wt. of asymmetric units*	V_m Å³/dalton	*Precipitant*	*pH*	*Ref.*
		$P2_12_12$	142	131.2	172.8	—	600 000(12)	300 000	2.7	ammonium sulphate	5.9	*r*
		*C*1	235.9	134.5	200.5	$\alpha = 90.17$ $\beta = 102.83$ $\gamma = 90.50$	600 000(12)	1 200 000	2.6	methyl-pentane diol.	7.0	*r*
		*C*2	235.3	135.6	199.1	102.83	600 000(12)	600 000	2.6	methyl-pentane diol.	7.0	*r*
		*P*312	244	244	298		600 000(12)	900 000	2.8	PEG 1000	7.0	*r*
Uteroglobin	rabbit	*P*1	36.36	37.40	53.28	$\alpha = 104.6$ $\beta = 97.0$ $\gamma = 111.3$	15 000(2)	30 000	—	ammonium sulphate	8.0	*s*
		$C222_1$	50.86	52.22	47.28	—	16 000(2)	—	—	ammonium sulphate	8.0	*s*
EcoRI endonuclease	*E. coli*	C2	209	129	50	98.4	116 000(4)	116 000	2.87	PEG	7.5	*t*
Citrate synthase	chicken heart[u]	$P2_12_12$	171.8	107.1	63.4	—	100 000(2)	100 000	2.92	PEG 4000	7.4	*w*
	chicken heart[v]	$P2_12_12$	154.9	106.9	64.0	—	100 000(2)	100 000	2.65	PEG 4000	7.4	*w*
	pig heart	$P4_12_12$	76.9	76.9	200.2	—	100 000(2)	50 000	2.98	PEG 4000	—	*w*
Tonin[x]	rat	$P3_121$ or $P3_221$	94	94	66	—	32 000(1)	32 000	2.60	ammonium sulphate	6.2	*y*
Transferrin	human	$P2_12_12_1$	78	94	112	—	81 000(1)	81 000	2.53	PEG 4000	7.5	*z*
Leucine, isoleucine, valine-binding protein	*E. coli* K12	$P2_12_12_1$	40.1	71.2	115.9	—	36 000(1)	36 000	2.30	PEG 6000	4.5	*aa*
Superoxide dismutase[bb]	*E. coli* B	$P2_12_12_1$	81.8	75.2	71.3	—	40 000(2)	40 000	2.83	ammonium sulphate	5.0	*cc*
Creatine kinase	rabbit muscle	*C*2	248	149	52	90.0	80 000(2)	120 000	4.0	methyl-pentane diol	8.2	*dd*

Toxic protein	scorpion	$P2_12_12_1$	52.2	42.0	28.5	—	7000(1)	7000	2.23	methyl-pentane diol	7.1	*ee*
Ricinus lectin	*Ricinus communis*	*P*622	166	166	341	—	125 000(4)	125 000	2.72	PEG 6000	<3.8	*ff*
Chloramphenicol acetylpentane transferase	*E. coli*	$P3_1$ or $P3_2$	116.3	116.3	147	—	100 000(4)	300 000	2.12	methyl-pentane diol	6·2	*gg*
K-type Bence-Jones protein	human	$P2_12_12_1$	132.0	93.3	42.3	—	23 000(1)	46 000	2.83	ammonium sulphate	6.5	*hh*
Ex foliative toxin	*Staph. aureus*	$P2_1$	49.75	67.14	82.84	93.4	26 000	52 000	2.66	PEG 4000	7.1	*ii*
D-Amino-acid oxidase	pig kidney	$C222_1$	325	138.8	200	—	39 600(1)	316 800	3.57	ammonium sulphate	8.3	*jj*
		$P3_112$	116	116	399	—	39 600(1)	237 600	3.26	ammonium sulphate	5.4	*jj*
Platelet Factor 4	human	$P2_12_12$	78.7	80.6	54.6	—	7800(1)	31 200[kk]	2.77	ammonium sulphate	6.5	*ll*
Cytochrome c_5	*Azotobacter vinelandii*	*C*2	45.0	38.4	41.3	101.0	24 000(2)	12 000	1.49	ammonium sulphate	6.7	*mm*
		*C*1	46.0	37.6	49.4	$\alpha = 87.33$ $\beta = 96.67$ $\gamma = 90.00$	24 000(2)	24 000	—	ammonium sulphate	6.7	*mm*
Modified fibrinogen	bovine	$P2_1{}^{nn}$	134	97.3	174	92.3	325 000(1)	319 000	3.55	low ionic strength	6.2	*pp*
		$P1^{oo}$	106	107	211	$\alpha = 97.9$ $\beta = 91.1$ $\gamma = 112.6$	325 000(1)	306 000	—	—	—	*pp*
Toxin II	North African scorpion	*C*2	51.9	33.3	40.4	113	7250(1)	7250	2.21	pH increase	—	*qq*
Haemoglobin II[rr]	white-tailed deer	$P2_1$	161.98	59.97	63.07	97.20	68 000(4)	68 000	2.23	phosphate buffer	7.3	*ss*
Oxyhaemoglobin	human	$P2_12_12^{tt}$	96	98	66	—	66 000(4)	66 000	2.39	PEG 6000	6.1–7.2	*uu*

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Table 4

Protein	*Source*	*Resolution*	*Comments*	*Ref.*
Southern bean mosaic virus	—	11 Å	data collection by screened precession photography. Rotation function shows that the virus has icosahedral symmetry and a mean diameter of 284 Å. The electron density present at a radius of 118 Å or greater obeys T = 3 symmetry and this is interpreted as the protein coat. There is little or no density at radii below 118 Å suggesting the RNA structure is disordered	*a*

Inorganic pyrophosphatase	yeast	5.5 Å	the dimeric molecule (mol. wt. 64 000) is ellipsoidal with dimensions 100 × 50 × 50 Å with an indentation at the equator. The two subunits appear to be related by a non-crystallographic two-fold axis. Several rod-like features, ranging in length from 25—35 Å, are tentatively identified as α-helices	*b*
γ-Crystallin fraction II	calf lens	5.5 Å	the molecule is ellipsoidal with dimensions 55 × 30 × 25 Å and is organized into two globular domains, each approximately 25 Å in diameter. Although the two domains have large areas of their surfaces close together, only a single strand of electron density connects them. Crystallographic data for two other calf γ-crystallins are also given	*c*
Met-myoglobin	gasteropod *Aplysia limacina*	5 Å	the molecule shows the myoglobin folding pattern and several helical regions can be easily seen. The haem group in the molecule seems to be more accessible than in the sperm-whale myoglobin. This is obvious on the E-helix side where the distal histidine is absent from the sequence	*d*
Lipovitellin-phosvitin complex	*Xenopus laevis* oocytes	20 Å	calculated from electron microscopy of microcrystals. Complex is an ellipsoid with dimensions 55 × 115 × 250 Å and the local two-fold axis has been identified. Tenuous assignments of the known protein and lipid components to the different structural domains have been made.	*e*
Aspartate transaminase	chicken heart	5 Å	the molecule consists of 2 closely packed subunits, each an ellipsoid 40 × 48 × 60 Å, related by a non-crystallographic two-fold axis. Nine segments of chain have been assigned as helices. Attempts have been made to identify the catalytic site and the co-factor, pyridoxal-5′-phosphate	*f*

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Table 5 *Low angle X-ray scattering*

Protein	*Source*	*Mol. wt.*	R_G/Å	*V*/Å^3	*Comments*	*Ref.*
Malate synthase	yeast	187 000	39.6	33 800	seems to have very anisometric shape. Substrate binding gives a decrease in this anisometry	*a*
Chloroplast coupling factor CF_1	*Vicia faba*	320 000	56.5	1.2×10^6	very high experimentally determined volume: dry volume ratio may show that channels exist within the protein	*b*
Ribonuclease A	bovine pancreas	13 300	15.9	—	comparison of calculated and measured scattering curves shows solution structure is the same as the crystal structure	*c*
Lysozyme	bacteriophage T4	18 000	18.6	—	calculated and measured scattering curves, with and without inhibitor, shows that the structure in solution differs noticeably from that in the crystal	*d*
Myoglobin	sperm whale	—	—	—	calculated and measured scattering curves show that the solution and crystal structures may differ by a shift of the GH hairpin bend	*e*
11*S* Globulin	sunflower seed	300 000	40.5	480 000	molecule appears to be an oblate ellipsoid ($a/b = 0.6$)	*f*
Phosphofructokinase	yeast	742 000	74.5	1.52×10^6	proposed model has 8 subunits arranged with dihedral point group symmetry D_2	*g*
Phosphofructokinase	*Dunaliella salina*-green alga	325 000	52.9	815 000	in the presence of fructose-1,6-di-phosphate the molecule appears to become more compact (R_G49.6 Å. Volume = 675 000 Å^3)	*h*
α-Crystallin	calf lens	837 000	6.2	1.55×10^6	native protein	*i*
		420 000	5.1	805 000	reassociated protein	
Immunoglobulin M	human	840 000	121	1.9×10^6	overall shape can best be fitted by a flat star-shaped model, with 5 Y-shaped arms	*j*

Immunoglobulin and antibody	pig	—	114.8 early complex	—	distance between the two bound antigens calculated to be 358 Å for the	*k*
		—	88.7 later complex	—	early formed complex and 273 Å for the complex formed later	
Low-density lipoprotein B	human plasma	$2{\cdot}4 \times 10^6$	90	6.4×10^6	best model is a sphere with radial symmetry. Internal structure seems to be arranged in layers with a central core of neutral lipids	*l*
Low-density lipoprotein	human serum	—	—	—	different levels of triglyceride relative to cholesteryl ester shift the thermal transition	*m*
Low-density lipoprotein	human serum	2.7×10^6	—	—	comparison of native and trypsin treated low-density lipoprotein. Trypsin treatment affects the outermost electron-dense shell in a uniform manner	*n*
Rhodopsin in detergent micelles	bovine	38 000	19	47 000	Neutron small-angle scattering study	*o*
S3 Ribosomal protein	*E. coli*	28 000	31	85 000		*p*
S4 Ribosomal protein	*E. coli*	23 000	26	55 000	all four proteins show an extended structure in solution	*p*
S7 Ribosomal protein	*E. coli*	21 000	27	65 000	but detailed analysis is hindered	*p*
S20 Ribosomal protein	*E. coli*	10 000	27	35 000	by their low solubilities	*p*
S1 Ribosomal protein	*E. coli*	75 000	72	—	all four proteins are highly elongated	*q*
S8 Ribosomal protein	*E. coli*	10 600	23	—	with axial ratios from 1 : 3 to 1 : 10.	*q*
S15 Ribosomal protein	*E. coli*	9500	26.5	—	S1 protein has a maximum dimen-	*q*
S16 Ribosomal protein	*E. coli*	10 000	21	—	sion of about 260 Å while the other three proteins all have a maximum dimension of about 100 Å. S15 has the most compact structure com-	*q*
S1 Ribosomal protein + 30*S* ribosomal subunit	*E. coli*	—	67.2	—	parison of *X*-ray and neutron small-angle scattering studies show (i) the alterations of structure in the 30*S* subunit induced by S1 binding are too small to be detected and (ii) the centre	*r*

Table 5 *(cont.)*

Protein	*Source*	*Mol. wt.*	R_G/Å	*V*/Å^3	*Comments*	*Ref.*
					of gravity of Sl bound to the 30*S* subunit is 75 Å from the centre of the particle	
S4 Ribosomal protein	*E. coli*	24 000	17.5	—		*s*
S7 Ribosomal protein	*E. coli*	16 000	14.5	—	small-angle neutron scattering	
S8 Ribosomal protein	*E. coli*	12 000	13.0	—	results from experiments	
S16 Ribosomal protein	*E. coli*	11 000	12.2	—	performed in deuterium oxide	
Polysomes	rat liver	—	340	polysomes	arrangement of ribosomes on the	*t*
		—	115	80*S* ribosomes	polysome is compact, the best model being a solenoid with six ribosomes per turn and a pitch of 330 Å. The distance between neighbouring ribosomes on the mRNA molecule is 350 Å, 550 Å for next-but-one neighbours	
Methionyl-tRNA synthetase + $tRNA^{Met}$	*E. coli.*	—	—	—	neutron scattering investigation of the interaction of the two compounds. Two-step binding has been observed: one tRNA binding close to the centre of mass of the protomer it is associated to and the second one giving an increase in the radius of gyration of the complex	*u*
Adenovirus type 2 hexon	—	310 000	49	630 000	an analysis of the shape of the virus hexon by small-angle scattering and a model-building study using known crystallographic data	*v*
Bacteriophage T4	—	—	—	—	comparison of the structures of isometric headed mutant bacteriophage T4 with the native form	*w*

Coat protein aggregates	bacteriophage P22	—	—	—	studies on the three morphological classes of aberrant particles formed in the absence of the scaffolding protein	*x*
Bacteriophage PM2	—	—	250	—	neutron small-angle scattering study. An outer shell contains all of protein II and a significant amount of protein I. The lipid bilayer occupies the next shell and contains protein III. Protein IV is at the interface of this bilayer and the central, highly solvated, DNA core	*y*
Tomato bushy stunt virus	—	8.7×10^6	132	—	neutron small-angle scattering study. The protein is concentrated in two shells 30 Å apart with a low electron density region in between. Most of the RNA is located in between these two shells	*z*

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influence of solvent conditions on the derivatives of the chloroplatinite ion produced and the reactions of the various derivatives are discussed. The means by which the reactions may be controlled are also considered.

The application of the direct methods program, MULTAN, to the analysis of isomorphous derivatives in protein crystallography has been reported by Wilson.[15] The method requires a minimum of user intervention and has been tested on a large number of isomorphous derivatives. It seems that the method is generally useful in the rapid elucidation of the heavy-atom binding positions in isomorphous derivatives.

A method for locating protein thiol residues using an electron microscope has been published.[16]

General Methods.—Karle[17] has presented a general review of the philosophical and mathematical aspects of the techniques used in single crystal structure determinations. He traces the development of structure determination by diffraction methods and shows the special problems involved with biological macromolecules.

Herbert[18] has described a simple method for obtaining the triclinic unit-cell parameters from Weissenberg photographs with only one crystal setting. Mathieson[19] has reported a method for eliminating the polarization ratio of a crystal monochromator when converting measured intensities to structure factors.

Main and Hull[20] have reported steps in the process of automating structure determinations. Procedures for peak searching and isolating and recognizing molecular fragments in E maps are described. Some of these methods are to be incorporated into MULTAN. Possible errors in the analysis of *X*-ray–neutron density maps when the diffraction data are collected at room temperature have been published.[21]

Further papers in the series on the use of magic integers in phase determination and on the application of phase relationships to complex structures have appeared.[22—26] Karle[27] has described a joint probability distribution of the invariants which comprise the determinantal inequalities while Karlsson[28] has reported how the use of direct methods can destroy pseudocentro symmetry. Frazer and co-workers[29] have presented an improved method for calculating the solvent contribution to the *X*-ray diffraction patterns from crystals or fibres

[15] K.S. Wilson, *Acta Cryst.*, 1978, **B34**, 1599.
[16] M. Stewart and V. Diakin, *Nature*, 1978, **274**, 184.
[17] J. Karle, *Proc. Nat. Acad. Sci. U.S.A.* 1978, **75**, 3540.
[18] H. Herbert, *Acta Cryst.*, 1978, **A34**, 964.
[19] A. McL. Mathieson, *Acta Cryst.*, 1978, **A34**, 404.
[20] P. Main and S.E. Hull, *Acta Cryst.*, 1978, **A34**, 353.
[21] C. Scheringer, A. Kutoglu, and D. Mullen, *Acta Cryst.*, 1978, **A34**, 481.
[22] P. Main,, *Acta Cryst.*, 1978, **A34**, 31.
[23] S.E. Hull, *Acta Cryst.*, 1978, **A34**, 38.
[24] S.E. Hull and M.J. Irwin. *Acta Cryst.*, 1978, **A34**, 863.
[25] D.J. Taylor, M.M. Woolfson, and P. Main. *Acta Cryst.*, 1978, **A34**, 870.
[26] R. Baggio, M.M. Woolfson, J.P. Declercq, and G. Germain, *Acta Cryst.*, 1978, **A34**, 883.
[27] J. Karle, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2545.
[28] R. Karlsson. *Acta Cryst.*, 1978, **A34**, 698.
[29] R.D.B. Fraser, T.P. MacRae, and E. Suzuki. *J. Appl. Cryst.*, 1978, **11**, 693.

of biological molecules. They show how use of this correction can reduce the *R*-factor when applied to the structure of hydrated collagen.

Baianu[30] has presented a method for analysing diffraction patterns from imperfect membrane lattices with stacking disorders between 2—6 %. The method is generally applicable to partially disordered centrosymmetric membranes but in some cases can be applied to asymmetric membranes. One consequence of the method is that refinement of membrane electron density projections becomes feasible and an example is presented.

Rae has reported an optimized conjugate gradient solution for least-squares equations[31] and has also presented methods for calculating overlap electron density scattering factors.[32, 33] Nixon[34] in a discussion on the use of direct methods to solve the phase problem suggests that overlapping vectors in the Patterson function may often be the cause of failures. A simple modification to remove these overlapping vectors is proposed and the use of the method to solve the structure of prostratin is described. Wilson[35] has discussed the probability of measuring the intensity of a reflection as negative. French and Wilson[36] have described a method which produces sensible estimates of the structure factor moduli from intensity observations, whether the latter are negative or positive. The applications of the method to data from the protein phosphorylase b are summarized. In another paper French[37] presents a Bayesian method of statistical analysis in general terms, and then discusses its applications to crystallographic refinement and the primary reduction of diffractometer data.

A comparison of the results from *X*-ray small-angle scattering and crystal structure analysis has been presented by Kratky and Pilz.[38] Laggner and Müller[39] have reviewed the *X*-ray small-angle scattering work carried out on the serum lipoproteins. The proceedings of a meeting on small-angle scattering methods and results have been published.[40]

Structure Refinement.—Several improved methods of refinement have been published in 1978 together with their applications to structures.[41—45] Smith and Arnott[41] describe a linked-atom least-squares method primarily intended for helical macromolecules. The refined parameters are the torsion angles around single bonds, with bond lengths and angles being fixed. This gives a greatly reduced number of parameters to refine, which is necessary in systems where the observed data are sparse. The authors list the polynucleotide, polysaccharide, and other fibrous materials which the method has been used to refine successfully.

[30] I.C. Baianu, *Acta Cryst.*, 1978, **A34**, 757.
[31] A.D. Rae, *Acta Cryst.*, 1978, **A34**, 578.
[32] A.D. Rae, *Acta Cryst.*, 1978, **A34**, 719.
[33] A.D. Rae and R.A. Wood, *Acta Cryst.*, 1978, **A34**, 724.
[34] P.E. Nixon, *Acta Cryst.*, 1978, **A34**, 450.
[35] A.J.C. Wilson, *Acta Cryst.*, 1978, **A34**, 474.
[36] S. French and K. Wilson, *Acta Cryst.*, 1978, **A34**, 517.
[37] S. French, *Acta Cryst.*, 1978, **A34**, 728.
[38] O. Kratky and I. Pilz, *Quart. Rev. Biophys.* 1978, **11**, 39.
[39] P. Laggner and K.W. Müller, *Quart. Rev. Biophys.* 1978, **11**, 371.
[40] Various authors, *J. Appl. Cryst.*, 1978, **11**, No. 5, pp. 295—674.
[41] P.J.C. Smith and S. Arnott. *Acta Cryst.*, 1978, **A34**, 3.

Parthasarathy[42] has considered the relative merits of refinement using all the observed data at the same time and stepwise refinement using data of increasing resolution. He has shown that the first strategy only works well when the trial structure is sufficiently accurate. For structures with large mean positional errors only the second method gives a useful refinement.

Agarwal[43] has presented a new least-squares refinement technique based on the fast Fourier transform algorithm and Isaacs and Agarwal[44] have described the application of the method to the structure of rhombohedral two-zinc insulin at 1.5 Å resolution. For large structures, the amount of computation time is almost proportional to the size of the structure making it very useful for work on proteins. The method also has a radius of convergence of 0.75 Å making it applicable at an early stage of the structure determination process. The method has been tested on hypothetical structures as well as insulin at 1.5Å resolution, barium beauvericin at 1.2Å resolution, and myoglobin at 2Å resolution.[43] A more detailed account of the insulin refinement[44] shows that the refinement requires three minutes per cycle on an IBM 370 computer. After 67 cycles of refinement the *R*-factor had dropped from 0.282 at 1.82 Å resolution to 0.113 for the 11 890 terms at 1.5Å resolution. The procedures are described in detail and from the experience gained from this refinement a strategy which would allow faster convergence with fewer cycles is proposed.

Jack and Levitt[45] have described an improved refinement method which is based on a simultaneous refinement of a realistic potential-energy function and the crystallographic *R*-factor. The method has been applied to pancreatic trypsin inhibitor and has been shown to correct positional errors of up to 3 Å. Errors larger than this are not corrected but the overall improvement in the model means that difference electron density maps are much easier to interpret.

Structure Storage and Computer Graphics.—Crippen and Havel[46] have described a new method for calculating the cartesian co-ordinates for a set of points given the matrix of interpoint distances. The results of trials of the method are presented.

Three papers on the storage and retrieval of molecular structure data have been published.[47, 48, 49] The first paper[47] covers the general principles and methods for the efficient retrieval of structural information from the Cambridge Crystallographic Data File. The statistical analysis of the retrieved molecular structures by multivariate techniques is outlined. The second paper[48] considers the causes of variance in the observed geometrical parameters of a molecular fragment. The authors show that using multivariate techniques, such as factor analysis, the variance can be partitioned between experimental errors and genuine structural variation. The third paper in the

[42] S. Parthasarathy, *Acta Cryst.*, 1978, **A34**, 512.
[43] R.C. Agarwal, *Acta Cryst.*, 1978, **A34**, 791.
[44] N.W. Isaacs and R.C. Agarwal, *Acta Cryst.*, 1978, **A34**, 782.
[45] A. Jack and M. Levitt, *Acta Cryst.*, 1978, **A34**, 931.
[46] G.M. Crippen and T.F. Havel, *Acta Cryst.*, 1978, **A34**, 282.
[47] P. Murray-Rust and S. Motherwell, *Acta Cryst.*, 1978, **B34**, 2518.
[48] P. Murray-Rust and R. Bland, *Acta Cryst.*, 1978, **B34**, 2527.

series[49] describes how the automatic search procedure was used to find the occurrences of the nucleoside fragment, β-1′-aminofuranoside. The sequences of searches and analyses involved are described in detail together with the specific stereochemical results found for this particular molecular fragment.

Tsernoglou and co-workers[50] have reported the use of a computer graphics system to fit a putative amino-acid sequence to an electron density map of sea snake neurotoxin at 2.2 Å resolution. The graphics system displays the electron density map and the molecule which can be adjusted by user intervention. The process allowed the complete set of co-ordinates to be obtained in 60 hours without the necessity of building a physical model.

Wodak and co-workers[51] have reported the structure determination of ribonucleases in the presence of the dinucleoside monophosphate, cytidilyl (2′,5′)adenosine. The interpretation of the electron density map was carried out on a graphics system using, as starting co-ordinates for the dinucleoside, those obtained from crystal structure determinations of similar compounds. The great speed of the method and the ready availability of co-ordinates show that graphics systems are ideally suited to the interpretation and analysis of difference Fourier maps.

The use of a computer graphics system to interpret the averaged electron density map of methaemerythrin at 2.8 Å resolution has been described.[52] The graphics system used has been used for several other structures[50] but in this work[52] Stenkamp and co-workers found that the quickest way to fit the electron density map was to generate several ideal α-helices and then to fit the entire helix into the electron density. Again the availability of co-ordinates and diagrams from the graphics system is apparent in the clarity of the figures presented.

Hagler and Honig[53] have used a computer to simulate the folding of a protein. They used a sequence of only alanine and glycine residues and have shown that glycines play a central role in stabilizing globular structure by facilitating the formation of turns and by destabilizing helical conformations.

Feldmann and co-workers[54] have described an exceedingly versatile graphics system which must currently represent the 'state of the art'. The molecular surface of proteins can be displayed and a colour code is employed to show the various structural or chemical features of the protein. As an example the authors show the binding of benzamidine derivatives to the trypsin molecule. These molecular-surface displays are, however, only a translation of a conventional stick-model graphics display which is also available. The figures shown in the paper were obtained directly from the colour display screen and allow a unique insight into the surface topography of protein molecules.

Jones[55] has described a graphics system capable of model building and refine-

[49] P. Murray-Rust and S. Motherwell, *Acta Cryst.*, 1978, **B34**, 2534.
[50] D. Tsernoglou, G.A. Petsko, and A.T. Tu, *Biochim. Biophys. Acta*, 1977, **491**, 605.
[51] S.Y. Wodak, M.Y. Liu, and H.W. Wyckoff. *J. Mol. Biol.*, 1977, **116**, 855.
[52] R.E. Stenkamp, L.C. Sieker, L.H. Jensen, and J.E. McQueen, jun., *Biochemistry*, 1978, **17**, 2499.
[53] A.T. Hagler and B. Honig, *Proc. Nat. Acad. Sci. U.S.A.* 1978, **75**, 554.
[54] R.J. Feldmann, D.H. Bing, B.C. Furie, and B. Furie. *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 5409.
[55] T.A. Jones, *J. Appl. Cryst.*, 1978, **11**, 268.

ment. The system can display electron-density maps and/or molecules and allows the user to orient the views obtained by direct interaction with the 24 potentiometers. Residues can be added to or subtracted from the displayed polypeptide chain and the system uses intensity depth cueing to remove the necessity for showing stereo pairs. The application of the system to three protein structures is discussed.

Rein and co-workers[56] have described their graphics system and have discussed their programs for displaying and manipulating molecules. The authors emphasise the ease of adapting their system to run on other computers together with its relatively low cost. They also consider in some detail the necessary computations for geometrical manipulations such as translations, rotations, and juxtapositioning. Several examples of the application of the programs to the study of the interactions of proteins with DNA are given.

Doolittle and co-workers[57] describe briefly a computer graphics system which they have used to analyse possible structures for the fibrinogen molecule.

3 Immunoglobulins and Lectins

Immunoglobulins.—N.m.r. studies on immunoglobulins have been reported[58] and the known three-dimensional structure of the immunoglobulins has allowed details of antigen binding to be elucidated.

Two structural analyses of Fab fragments have been published.[59,60] The structure of Fab from immunoglobulin New has been refined at 2 Å resolution using model building and real-space procedures.[59] The average shift of all atoms during real-space refinement was 0.62Å and the final model gives good agreement between the co-ordinates and the electron-density map. The *R* factor for the final model is 0.46.

Ramachandran plots show the predominant β-sheet conformation of the protein with glycine residues appearing in some disallowed regions for L-amino-acids. The structural homology of the V_H, V_L, C_H1 and C_L domains was examined and found to be even closer than that seen for Fab from McPC603. The number of alpha carbons which superimpose within 1.5 Å is greater when comparing V_H to V_L and C_H1 to C_L. It is also seen that even though the amino sequence is not used in determining the superimposition, the procedure leads to sequence alignments that reflect the known homologies of the domains. There is also considerable homology between the variable and constant regions. The distances of close contacts between the homologous domains have been determined and the results show that most of the residues which determine the closer contacts between V_H and V_L and between C_H1 and C_L are structurally homologous and highly conserved, or conservatively replaced, in immunoglobulin sequences. The implications of the refined model for the stereochemistry of the hypervariable regions and the antigen combining site are discussed.

[56] R. Rein, S. Nir, K. Haydock and R.D. MacElroy. *Proc. Indian Acad. Sci.*, 1978, **87A**, 95.
[57] R.F. Doolittle, D.M. Goldbaum, and L.R. Doolittle. *J. Mol. Biol.*, 1978, **120**, 311.
[58] S.B. Easterbrook-Smith, P. Zavodszky, K.J. Willan, P. Gettins, and R.A. Dwek, *Biochem. Soc. Trans.*, 1978, **6**, 1126.
[59] F.A. Saul, L.M. Amzel, and R.J. Poljak. *J. Biol. Chem.*, 1978, **253**, 585.

The structure of the Fab fragment of immunoglobulin Kol has been determined from a 3Å resolution electron-density map.[60] Four heavy-atom derivatives were used to determine the phases and data collection was by screenless precession photography. Examination of the electron-density map showed the location of the V and C domains. To locate the heavy and light chains α-carbon co-ordinates of known heavy and light chains were fitted into the electron density. The lack of sequence data has led to side-chain assignments being only tentative. Final model-fitting using the known structures was greatly aided by the use of a computer graphics system.[55] The Fab fragment of Kol has also been fitted into the electron density map of the intact Kol IgG.

The *R*-factor for the refined Fab Kol fragment is 0.36 and it is interesting to note that its quaternary structure is quite different to that seen for Fab fragments in McPC603 and New. Comparisons of the three Fab fragments are described showing that the elbow angles in Fabs McPC603 and New are much more bent than the angle in Fab Kol. In contrast, the elbow angle in Fab Kol is only 8° more bent than in the intact Kol IgG. The intramolecular longitudinal V–C contacts are essentially similar in Fab Kol and Kol IgG but considerably fewer than in other Fab fragments. The lateral associations are identical in Fab Kol and Kol IgG and, although they crystallize in different lattices, they show almost identical head to tail packing of the Fab parts, with the C domains touching the hypervariable regions of the V domains. It is suggested that this strongly conserved mode of aggregation might be reflected in the property of cold precipitation shown by the Kol cryoglobulin.

Two papers have been published describing conformational isomers of the light chains in the Mcg immunoglobulins.[61,62] The λ-type light chains were dissociated from the IgG immunoglobulin isolated from patient Mcg.[61] These light chains were then reassociated into non-covalently bound dimers. The reassembly was completed by allowing the two residues to form an interchain disulphide band and this covalently linked dimer was compared with the Mcg urinary Bence–Jones dimer whose structure is known at 2.3 Å resolution. Comparison of c.d. spectra from the two crystal forms of the Bence-Jones dimer and the light chain dimer shows that they can both exist in at least two conformations and these conformations have been analysed on the basis of the known structure of the Bence–Jones dimer.

The light-chain dimers have also been crystallized[62] both in the non-covalently and covalently linked forms. When crystallized from ammonium sulphate, the light chain dimers adopt the trigonal crystal form characteristic of the native Bence–Jones protein, and an abnormal needle form associated with conformational changes in the vicinity of the interchain disulphide bond. The trigonal crystal forms were compared by difference Fourier syntheses at 3.5 Å resolution. Difference Fourier maps for reassembled covalent dimers showed that the most prominent features were situated along the V domain interface

[60] M. Matsushima, M. Marquart, T.A. Jones, P.M. Colman, K. Bartels, and R. Huber, *J. Mol. Biol.*, 1978, **121**, 441.

[61] J.R. Firca, K.R. Ely, P. Kremser, F.A. Westholm., K.J. Dorrington, and A.B. Edmundson, *Biochemistry*, 1978, **17**, 148.

[62] K.R. Ely, J.R. Firca, K.J. Williams, E.E. Abola, J.M. Fenton, M. Schiffer, N.C. Panagiopoulos, and A.B. Edmundson, *Biochemistry*, 1978, **17**, 158.

and suggested that this region is susceptible to distortion during the reassembly process. In contrast, the solvent-free C domain interface showed hardly any structural alterations. If covalently-linked dimers were dissociated and recrystallized in the unlinked state the native structure was returned. This means that removal of constraints at the *C*-terminal end of the molecule allows the V domain interface, 50 Å away, to undergo readjustments of conformation.

The non-covalent dimers, with blocked sulphydryl groups, showed as a major feature in their difference Fourier maps a negative region in the position of the interchain disulphide bond in the native dimer. The simplest explanation of all the features seen is a 2 Å movement of the carboxyl terminal segment of monomer-2 when the interchain disulphide bond is cleaved and derivatized. There seems to be little movement of this region for monomer 1.

Binding of two molecules of bis(dinitrophenyl)lysine and subsequent removal of the ligands by dialysis led to the dimer crystallizing only as needles. Cleavage and reoxidation of the interchain disulphide bond allowed the protein to crystallize in the trigonal form. This suggests that binding at the V domain can cause conformational changes 50 Å away at the distal regions of the C domain. In functional antibodies binding of antigens at the V domains causes interaction of the complement system with the C_H2 domain. The allosteric effects described in this work may act as a model for the functional antibody system.

Crystallizations of the intact immunoglobulin Zie IgG2 and of the bivalent antigen-binding fragment, $F(ab')_2$, derived from it have been reported.[63] Both the parent protein and the fragment have cryoglobulin properties and the crystals of the two proteins are nearly isomorphous. The intensity distributions of the two crystal forms are strikingly similar indicating that the conformations of the two proteins are very similar. The conclusion that has to be drawn is that the Fc region does not contribute significantly to the diffraction pattern of the IgG2 protein and that the Fab′ regions are very similar in the fragment and parent molecule. Since the two crystal forms are practically isomorphous the Fc region in the intact IgG2 must occupy a volume which contains solvent in the $F(ab')_2$ crystals and can adopt more than one orientation within that volume.

Concanavalin A—Two papers examining the effects of demetallization on concanavalin A[64,65] have been published. Reeke and co-workers[64] have examined the changes occurring on demetallization using the molecular replacement method. Refinement of the structure was carried out by constrained difference Fourier methods with automated calculations of the atomic shifts at each cycle.

Only minor differences are seen between the structures of the two monomers in the crystallographic asymmetric unit. The significant differences between the holo and apoprotein were confined to the metal-binding regions. These differences include a reorganisation and disordering of the loop consisting of

[63] K.R. Ely, P.M. Colman, E.E. Abola, A.C. Hess, D.S. Peabody, D.M. Parr, G.E. Connell, C.A. Laschinger, and A.B. Edmundson, *Biochemistry*, 1978, **17**, 820.

[64] G.N. Reeke, jun., J.W. Becker, and G.M. Edelman. *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2286.

[65] M. Shoham, J.L. Sussman, A. Yonath, J. Moult, W. Traub, and A.J. Kalb, *F.E.B.S. Letters*, 1978, **95**, 54.

residues 7—25. This loop contains all of the direct metal ligands of the protein. In some of the molecules the side-chain of arginine residue 228 appears to move into the metal-binding region where it may compensate in part for the missing positive charge of the metal ion. The *cis*-peptide group observed in the native protein at Ala-207 to Asp-208 is apparently not present in the demetallized protein. The change in conformation of this peptide may be rate-determining in the activation of the demetallized protein and, since the area of Ala-207 is associated with the saccharide-binding site, it seems that the conformational changes induced by metal-ion binding are essential for producing a functional binding-site.

Shoham and co-workers[65] have also reported the crystallization of demetallized concanavalin A. They have determined the structure at 3.2Å resolution with a final *R*-factor of 0.26. They also show the movement of the loop of residues 7—25 and the movement of the side-chain of Asp-19 away from the calcium site. The separation of the side-chain of Asp-19 from that of Asp-10, another of the calcium ligands, renders the site incapable of binding calcium ions and shows that a single carboxy-group is evidently not capable of binding calcium ions. The side-chain of Glu-8 in the absence of a transition-metal ion cannot be seen in the electron-density map suggesting it is capable of free movement. The *cis–trans* conversion reported earlier[64] at 2.8Å is not observed at 3.2Å in this study[65] although it is not ruled out.

4 Oxygen-carrying and Electron Transport Proteins

Haem-protein Chemistry.—Several papers have been published in 1978 covering general aspects of haem-protein stereochemistry.[66,67,68] The synthesis and crystal structure determination of a six-co-ordinate high-spin ferric porphyrin complex have been reported.[66] The compound shows many properties in parallel with those of aquomethaemoglobin but, in contrast to all other known high-spin ferric porphyrins, the iron atom is precisely located in the plane of the porphyrin ring.

The binding of carbon monoxide to myoglobin and haemoglobin has been considered to determine the cause of the deviation of the iron–carbon monoxide geometry from that observed in model systems.[67] Energy calculations have been carried out and possible distortions due to protein–ligand interactions analysed.

Stellwagen[68] has determined the accessibility of the haem group in several haem proteins and shown that the reduction potential is inversely dependent on the exposure of the haem to an aqueous solvent. In all the cases examined the apolarity of the haem crevice is nearly identical and cannot therefore be the primary determinant of the reduction potential as had previously been proposed.

[66] T. Mashiko, M.E. Kastner, K. Spartalian, W.R. Scheidt, and C.A. Reed, *J. Amer. Chem. Soc.*, 1978, **100**, 6354.
[67] D.A. Case and M. Karplus, *J. Mol. Biol.*, 1978, **123**, 697.
[68] E. Stellwagen, *Nature*, 1978, **275**, 73.

Myoglobin.—The structure of myoglobin from the common seal has been reported at 2.5 Å resolution[69] and compared to the protein isolated from sperm whale.[70] The structure determination of seal metmyoglobin[69] has utilized phases determined by the isomorphous replacement method. Data for the native and two heavy-atom derivative crystals were measured using a four-circle diffractometer and refinement of the heavy-atom positions has led to a final R-factor of 0.62 for all the data. The electron-density map could be easily interpreted, the course of the backbone chain and the haem group being very clear. The two histidine residue side-chains, His-E7 and His-F8, were easily located and model-building started at these residues. Although the amino-acid sequence was used in fitting the map, one third of the 153 residues could be identified with certainty from the electron density. For 25 residues, one or more atoms do not fit the observed electron density.

The overall folding is very similar to that of sperm whale metmyoglobin, amino-acid substitutions occurring mainly at or near the non-helical segments of the molecule. In an accompanying paper[70] a preliminary comparison of the sperm whale and seal metmyoglobin molecules is presented. The comparison has included the 127 invariant residues and the backbone chain and β-carbon atoms for the 26 altered residues. Three comparisons were performed: (i) α-carbon atoms, iron atom, and 4 pyrrole nitrogens only; (ii) all the globin atoms with and without the haem atoms; (iii) all the atoms of the helical segments together with the haem atoms. In these comparisons the r.m.s. deviations in atom positions ranged from 1.374—2.27 Å. Several stereo-drawings of the superimposed molecules are presented showing the external location of the 26 altered amino-acid side-chains.

The structure of sperm whale oxymyoglobin has been determined at 2.0 Å resolution.[71] The oxymyoglobin crystals were prepared by exposing deoxy-crystals to air before mounting, and after data collection the crystals were dissolved and the visible spectrum recorded. Data from crystals showing more than a trace of metmyoglobin were rejected. The difference electron density maps using the metmyoglobin or deoxymyoglobin data as native both clearly show the oxygen ligand bound in the bent end-on configuration. The maps also show widespread small changes in the protein structure. Refinement of the structure has preceded to an R-factor of 0.20 and the oxygen site is seen to be about 90 % occupied. The iron atom is displaced by 0.015 Å from the mean porphyrin plane towards the oxygen ligand in a model compound but in the myoglobin structure the iron atom is displaced 0.33 Å towards the proximal histidine, His-F8. The results show that the transition from deoxy- to oxymyoglobin is associated with a movement of the iron atom towards the porphyrin plane, but the magnitude of the movement is smaller than expected. The oxymyoglobin molecule has also been studied by polarized optical spectroscopy of single crystals.[72] Differences are seen in the structure of the complexes with sperm whale and *Aplysia* myoglobins.

[69] H. Scouloudi and E.N. Baker, *J. Mol. Biol.*, 1978, **126**, 637.
[70] H. Scouloudi, *J. Mol. Biol.*, 1978, **126**, 661.
[71] S.E.V. Phillips, *Nature*, 1978, **273**, 247.
[72] M.W. Makinen, A.K. Churg, and H.A. Glick, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2291.

Haemoglobin.—The structures of two mutant haemoglobins have been reported.[73] In haemoglobin Zurich, His-E7(β) is replaced by an arginine residue while in haemoglobin Sydney, Val-E11(β) is replaced by alanine. In haemoglobin Zurich the arginine side-chain is salt-bridged to one of the propionate-group oxygens and not only leaves a large hole where the His-E7 was, but also leaves the entrance to the haem pocket open. Sulphonamide can be model-built into this hole in such a way that the amino-group of the drug can interact with the iron atom. In the haemoglobin Sydney structure a water molecule hydrogen-bonded to the His-E7 is seen together with negative peaks for the missing valine atoms. The water molecule partly fills the gap caused by the replacement of the valine side-chain by alanine and the water seems to be in contact with the porphyrin but not the iron atom. This binding mode is similar to that seen in the α-subunits. The chemical properties of the two mutant proteins are discussed in depth.

A three-dimensional reconstruction of the fibres of sickle cell haemoglobin has been performed using electron microscopy[74] and extended *X*-ray absorption fine-structure measurements have been made on oxy- and deoxyhaemoglobin and of the 'picket fence' porphyrin.[75] Experiments with model compounds have been carried out to determine the effects of the proximal base and ligand reactivity on the haem group[76] and paramagnetic measurements have been performed on different methaemoglobin derivatives to determine the effect of the quaternary structure of the globin on the thermal-spin equilibria.[77]

Haemerythrin.—Three papers concerning the structure of haemerythrin from the marine worm *Themiste dyscritum* have been published[52, 78, 79] The structure of the octameric protein at 2.8 Å resolution has been determined by isomorphous replacement methods.[52] A single derivative, combining isomorphous replacement techniques with anomalous scattering data, gave an overall figure of merit of 0.807. The four subunits in the crystallographic asymmetric unit are distributed between the two octamers in the unit cell and the four subunits in the asymmetric unit were averaged to improve the map. The averaged map showed considerable improvement over the initial map with no breaks appearing in the backbone chain. This averaged map was used in a model-building procedure on a computer-controlled graphics system.

The subunit, 113 amino-acid residues in length, is folded into overall dimensions of 20 × 25 × 40 Å and consists of four nearly parallel $\dot{\alpha}$-helices linked by stretches of extended chain. Eight ligands to the two iron atoms have been identified: 5 histidines, 1 glutamate, 1 aspartate, and 1 tyrosine. The tertiary structure of each subunit appears to be maintained by the iron ligands and hydrophobic interactions between the helices.

[73] P.W. Tucker, S.E.V. Phillips, M.F. Perutz, R. Houtchens, and W.S. Caughey, *Proc. Nat. Acad. Sci. U.S.A.* 1978, **75**, 1076.
[74] G. Dykes, R.H. Crepeau, and S.J. Edelstein, *Nature*, 1978, **272**. 506.
[75] P. Eisenberger, R.G. Shulman, B.M. Kincaid, G.S. Brown, and S. Ogawa, *Nature*, 1978, **274**, 30.
[76] V.S. Sharma, J.F. Geibel, and H.M. Ranney, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3747.
[77] C. Messana, M. Cerdonio, P. Shenkin, R.W. Noble, G. Fermi, R.N. Perutz, and M.F. Perutz, *Biochemistry*, 1978, **17**, 3652.

The structure of the haemerythrin molecule has been refined at 2.8 Å and 2.5 Å resolution.[78] The initial *R* factor at 2.8 Å was 0.385 and has been reduced to 0.253 for the data to 2.5 Å. Difference Fouriers were calculated and interpreted in an automatic process with occasional visual inspection to locate solvent molecules. In the course of the refinement, 49 water molecules have been positioned in the cell. The authors describe the course of the refinement in detail.

Complexes of haemerythrin with azide, thiocyanate, and perchlorate have been examined at 4.0, 3.5, and 3.5 Å resolution respectively.[79] The phases used in the calculation of the difference Fourier maps were calculated from the refined 2.5 Å resolution structure.[78] Azide and thiocyanate are both seen to bind directly to the iron complex from which they both displace a water molecule. Their mode of binding is probably the same as that for oxygen.

The perchlorate ions bind in two different sites separated from the iron complex by polypeptide backbone chain. Both of these sites are on the non-crystallographic two-fold axes which pass through the corners of the square doughnut-shaped octamers. The major perchlorate binding site is near to the peptide amides of Asp-30 which could make hydrogen bonds to the oxyanion. The minor site, near the inner cavity of the molecule, seems to involve the side-chains of Lys-53 and the bound oxyanion blocks access to Cys-50. The perchlorate binding also gives structural changes in the region near the iron complex some 12—15Å away from the oxyanion-binding site. Negative electron density is seen at all four azide-binding sites but no obvious structural model for this process is available.

Erythrocruorin.—The structure of oxy-erythrocruorin at 1.4 Å resolution has been determined using a novel procedure for storing the crystals during data collection.[80] Crystals were mounted in a flow cell and a 1 : 6 mixture of hydrogen sulphide–air was passed over the crystal. Preliminary experiments had shown that this technique converts met-erythrocruorin completely to the oxy- form without any direct hydrogen sulphide–erythrocruorin ligation. Data were collected by rotation photography.

The difference Fourier map calculated between the oxy- and deoxy- forms showed the single oxygen atom bound to the iron atom. The iron atom itself is moved 0.17 Å, and a water molecule is seen 2.5 Å away from the bound oxygen.

The oxygen molecule is bound end-on with an Fe–O–O angle of about 170°, with the outermost oxygen atom being hydrogen-bonded to a water molecule. The iron atom is displaced even more towards the proximal side in the oxy-form but the puckering of the ring does not alter and there is no movement of the haem group parallel to its plane as seen for carbonmonoxyerythrocruorin and cyano-erythrocruorin. The water may be involved in the auto-oxidation process and is also seen in the cyano-erythrocruorin complex.

[78] R.E. Stenkamp, L.C. Sieker, and L.H. Jensen, *Acta Cryst.*, 1978, **A34**, 1014.
[79] R.E. Stenkamp, L.C. Sieker, and L.H. Jensen, *J. Mol. Biol.*, 1978, **126**, 457.
[80] E. Weber, W. Steigemann, T.A. Jones, and R. Huber, *J. Mol. Biol.*, 1978, **120**, 327.

Cytochromes.—The crystal structure of cytochrome c_{551} from *Pseudomonas aeruginosa* has been determined at 2.0 Å resolution.[81] Native data were collected to 2.0 Å resolution and derivative data, for three heavy atom derivatives, were collected to 2.4 Å resolution. An electron density map was calculated using the phases determined at 2.4 Å and a molecular model built in an optical comparator. The co-ordinates of this molecule were used as a starting point for a refinement at 2.0 Å resolution. The refinement involved automated interpretation of difference Fouriers, with occasional manual shifts being applied, alternating with regularization of bond lengths and angles. With the addition of 42 water molecules the final *R*-factor is 0.162.

Comparison of the c_{551} molecule with tuna cytochrome *c* shows several areas of homology. The main area of difference is the deletion of 15 residues seen in the tuna molecule. This deletion causes a tilting of the haem within the polypeptide cage when compared with cytochromes *c*, c_2, and c_{550}. This rotation is about 11° and is similar to that seen in cytochrome c_{555}. The hydrophobic environment around the haem group is almost identical despite the c_{551} having only 82 residues compared to 103 in the tuna protein. In several positions sequentially unrelated residues take up topologically equivalent positions.

The authors outline the possible evolutionary relationships between the cytochrome *c* molecules from the wide range of species studied. The high degree of structural and, in some cases, sequential, homology suggests that this class of cytochromes arose soon after bacteria developed the ability to trap light and use it for chemical purposes. The molecule has been retained in the divergent evolution of present-day photosynthesis and respiration.

The haem ligands in cytochrome b_{562} from *E. coli* have been identified by *X*-ray and n.m.r. methods.[82] In a preliminary report on the structure at 2.5 Å resolution, His-106 is identified as a haem ligand on one face of the haem group. From the electron density map the ligand on the other haem face could be a histidine or methionine, as seen in other cytochromes, or any other side-chain. N.m.r. measurements show that the ligand is in fact a methionine, and knowing this the electron density is good enough to show which of the three methionines it is (Met-7). Further analysis of the structure must await refinement at higher resolution.

Cytochrome *c* Peroxidase.—The location of the haem group and its orientation in yeast cytochrome *c* peroxidase has been determined by *X*-ray methods at 2.5 Å resolution.[83] Data collection for the native and two heavy atom derivatives were collected on a multiwire area detector diffractometer.[3] The overall figure of merit is 0.60.

The electron density map is clear enough to show the molecular boundary and at least 60 % of the polypeptide chain can be fitted unambiguously. The interior of the molecule, including the stretches of chain near the haem group, are more readily interpretable and the ambiguity of some of the chain connections has led the authors to delay full interpretation until the amino-acid

[81] R.J. Almassy and R.E. Dickerson, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2674.
[82] A.V. Xavier, E.W. Czerwinski, P.H. Bethge, and F.S. Matthews, *Nature*, 1978, **275**, 245.
[83] T.L. Poulos, S.T. Freer, R.A. Alden, N.H. Xuong, S.L. Edwards, R.C. Hamlin, and J. Kraut, *J. Biol. Chem.*, 1978, **253**, 3730.

sequence is known. Several features are apparent however. The fifth and sixth haem ligands are the NE2 of a histidine side-chain and a water molecule. One side of the haem crevice is open to solvent and the crevice itself appears to be lined with hydrophobic residues. The haem orientation is similar to that for other haem proteins where the haem group is non-covalently bound.

A difference Fourier map with a crystal soaked in potassium cyanide shows the cyanide ion bound to the iron atom. The Fe–C–N angle is 130° and this is characteristic of cyanide ligands bound to other haem proteins. The binding of cyanide is accompanied by a movement of the iron atom together with a distortion of the haem ring but is not at high enough resolution for the movements to be characterized fully.

Movements of side-chains which may be involved in substrate binding are also seen. Cyanide binding causes movement of a tyrosine side-chain away from the haem by 0.3 Å. This side-chain forms part of a channel connecting the sixth co-ordination position with the surface of the enzyme, and cyanide therefore causes a widening of this channel. In the binding of the bulkier true substrates the tyrosine residue would have to move further to accommodate the sixth ligand.

Thioredoxin.—The molecular structure of the thioredoxin induced by bacteriophage T4 when it infects *E. coli* has been determined at 2.8 Å resolution.[84] Phase determination was carried out using the anomalous native differences, the isomorphous differences of a platinum derivative and the anomalous differences of the platinum derivative.

The electron density map clearly shows the two independent molecules in the asymmetric unit and there seems to be no differences between these two molecules. The molecule is built up from a central β-sheet structure of two parallel strands and two anti-parallel strands and the overall topology is very similar to that of the *E. coli* protein. The *N*-terminal third of the protein folds into the parallel β-sheet strands joined by a helix at the *N*-terminal end of which is the catalytically active disulphide group. The *C*-terminal third of the chain folds into the anti-parallel β-strands and a *C*-terminal α-helix. The central third of the chain joins these two domains and contains an α-helix which lies perpendicularly to the strands of the helix.

Comparison of the T4 and *E. coli* thioredoxins shows that the T4 enzyme is lacking the first 22 residues of the *E. coli* enzyme. Other minor changes including both insertions and deletions are seen at the various external loops.

The locations of the cadmium ions and their role in the production of crystals is discussed in detail. The externally protruded loop region containing the catalytically active disulphide bridge may well be the site of interaction with the ribonucleotide reductase, and the insertion in this loop seen for the T4 protein could explain why it cannot interact properly with the *E. coli* reductase.

Rubredoxin.—The water structure in crystals of rubredoxin has been deter-

[84] B.-O. Söderberg, B.-M. Sjöberg, U. Sonnerstam, and C.-I. Brändén, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 5827.

mined from refinement of a 1.2 Å resolution data set.[85] Starting from a 1.5 Å resolution refinement in which 130 water molecules were positioned the new data set has positioned 127 water molecules. During the refinement, water molecules were removed from the structure if their occupancy fell below 0.3 or their temperature factors exceeded 50 Å^2. Of the 127 water molecules 91 are in positions determined at 1.5 Å resolution. The most tightly bound water molecules are hydrogen-bonded to two or more main-chain nitrogen or oxygen atoms. The water forms extensive hydrogen bond networks bridging the crevices on the molecular surface and between adjacent molecules. The minimum distances of the water sites from the protein surface are distributed about two distinct maxima, the major one at 2.5—3.0 Å and a minor one at 4.0—4.5 Å. Beyond about 6 Å the discrete water structure is seen to merge into the aqueous continuum.

Fluorescent *X*-ray absorption spectra of rubredoxin and its model compounds have been measured using *X*-rays from synchrotron radiation.[86] These measurements have allowed the geometry of the iron–sulphur centres to be determined.

Azurin.—The structure of azurin, a blue copper-containing protein from *Pseudomonas aeruginosa,* has been determined at 3.0 Å resolution.[87] The electron density map was calculated with phases based on a uranyl derivative to 3.0 Å resolution and a platinum derivative to 3.7 Å resolution. The overall figure of merit is 0.75.

The asymmetric unit contains four azurin molecules of 128 amino-acid residues and the shape of the molecule is best described as a flattened pear of dimensions 25 × 30 × 45 Å with the copper atom located at the small end of the molecule.

Although the overall shapes of the molecules were clearly distinguished the quality of the map was not good enough to follow the polypeptide chain in any molecule. Averaging of the electron density has helped to determine the folding of the protein. The molecule is an eight-stranded β-barrel flared at one end with an external flap containing a short stretch of α-helix. Several side-chains are clearly visible including the single tryptophan residue.

The location of the copper atom is unambiguous and Cys-112 and His-117 are identified as copper ligands. Three other potential copper ligands are identified tentatively; Met-121, His-46, and Gln-18. The copper atom is shielded from the solvent by His-117 and Phe-114.

The overall folding of the azurin molecule is topologically equivalent to that of Cu, Zn superoxide dismutase and the authors discuss the similarities of the two proteins.

Plastocyanin.—Plastocyanin, like azurin, is a blue copper-containing protein. The structure of the protein isolated from Poplar leaves has been determined

[85] K.D. Watenpaugh, T.N. Margulis, L.C. Sieker, and L.H. Jensen, *J. Mol. Biol.*, 1978, **122**, 175.
[86] R.G. Shulman, P. Eisenberger, B.K. Teo, B.M. Kincaid, and G.S. Brown, *J. Mol. Biol.*, 1978, **124**, 305.
[87] E.T. Adman, R.E. Stenkamp, L.C. Sieker, and L.H. Jensen, *J. Mol. Biol.*, 1978, **123**, 35.

at 2.7 Å resolution,[88] the asymmetric unit containing one molecule. Diffraction data for the native and three heavy-atom derivative crystals were measured on a diffractometer. The overall figure of merit for the reflections to 2.7 Å resolution is 0.83.

The boundary of the molecule was clearly visible in the electron density map and connected density accounted for all 99 residues of the peptide backbone. The highest peak in the map was assigned to the copper atom and was seen to be joined to four points in the protein. The overall shape of the molecule resembles a flattened cylinder with dimensions 40 × 32 × 28 Å, the walls of the cylinder being formed by an eight-stranded β-sheet parallel to the cylinder axis. The core of the molecule is hydrophobic and contains a cluster of six phenylalanine residues. Polar groups are distributed unevenly on the surface of the molecule and there is a hydrophobic patch on the surface surrounding an opening beneath which the copper site is located.

The copper atom is co-ordinated by the sulphur atoms of Cys-84 and Met-92 and by the δ-nitrogen atoms of the imidazole groups of His-37 and His-87. Three of these residues are located on a tight loop connecting two of the β-strands. At the resolution reported the co-ordination is interpreted as distorted tetrahedral. The authors discuss the biological implications of the structure and analyse the structure in terms of the amino-acid sequences of other species of plastocyanin. A comparison of the plastocyanin molecule with azurin[87] has not yet been performed but an interpretation of the known chemical properties in light of the structure is presented.

5 Lysozyme

The three-dimensional structure of the lysozyme induced by bacteriophage T4 has been determined at higher resolution and, with the inclusion of additional data, more accurately.[89] The electron density map was calculated at 2.4 Å resolution with phases determined from isomorphous replacement and anomalous differences for two derivatives. Full details of the data collection and processing are included in the paper.

Examination of the electron density map allowed the protein backbone to be followed unambiguously and confirmed the preliminary description of the molecule. The molecule is folded into two distinct lobes joined by a long helix. The carboxyl terminal lobe is composed predominantly of α-helices surrounding a hydrophobic core while the *N*-terminal lobe is composed of an irregular region of extended antiparallel β-sheet and some helix. The strong helical character of the protein can be readily seen in a plot of the backbone torsion angles. There are 4 β-bends in the structure, the most obvious being at the ends of the extended β-sheet structure.

The reinterpretation of the electron density map led to some changes in the assigned secondary structure so the authors have compared the new structure

[88] P.M. Colman, H.C. Freeman, J.M. Guss, M. Murata, V.A. Norris, J.A.M. Ramshaw, and M.P. Venkatappa, *Nature*, 1978, **272**, 319.

[89] S.J. Remington, W.F. Anderson, J. Owen, L.F. Ten Eyck, C.T. Grainger, and B.W. Matthews, *J. Mol. Biol.*, 1978, **118**, 81.

with the results obtained from 11 structure predictions. The conclusion from this comparison is that current predictive methods can be very misleading.

Several mutants of the bacteriophage T4 are known which produce altered lysozymes and in some cases the changes in the amino-acid sequence have been determined. The position of these mutations with respect to the stereochemistry of the enzyme have been analysed on the basis of their position in the amino-acid sequence. The *N*-terminal pentapeptide is located on the surface of the molecule and this is reflected in the fact that several insertions are known in this region which have little or no effect on activity. In contrast, deletion of Phe-4 substantially reduces the catalytic effectiveness of the enzyme. This effect seems to arise not from the loss of Phe-4, which is totally in the solvent, but from the consequential replacement of the partially buried Ile-3 by the hydrophilic side-chain of Asn-2.

The cleft in the molecule has been shown to be the active-site region and studies of mutant enzymes have shown that Asp-20 is located in this cleft and is essential for catalysis. Residues 34—41 form an open irregular loop on the surface of the molecule and are not involved in any obvious catalytic or structural role. Many changes to the amino-acid sequence in this region are known, including an insertion, with little effect on enzyme activity.

The long helix, residues 60—79, is as expected a site of few alterations. Replacement of Gln-69, which is fully exposed to the solvent, by serine or tyrosine in two mutants would not be expected to affect the properties of the enzyme.

Gln-105 is located in the wall of the active-site cleft and seems to make van der Waals contact with the substrate when it binds to the enzyme. In a mutant where Gln-105 is replaced by a serine residue the enzyme is fully active suggesting that Gln-105 may be involved in substrate binding rather than catalysis. In a mutant where Gln-105 is replaced by tyrosine the enzyme is almost totally inactive.

The *C*-terminal residues 162—164 are fully exposed to the solvent and residues 163 and 164 in particular appear to be disordered in the crystal. It is seen that removing these two residues using carboxypeptidase A has little effect on the enzyme.

Warshel has reported quantitative studies on the energetics of the lysozyme reaction[90] based on the effects of charge stabilization in aqueous solutions and in the active-site of enzymes.

6 Proteolytic Enzymes

Trypsin and Trypsinogen.—The crystal structures of bovine trypsinogen in a complex with pancreatic trypsin inhibitor (PTI) and with and without the dipeptide isoleucylvaline (Ile-Val) have been reported.[91] The trypsinogen–PTI complex crystallizes in large crystals isomorphous with trypsin–PTI crystals. *X*-ray data to 1.9 Å resolution were measured by rotation photography and after merging the final data set, contained 69 % of all the data to 1.9 Å

[90] A. Warshel, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 5250.
[91] W. Bode, P. Schwager, and R. Huber, *J. Mol. Biol.*, 1978, **118**, 99.

resolution. For the ternary complex, trypsinogen–PTI–Ile-Val, 66 % of the data to 1.9 Å resolution were present in the final merged set.

Both crystal structures have been refined crystallographically, using the refined trypsin–PTI complex as a starting model. The final *R*-factors are 0.25 and 0.26 for the binary and the ternary complexes respectively. The mean main-chain atom deviations for the three complex structures are about 0.15 Å. In contrast, the mean deviation between the complexed and free trypsinogen was 0.28 Å, reflecting the influence of crystal packing and the changes upon complex formation. The binding of PTI causes the trypsinogen to adopt a trypsin-like conformation with the side-chain of Asp-194 turning round as is seen in the zymogen activation process. This movement leads to the activation domain becoming more rigid, the formation of the specificity pocket and of the Ile-16 binding cleft. Interactions between PTI and trypsinogen are the same as seen in the trypsin complex and, as in free trypsinogen, the *N*-terminus, including residues Val-10 to Gly-18, is mobile and sticks out into the solution. The autolysis loop in the trypsinogen–PTI complex is more mobile than in the trypsin–PTI complex, but upon binding of the dipeptide Ile-Val causes the trypsinogen–PTI complex to become almost indistinguishable from the trypsin–PTI complex. Difference Fouriers between trypsinogen–PTI–Ile-Val and trypsin–PTI show no interpretable features whereas the trypsinogen–PTI–Ile-Val/trypsinogen–PTI difference Fourier clearly shows the position of the dipeptide in the Ile-16 cleft.

The use of an interactive graphics facility has been used to examine the active-site of bovine trypsin and its interaction with benzamidine[54] The system, which can use different colours to represent different areas of a molecule, allows a unique ability to determine the identity and the chemical properties of the groups involved in the interactions and can also carry out a quantitative calculation of the physico-chemical forces involved in the binding.

Bode and Huber[92] have compared the structure of trigonal trypsin with orthorhombic trypsin and polyethylene glycol-grown trypsinogen with trigonal trypsinogen. The structures have been compared by difference Fourier methods in an attempt to provide information on the structural changes due to the activation process (trigonal trypsin–trigonal trypsinogen) and those due to lattice forces (orthorhombic trypsin–trigonal trypsin). Although there are a limited number of significant alterations in structure, the authors could find no way to correlate the changes seen with the two processes. Even more striking is that there is very little difference in the structures of trypsinogen grown in 2.4 M magnesium sulphate and 30 % polyethylene glycol (mol. wt. >2000) despite the large differences in ionic strength and dielectric constant. An identical binding-site for calcium has been confirmed for trypsin and trypsinogen and the calcium ions can be replaced by ytterbium ions.

Chymotrypsin.—The binding of toluenesulphonamide and of pipsylamide to α-chymotrypsin has been studied.[93] Each compound binds at the aromatic specificity binding-site but when bound their orientations vary by 90° from that

[92] W. Bode and R. Huber, *F.E.B.S. Letters*, 1978, **90**, 265.
[93] A. Tulinsky, I. Mavridis, and R.F. Mann, *J. Biol. Chem.*, 1978, **253**, 1074.

usually associated with substrate-like molecules suggesting a non-productive binding mode. The sulphonamide group of toluenesulphonamide, for example, does not approach the side-chain of the active-centre serine, Ser-195. In addition the pipsylamide derivative displays a second binding-site 22Å from the active-site. This site is intramolecular in the vicinity of three tryptophan residues and two prolines. Unlike previously reported inhibitors, neither toluenesulphonamide nor pipsylamide cause any significant changes in the native protein structure. The authors display the changes caused by tosylation and transition state analogues in a new way.

The effect of denaturants on the structure of α-chymotrypsin has been examined at 2.8 Å resolution.[94] The effects of 2.0 M guanidine hydrochloride and of 3.0 M urea on the chymotrypsin molecules in ammonium sulphate-grown crystals were observed. The guanidine hydrochloride derivative displayed changes which occurred exclusively on the surface of the protein. The difference Fourier peaks represented mostly small changes in the protein structure of surface groups and in the adjacent solvent regions, but some showed convincing evidence for bound guanidinium-ions. The urea difference map showed not only surface changes to the protein structure but also numerous changes to the hydrophobic interior of the chymotrypsin molecules. Urea binding was seen to occur in one of two modes, either causing or accompanying structural changes in one mode, or binding with no accompanying protein changes in the second mode. Examples of both these modes of urea binding were seen in both the surface regions and in the hydrophobic interior of the molecule.

Elastase.—The atomic structure of porcine pancreatic elastase at 2.5 Å resolution has been published.[95] The data from 3.64—2.50 Å resolution were measured with a four-circle diffractometer for the four derivatives used in the 3.5 Å resolution structure determination. The enzyme inhibited with *p*-toluene sulphonyl fluoride was used as the native structure to aid isomorphism. The mean figure of merit averaged over all the high resolution data was 0.858, the excellent phasing being aided by full occupancy of the heavy-atom sites, the high degree of isomorphousness between derivatives and the need for only two crystals of each derivative avoiding scaling errors.

Examination of the 2.5Å resolution electron-density map showed greatly increased clarity in all areas. Unlike the 3.5 Å resolution map carbonyl oxygens could be seen for nearly all the peptide groups, only four being absent. The inserted loop of four residues is seen to project from the molecular surface and has no contacts with neighbouring molecules. In parts its electron density is diffuse suggesting a greater degree of thermal motion for this loop.

Amino-acid side-chain density can be clearly seen and the dimpling of six-membered rings and forking of branched side-chains is obvious evidence of the better quality of the 2.5 Å resolution map. The sulphur atoms of the disulphide bridges are just resolved and appear at twice the main-chain density.

[94] L.S. Hibbard and A. Tulinsky, *Biochemistry*, 1978, **17**, 5460.
[95] L. Sawyer, D.M. Shotton, J.W. Campbell, P.L. Wendell, H. Muirhead, H.C. Watson, R. Diamond, and R.C. Ladner, *J. Mol. Biol.*, 1978, **118**. 137.

The tosyl group and the bound sulphate-ion appear with great clarity in the map. Many small isolated peaks are seen in the solvent spaces, at the protein's surface, and in small internal cavities in the protein itself. These have been interpreted as water molecules. In the case of eight surface-residues, the extremities of long side-chains are not represented by density, and in the case of ten others by only diffuse density, suggesting the different degrees of mobility for these side-chains.

The measured co-ordinates were standardized to generate a model in which the r.m.s. difference of the co-ordinates on the starting model was 0.188 Å. This model has been subjected to several cycles of real-space refinement and further refined by an energy minimization procedure.

The improved quality of the 2.5 Å resolution map has allowed much more accurate positioning of potential hydrogen-bonding groups. Most of the internal polar groups form hydrogen bonds either with other protein groups or with one or more of the 25 water molecules which lie inside the protein in positions inaccessible to free solvent.

Comparison of the elastase structure with chymotrypsin shows an r.m.s. difference between main-chain atoms of 1.80 Å, the major differences being located in areas where the insertions and deletions occur. Despite the close similarities in structure there is only 39 % identity of amino-acid sequence and 51 % homology of sequence.

The authors give a detailed description of the molecule's conformation and compare main-chain hydrogen-bonding patterns for elastase and chymotrypsin. They also detail the distribution of various classes of side-chains within the structure and compare the hydrogen bonding of side-chains in elastase and chymotrypsin. The positions of 111 external and 25 internal water molecules are also described.

The structure of the tosyl-enzyme derivative has been compared to that of the native enzyme at 2.5 Å resolution. The conformational differences seen are restricted to the active-site and the binding of the tosyl group displaces a sulphate ion and the imidazole side-chain of His-57 which further results in the displacement of two external water molecules. Comparison of the active-sites of elastase and chymotrypsin gives an r.m.s. difference between main-chain and side-chain atoms of the seven residues involved of 0.7 Å. The authors then describe in detail the interactions involved in the active-site and their consequences in relation to the function of elastase with respect to the other serine proteases.

Streptomyces Protease A.—An electron density map at 2.8 Å resolution of protease A from *Streptomyces griseus* has been reported.[96] The map has been calculated with phases determined using four heavy-atom derivatives and the phase determination has resulted in an overall average figure of merit of 0.82. The map shows each enzyme molecule clearly separated from its neighbours. Solvent regions up to 15 Å in width can be seen on the map.

Interpretation of the course of the polypeptide chain was carried in an optical comparator. The most pronounced features were the disulphide bonds

[96] G.D. Brayer, L.T.J. Delbaere, and M.N.G. James, *J. Mol. Biol.*, 1978, **124**, 243.

and the single methionine residue, and the single tryptophan residue was similarly easy to find. When all the non-hydrogen atoms had been located and their co-ordinates measured, a mathematical model-building procedure was used to regularize the structure. The r.m.s. difference for all non-hydrogen atom positions between the measured and idealized co-ordinates was 0.25 Å.

In a second paper[97] the authors describe the molecular architecture in detail and compare it with chymotrypsin. The overall structure is very similar to that of *Streptomyces griseus* Protease B (SGPB) as would be expected for two closely homologous proteins. Sequence homologies between the bacterial serine proteases and the pancreatic serine proteases are minimal, only 21 % identity at most, and would not be expected to give great structural homology. In fact very pronounced similarities are seen.

Streptomyces griseus Protease A (SGPA) is folded into two hydrophobic domains, the junction of which forms a shallow depression on the surface containing the active-site region. Each domain consists of a 6-stranded barrel-like structure of β-sheet in an overall conformation similar to that seen for chymotrypsin. All the peptide bonds are *trans* with one exception, Phe-94—Pro-99, this proline being located at a β-bend. A *cis*-peptide linkage has also been observed in this position in SGPB and α-lytic protease. The authors give a detailed analysis of the secondary structural features of the enzyme and also give a detailed comparison of the SGPA molecule with chymotrypsin. Topological homologies are seen to be much more extensive than sequence homologies and leave little doubt that these two enzyme families have a common ancestral origin. Residues near the active-site are the most similar and in fact the four catalytically important residues, Ser-214, Asp-102, His-57, and Ser-195, are in almost identical conformations. The differences in the active-site are of interest. Since there are no known zymogen precursors of the bacterial enzymes it is interesting that the residue involved in the activation process, Asp-194, is seen to be involved in a permanent ion-pair with the guanidinium group of Arg-138. The side-chain of Asp-102 is buried but in a highly polar environment being an acceptor of four hydrogen bonds. This may lead to a lowered p*Ka* as is seen for the buried aspartate residue in the acid proteases. The side-chain hydroxy-group of Ser-195 is not in a position to form a hydrogen bond to the imidazole group of His-57 and there is no evidence for Ser-195 adopting the conformation seen in chymotrypsin.

Endothia Protease.—Two papers describing work on the acid protease isolated from *Endothia parasitica* have been published.[98, 99] In the first paper[98] a technique for cross-linking the crystals using glutaraldehyde is described. The high ammonium sulphate concentrations necessary to produce the crystals had interfered with the search for useful heavy-atom derivatives and the glutaraldehyde cross-linking has allowed the crystals to be transferred to water. Three

[97] G.D. Brayer, L.T.J. Delbaere, and M.N.G. James, *J. Mol. Biol.*, 1978, **124**, 261.
[98] C. Wong, T.J. Lee, T.Y. Lee, T.H. Lu, and C.S. Hung, *Biochem. Biophys. Res. Comm.*, 1978, **80**, 886.
[99] C. Wong, T.J. Lee, T.Y. Lee, T.H. Lu, and C.S. Hung, *Biochem. Biophys. Res. Comm.*, 1978, **80**, 891.

derivatives have been prepared using these cross-linked crystals and have been used in the structure determination.

The structure determination at 3.5 Å resolution of the *Endothia* protease has also been published in 1978.[99] The three derivatives prepared using the cross-linked crystals[98] and a fourth derivative prepared in the normal mother liquor have been used in the phase determinations and give an overall average figure of merit of 0.85. The structures of both the cross-linked and the native crystals have been determined and compared. The main chain can be easily followed and most of the large side-chains are clearly visible. A break in the continuity of the chain near the *N*-terminus and an ambiguity of electron density near the base of the active-centre cleft are apparent in the electron-density map. The supposed active-site aspartate residues, 33 and 223, both show side-chains protruding into the active-centre cleft. Differences in structure amplitude between the cross-linked and native data averaged 10 % with a mean change in phase angle of 40°. These differences are reflected in changes in the protruding surface structure where the cross-linked map gave more detail extending further from the surface. The cross-linked map shows 326 residues whereas only 315 can be positioned in the native map. Only one disulphide could be identified in the cross-linked map. Comparison of the two structures shows that even though the environment of the protein molecules has been changed from 2.7 M ammonium sulphate to water, the folding of the polypeptide chain is not observably affected and the cross-linked enzyme still retains biological activity.

Acid Proteases.—Tang and co-workers[100] have analysed the structures of penicillopepsin, Rhizopuspepsin, and Endothiapepsin and shown r.m.s. deviations between topologically equivalent α-carbons of 1.35 Å. The sequences of pepsin and chymosin strongly suggest that they also have similar secondary and tertiary structures.

The acid proteases show a bilobal structure with a central cleft where the active-site is located. The two lobes, of 175 and 151 residues, form predominantly β-sheet structures. The structure shows a two-fold symmetry axis positioned between the antiparallel β-strands i and q and relates the structure in the *N*-terminal lobe to the *C*-terminal lobe. Several structural features are seen to be topologically equivalent in both the secondary and tertiary structures. The two-fold axis is found to be inexact, involving a rotation of 174.6° and a translation of 1 Å along the rotation axis. There are 61 topologically equivalent residues in penicillopepsin and many of the differences between the lobes correspond to extensions or deletions of β-loops connecting strands in the β-sheet, these changes occurring near the surface of the enzyme and not interfering with the tertiary fold. The authors conclude that the acid proteases evolved by gene duplication of an ancestral protein of about 150 residues. Before the gene duplication the protein may well have existed as a dimer, this explaining the very hydrophobic nature of the contact between the antiparallel sheets i and q. This analysis shows once more that sequence homology is not

[100] J. Tang, M.N.G. James, I.N. Hsu, J.A. Jenkins, and T.L. Blundell, *Nature*, 1978, **271**, 618.

essential for structural homology and shows that searches of topological homology are much more likely to succeed.

Martin[101] has re-examined the proposed mechanism for the acid proteases and points out that the amide nitrogen of the scissile bond is incapable of accepting the tyrosine proton.

Ribonuclease.—The structure of ribonuclease S complexed with 2′-deoxy-2′-fluoro-uridyl-3′,5′-adenosine (FUA) has been determined at 2.5 Å resolution.[102] Data collection was by diffractometer using a single crystal. Examination of the difference electron density map allowed the conformation of the dinucleoside phosphate to be determined. The uridine fragment is in the *anti* conformation while the adenosine fragment is in the *syn* conformation. Several hydrogen bonds can be inferred from the structure and others, known to exist in other complexes with substrates, are seen to be absent.

Binding of the FUA induces noticeable conformational changes in the protein some of which have been seen for the complexes with 3′-CMP and 2′-CMP. One change specific to FUA binding is the movement of the imidazole group of His-119 to a position where it can interact with the phosphate group of the dinucleoside. The results suggest that the structure of the complex corresponds well with that of the enzyme–substrate productive complex.

7 Glycolytic Enzymes

Phosphorylase *a*.—Four papers describing the binding of substrates and effectors to phosphorylase a have been published.[103—106] The binding of glucose, maltose, maltotriose, maltoheptaose and maltodextrin have been examined.[103] Glucose was found not to bind at the oligosaccharide site even when the crystals were soaked in 0.5 M glucose solutions. Maltose was seen to bind in subsites B and C of the oligosaccharide binding-site while maltotriose binds in subsites A, B, C, and D. Maltoheptaose at low concentration also binds at subsites A—D but at higher concentrations occupies all 5 subsites, A—E. The difference Fourier map shows the $\alpha(1\text{—}4)$ chain direction proceeds from site E—A. The binding of maltodextrin, chain length 11.2, is also seen only in the sites A—E suggesting that the enzyme can only positively locate 5 residues. Much of the interpretation of these studies awaits an accurate atomic model of the enzyme.

The binding of nucleotide derivatives to phosphorylase *a* has been examined by *X*-ray crystallography and kinetic studies.[104] Two binding-sites are located in the molecule: an AMP activator site which has a high affinity for nucleotide, and a nucleoside site which binds nucleotides with lower affinity than nucleosides. The AMP site also binds inorganic phosphate, glucose-1-phosphate, and other phosphoryl compounds. The binding at the nucleoside site inhibits the phosphorylase *a* activity by allosteric competition with glucose-1-phosphate. Both the positive and negative effector sites are distant from the active-site: the

[101] R.B. Martin, *Nature*, 1978, **271**, 94.
[102] A.G. Pavlovsky, S.N. Borisova, V.V. Borisov, I.V. Antonov, and M.Y. Karpeisky, *F.E.B.S. Letters*, 1978, **92**, 258.
[103] P.J. Kasvinsky, N.B. Madsen, R.J. Fletterick, and J. Sygusch, *J. Biol. Chem.*, 1978, **253**, 1290.

AMP activator site is 30 Å away while the nucleoside site is 10 Å away. The virtual substrate 5-thio-α-D-glucopyranosyl phosphate, a competitive inhibitor with respect to glucose-1-phosphate, binds at two sites per monomer at low concentration. These are the AMP and glucose sites. In double ligand studies, allosteric competition between inosine, bound at the nucleoside site, and 5-thio-α-D-glucopyranosyl phosphate, bound at the glucose site, is seen. This result is consistent with the glucose site, adjacent to the pyridoxal 5′-phosphate co-enzyme, being the binding locus for substrate. Nucleotides, such as adenosine, are effective at very low concentration in preventing substrate binding at the active-site, presumably by stabilizing an inactive conformer of the enzyme.

The allosteric transitions of phosphorylase *a* have been analysed in structural terms.[105] Crystals of glucose-inhibited phosphorylase *a* shatter when exposed to substrates, but cross-linking with glutaraldehyde can prevent this shattering. The crystals undergo the transition slowly and do not change space group. The subunits of the protein dimer move apart by about 2 Å and several long segments of the chain in the *N*-terminal half of the protein show significant movements. These movements tend to link the four major ligand-binding sites, either directly or by intersubunit excursions. For example, an α-helix, residues 49—75, which is near the subunit interface and forms part of the AMP-activator site, moves inward by 5 Å along its entire length. The phosphorylated serine residue, Ser-14, is located on the outer surface of this helix. All these changes bear obvious relationships to the known co-operative properties of the enzyme. The stereochemistry of the Ser-14 phosphate residue provides a molecular explanation for the action of phosphorylase *a* phosphatase. The phosphorylase *a* is complexed *in vivo* with AMP and substrates so that Ser-14 phosphate is tucked away into a fold of the protein where it is not available to the phosphatase. The phosphatase is, however, bound to the phosphorylase molecule. The binding of glucose causes the Ser-14 phosphate to be exposed to the action of the phosphatase and when phosphorylase *b* is formed the phosphatase, which binds poorly to phosphorylase *b*, is then free to activate glycogen synthetase.

The synergistic regulation of phosphorylase *a* by glucose and caffeine have been examined kinetically and interpreted in structural terms.[106] The caffeine binding-site is an extended hydrophobic slot on the side of the protomer, and this pocket shows fairly low specificity, being able to bind more than a dozen related fused-ring compounds. The synergism with glucose may be due to the fact that part of this pocket is formed by a loop of chain which moves 5 Å when glucose is replaced by the substrate, glucose-1-phosphate.

Phosphorylase *b*.—The binding of various metabolites to phosphorylase *b* has been examined at low resolution.[107] Five binding-sites have been identified using crystals of phosphorylase b in the presence of inosine monophosphate.

[104] P.J. Kasvinsky, N.B. Madsen, J. Sygusch, and R.J. Fletterick, *J. Biol. Chem.*, 1978, **253**, 3343.
[105] N.B. Madsen, P.J. Kasvinsky, and R.J. Fletterick, *J. Biol. Chem.*, 1978, **253**, 9097.
[106] P.J. Kasvinsky, S. Shechosky, and R.J. Fletterick, *J. Biol. Chem.*, 1978, **253**, 9102.
[107] L.N. Johnson, I.T. Weber, D.L. Wild, K.S. Wilson, and D.G.R. Yeates, *J. Mol. Biol.*, 1978, **118**, 579.

The active-site, which is seen to bind glucose-1-phosphate, maltotriose, and arsenate, is located close to the subunit interface of the phosphorylase dimer. The allosteric AMP-activator–ATP-inhibitor site is seen to be located very close to the active-site. The inhibitor, glucose-6-phosphate, also binds close to the active centre and causes extensive conformational changes in the protein. These are the largest changes seen for any metabolite but must await high resolution studies for a detailed analysis. A glycogen binding-site is located on the surface of the molecule and is 50—60 Å away from the active-site measured over the surface of the subunit. A second glucose-1-phosphate site has been located in the interior of the molecule. The significance of this site is not yet understood but its position suggests that it may have a key role in the enzyme's functioning. Several of the above sites are located in the same places as seen for phosphorylase *a*.[103—106]

High resolution studies on the structure of phosphorylase b and its complexes with glucose-1-phosphate, a substrate, and with maltotriose, a glycogen analogue, have been reported.[108] An electron density map at 3.0 Å resolution has been calculated and 801 of the 841 α-carbon positions have been located. The molecule is folded into three domains: the *N*-terminal domain containing residues 1—310; the glycogen-binding domain containing about 160 residues; the *C*-terminal domain of approximately 360 residues. The subunit shape is compact apart from two loops which cross the two-fold axis and extend into the symmetry-related subunit of the dimer. One of these loops contacts the nucleotide-binding site of the symmetry related subunit and may be concerned in the control properties of the enzyme. The overall folding appears to be very similar to that of phosphorylase *a* except for the *N*-terminal 19 residues which cannot be located in the phosphorylase *b* map.

The pyridoxal phosphate site is located in the interior of the molecule in a hydrophobic region in a position the same as that seen for phosphorylase *a*. Glucose-1-phosphate is seen to bind at the catalytic site which is located between the three domains at the surface of the molecule. Small conformational changes are seen in the molecule. Glucose-1-phosphate also binds at the nucleotide-binding site located near the subunit interface and the glucose is in contact with one of the loops from the symmetry related molecule. This site is 33 Å away from the catalytic site. Maltotriose is seen to bind on the surface of the molecule in the glycogen-binding domain. No conformational changes are seen at any distance from this site. This glycogen storage site is over 30 Å away from the catalytic site and 40 Å away from the nucleotide-binding site. These studies have been reported in a second publication.[109]

Hexokinase.—Three papers on the structure of yeast hexokinase B have appeared in 1978.[110, 111, 112] In consecutive papers the interpretation of a 2.5 Å resolution electron-density map[110] and the subsequent refinement of the atomic co-ordinates and sequence[111] have been reported.

[108] I.T. Weber, L.N. Johnson, K.S. Wilson, D.G.R.Yeates, D.L. Wild, and J.A. Jenkins, *Nature*, 1978, **274**, 433.

[109] L.N. Johnson, K.S. Wilson, I.T. Weber, D.L. Wild, J.A. Jenkins, and E.A. Stura, *Biochem. Soc. Trans.*, 1978, **6**, 1108.

[110] C.M. Anderson, R.C. McDonald, and T.A. Steitz, *J. Mol. Biol.*, 1978, **123**, 1.

[111] C.M. Anderson, R.E. Stenkamp, and T.A. Steitz, *J. Mol. Biol.*, 1978, **123**, 15.

The 2.5Å resolution electron-density map was calculated using phases determined from 8 isomorphous heavy-atom derivatives. The overall average figure of merit is 0.74. The map was readily interpretable in areas of regular secondary structure but in other areas there are breaks in the density or lower density than was expected. Some side-chain density is visible for about 80 % of the residues and for about 30 % of the residues the amino-acid could be determined unambiguously. The map was interpreted with 2944 atoms of the 3596 expected from the amino-acid analysis. The *R*-factor for this model was 0.48 and the overall folding is the same as that derived from earlier studies with the exception of a few insertions and deletions. The major difference is an area previously interpreted as a three-strand β-sheet which is now seen to contain one α-helix and two pieces of random chain. The previous interpretation required a parallel two-stranded β-sheet to be connected in a left-handed way, an exception to the general folding rule.

The refinement of the model at 2.1 Å resolution has been reported.[111] The atomic co-ordinates were adjusted by real-space refinement into the electron density map followed by automated difference Fourier refinement and restrained parameter structure factor least-squares refinement. Visual inspection was carried out periodically to evaluate any necessary sequence changes. The refined model contains 3293 atoms out of the expected 3596 atoms together with 152 water molecules and has an *R*-factor at 2.1 Å of 0.25. There is also better agreement between the *X*-ray and chemically determined amino-acid compositions.

Some preliminary identification of the binding-site of *o*-toluoylglucosamine has been possible. The three residues involved seem to be two aspartates or asparagines and a serine. A sulphate ion bound to serine residues lies near the inhibitor. Several criteria show an improvement of the model after refinement: the *R*-factor has decreased to 0.25 at 2.1 Å from 0.48 at 2.5 Å; there has been a significant decrease in the number of disallowed backbone torsion angles; the overall addition of protein atoms has allowed amino-acid sequences from chemical work to be fitted in the refined model.

The binding of the inhibitor *o*-toluoylglucosamine has been examined in greater detail.[112] The difference Fourier maps show that the sugar binds in the chair equatorial conformation with its 1-hydroxy-group axial (α-anomer). The carboxy-group of Asp-189 appears to be hydrogen bonded to both the 6- and 4-hydroxy-groups of the sugar. Since the chemical sequence is not known the rest of the binding is seen to be Asx-188 and Asx-215 hydrogen bonded to the sugar 4-hydroxy-group while the 3-hydroxy-group interacts with both Asx-188 and Asx-245. In the presence of the sugar, the side-chain carboxy-group of Asp-189 is completely excluded from solvent and may act as a general base to promote the attack of the 6-hydroxy-group on the γ-phosphate of ATP.

Glucose binds to the enzyme in the same orientation and configuration as the sugar moiety of the inhibitor. The fact that *o*-toluoylglucosamine is not a substrate may be due to the fact that the glucose-induced rotation of the subunits cannot be produced because of the bulky toluoyl group. These conformational changes must be essential for catalytic activity.

[112] C.M. Anderson, R.E. Stenkamp, R.C. McDonald, and T.A. Steitz, *J. Mol. Biol.*, 1978, **123**, 207.

Thiol groups do not apear to have a direct role in catalysis or substrate binding. One thiol is 5.5 Å away from the 3-hydroxy-group and hydrogen bonded to three of the substrate-binding Asx residues. Modification of this thiol would disrupt the sugar-binding site explaining known inhibition results. A sulphate ion, bound by two serine side-chains, is located 5.5 Å away from the glucose 6-hydroxy-group. If the γ-phosphate of ATP was positioned here it would still be too far from the glucose 6-hydroxy-group for direct phosphoryl transfer.

The glucose-induced conformational changes in hexokinase A from yeast have been examined at 4.5 Å resolution.[113] The structure was solved by the multiple isomorphous replacement method using two heavy-atom derivatives. This model was then extended in resolution using the refined structure of the hexokinase B molecule. The changes induced by glucose are caused by a rotation of one lobe of the monomer by 12° with respect to the other lobe. Backbone chain movements of up to 8 Å are seen which close the intersubunit cleft where glucose binds. Since this change is essential for catalysis[112] it provides an example of induced fit. The surface area of the hexokinase A–glucose complex exposed to solvent is smaller than that seen for the native hexokinase B molecule. Using these differences it has been calculated that the hydrophobic effect alone favours the active conformation of hexokinase in the presence and absence of glucose.

6-Phosphogluconate Dehydrogenase.—The 2.6 Å resolution structure of 6-phosphogluconate dehydrogenase and binding studies at 6.0 Å resolution have been reported.[114] A preliminary examination of the map confirms the molecular shape determined at 6.0 Å resolution. The binding of $NADP^+$, NADPH and halogenated derivatives of $NADP^+$ has been examined and binding was observed in the cleft region of the molecule. The location of 6-phosphogluconate in the abortive enzyme–NADPH–6-phosphogluconate complex was also possible. At this low resolution stage the conformation for the bound co-enzyme seems to be different from that of NAD^+ in lactate dehydrogenase.

Triose Phosphate Isomerase (TIM).—The three-dimensional structure of chicken triose phosphate isomerase has been analysed and compared to the structure of lactate dehydrogenase.[115] The structure of TIM contains a high percentage of secondary structure: 55 % α-helix; 23 % β-sheet; 3 % β-bends. The eight β-strands in the structure run parallel to each other and the strands form consecutively in the sheet from a to h. The final strand is hydrogen bonded to g and a, forming a β-barrel structure. Strands vary in length from 4 to 9 residues. The structure contains 12 helices, 11α-helices and one 3_{10} helix, eight of which run roughly antiparallel to the β-strands. The residues connecting the β–α structures form loops, β-bends, or additional shorter helices at right angles to the main helices and sheet.

[113] W.S. Bennett jun., and T.A. Steitz, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4848.
[114] M.J. Adams, I.G. Archibald, J.R. Helliwell, and S.E. Jenkins, *Biochem. Soc. Trans.*, 1978, **6**, 1112.
[115] D.C. Phillips, M.J.E. Sternberg, J.M. Thornton, and I.A. Wilson, *J. Mol. Biol.*, 1978, **119**, 329.

The topology of the $\beta\alpha$ and $\beta\alpha\beta$ units are considered together with α–α and β–β interactions. It is seen that the eight-fold repeat of the $\beta\alpha$ secondary structure does not extend to the details of the tertiary interactions between the strands and helices. Quantitative comparisons of the individual $\beta\alpha$ units within the TIM molecule were performed and are discussed in detail. The TIM structure has also been compared with secondary structural elements of LDH in terms of topological equivalence and amino-acid sequence relatedness. The results overall indicate no evidence for gene replication with TIM even though there is an overall repeating fold of eight $\beta\alpha$ and the eight $\beta\alpha\beta$ units. There seems to be a closer similarity between some structural features of TIM and LDH and together with some sequence similarities suggest a possible divergent evolution from a common ancestor. The evidence for this however is far from conclusive.

Pyruvate Kinase.—The structure of cat-muscle pyruvate kinase has been reported at 2.6 Å resolution[116] and its topology compared with related proteins. Each of the four subunits is composed of about 500 amino-acid residues and the polypeptide chain is folded into three distinct domains. Domain A is the largest and is composed of about 220 residues which form a β-barrel of eight parallel strands. Adjacent strands are connected by α-helices which form an outer cylinder co-axial with the β-barrel. Domain B is composed of about 100 residues with an antiparallel β-sheet as its main secondary structural feature. Domain C consists of the *C*-terminal 120 residues of the molecule, the first part of which comprises two long antiparallel α-helices. The rest of domain C is folded up into a five-stranded β-sheet flanked by α-helices. The first three strands of sheet and two helices are folded into the well known mononuleotide-binding fold. Domain A shows close similarity with the folding pattern seen in triose phosphate isomerase. Domain B occurs at the position of the largest loop in triose phosphate isomerase while domain C is an extension at the *C*-terminus.

Quantitative comparisons show that domain A and triose phosphate isomerase folding similarities are more significant than the similarities between dehydrogenases. Part of domain C agrees well with the mononucleotide-binding fold of lactate dehydrogenase.

The phosphoenolpyruvate-binding site is located at one end of the barrel at a position similar to that seen for dihydroxyacetone phosphate in triosephosphate isomerase. The ADP-binding site lies between domains A and C and the nucleotide interacts with the amino ends of β-strands in the barrel and the carboxy-end of the second helix of the mononucleotide-binding fold in domain C. It is interesting to note that this is not the usual binding position of nucleotides to this fold. The authors discuss the possible implications of these results in evolutionary terms.

Alcohol Dehydrogenase.—The binding of substrates and substrate analogues to horse-liver alcohol dehydrogenase has been examined at 4.5 Å resolution.[117] Crystals of the complexes: alcohol dehydrogenase–NAD^+–*p*-bromobenzyl

[116] M. Levine, H. Muirhead, D.K. Stammers, and D.I. Stuart, *Nature*, 1978, **271**, 626.
[117] B.V. Plapp, H. Eklund, and C.-I. Brändén, *J. Mol. Biol.*, 1978, **122**, 23.

alcohol; alcohol dehydrogenase–NAD^+–trifluoroethanol; and alcohol dehydrogenase–NAD^+ (or NADH)–2-methylpentane-2,4-diol; were isomorphous with the complex of enzyme–NADH–dimethylsulphoxide previously determined at 4.5 Å resolution.

Difference Fourier maps show that *p*-bromobenzyl alcohol and trifluoroethanol bind in a pocket close to the position occupied by dimethyl sulphoxide. The protein and co-enzyme conformations are the same as seen in the dimethyl sulphoxide complexed protein. A provisional model for the active centre suggests that the oxygen atom of either alcohol is ligated to the catalytic zinc atom (2 Å away) while the C-1 carbon atom of the alcohol is 3.5 Å away from C-4 of the nicotinamide ring. More detailed interpretation is awaiting higher resolution electron-density maps.

The subunit conformation of yeast alcohol dehydrogenase has been examined[118] by comparison of the known amino-acid sequence for the yeast enzyme with the known structure of the horse-liver enzyme. Amino-acid changes affect 75 % of the positions but in spite of these differences, 90 out of 93 strictly internal residues are similar, 18 space-restricted glycines are conserved, and 16 structurally compensated exchanges occur. All functionally essential residues are similar or identical and 41 gaps in either sequence can be accommodated in the model. Overall these results show that the general subunit conformations and enzymatic mechanism of the two enzymes are largely identical. The surface changes that are seen may well correlate with differences in the quaternary structures of the two proteins.

8 Hormones

Insulin.—The structure of zinc-free insulin from pig has been determined.[119] The protein crystallizes in a cubic space-group and data were collected to 1.7Å ordinates of a monomer from the two-zinc insulin structure. A set of structure factors were calculated from the atomic co-ordinates of the two-zinc insulin monomer. The co-ordinates for residues B1—B4, B29, B30, and the side-chains of residues B5, B6, and B10 were omitted because they are the areas most likely to be affected by the different aggregation and packing in the cubic cell. In total, one eighth of all non-hydrogen atoms were left out of the calculation and no account was taken of solvent molecules.

Three electron-density maps were then calculated; an $F_{obs}\alpha_{calc}$, an $(F_{obs} - F_{calc}\ \alpha_{calc}$, and a $(2F_{obs} - F_{calc})\alpha_{calc}$. All these maps were calculated using a Sim-type weighting scheme. From these maps the course of the peptide backbone could be followed quite easily and in many areas side-chains are visible. Model-building and refinement of the structure are being carried out.

The packing of the dimeric molecules in the cubic cell is interesting. The insulin dimers form continuous chains throughout the crystals in three mutually perpendicular directions and this leads to uninterrupted channels of solvent, parallel to the protein molecule chains, running throughout the crystal.

[118] H. Jörnvall, H. Eklund, and C.-I. Brändén, *J. Biol. Chem.*, 1978, **253**, 8414.
[119] E.J. Dodson, G.G. Dodson, A. Lewitowa, and M. Sabesan, *J. Mol. Biol.*, 1978, **125**, 387.

Residues responsible for the negative co-operativity of the receptor-binding region of insulin have been determined[120] by examining the activity of 29 insulins and insulin analogues.

A transformation of rhombohedral two-zinc insulin crystals to rhombohedral four-zinc insulin crystals has ben described.[121] This transformation occurs when two-zinc insulin crystals are transferred to four-zinc insulin crystal mother liquor. This transformation is reversible and can be monitored by following the degree of cracking of the crystal. Excessive soaking after the transformation leads to the pattern becoming disordered and the transformed crystals are more sensitive to radiation damage than the native crystals. The authors analyse the difference between the two-zinc and four-zinc insulin crystals in structural terms and describe the changes in intermolecular packing that occur during the transformations.

Insulin-like Growth Factor.—Blundell and co-workers[122] have proposed a structure for the insulin-like growth factor isolated from human serum. The model has been proposed on the basis of the close sequence homology of insulin and the growth factor and shows that the growth factor can form a hydrophobic core very similar to that of insulin. The conformation for the residues equivalent to B6—B27 and A1—A21 can adopt an insulin conformation. A short connecting peptide of twelve residues and an extension of the *C*-terminal end of the molecule can be easily accommodated on the surface of the molecule. The area responsible for dimer formation in insulin is largely conserved but the zinc-binding residue and those involved in hexamerization are very different, suggesting that the growth factor is unlikely to form zinc hexamers. The model provides an explanation for the inability of the growth factor to bind antibodies to insulin and for its ability to bind insulin receptors with a low affinity.

9 Viruses

Tobacco Mosaic Virus (TMV).—Graham and Butler[123] have described experiments to locate the tyrosine residues in the protein disk of TMV. The tyrosine side-chains were labelled by iodination and their positions determined by difference Fourier calculations. These experiments were hindered by the variability in iodine binding seen from crystal to crystal in a single vial and by fairly large changes in the diffraction pattern caused by the higher salt concentration in the iodination experiments. Protein movements are caused by the salt changes alone but iodine binding seems to cause slight alterations in the protein structure. The iodine atoms bind at a radius of 75 Å in one ring of the disk only, and at 80 Å in both rings but with variable occupancy. Chemical studies suggest that iodine reacts mainly with Tyr-139 and to a lesser extent with Tyr-2.

[120] P. De Meyts, E. Van Obberghen, J. Roth, A. Wollmer, and D. Brandenburg, *Nature*, 1978, **273**, 504.
[121] G. Bentley, G. Dodson, and A. Lewitowa, *J. Mol. Biol.*, 1978, **126**, 871.
[122] T.L. Blundell, S. Bedarkar, E. Rinderknecht, and R.E. Humbel, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 180.
[123] J. Graham and P.J.G. Butler, *European J. Biochem.*, 1978, **83**, 523.

In consecutive papers the structure of the TMV protein disk at 2.8 Å[124] resolution and tomato bushy stunt virus (TBSV) at 2.9 Å[125] have been described.

The structure determination of the TMV protein disk at 2.8 Å has involved the collection of 1.4 million reflections.[124] The derivative data extend to only 2.95 Å resolution but use of the 17-fold non-crystallographic symmetry compensates for this.

The molecular boundary, the course of the polypeptide chain, and the very close similarity between subunits in the two rings of the disk are all clearly seen. The central part of each subunit consists of four α-helices arranged closely parallel or antiparallel to each other. These helices extend from 45—65 Å radius. Two of the helices are joined at low radius by a hairpin bend in the structure while the density from the other two helices becomes very weak at low radius, indicating the flexibility of this loop region of the protein in the absence of RNA. The high radius ends of all four helices are joined by protein strands which form a short four-stranded anti-parallel β-sheet. In the central part of each subunit, on the distal side of the β-sheet there is a cluster of six hydrophobic residues. This cluster together with several other side-chains gives a continuous belt of hydrophobic interactions encircling each ring of the disk.

Several extensive salt-bridge systems are involved in ring–ring interactions and in subunit–subunit interactions within each ring. These interactions account for nine out of the thirteen lysine and arginine residues. The remaining four arginine residues all lie at low radius in the area where interaction with RNA may occur in the virus.

Comparison of the disk structure with that of the virus has shown several interesting alterations. One of the radial helices seems to continue for a further four residues at low radius before turning upwards into the vertical column of density seen in the virus map. The structure of the subunit explains not only how the protein can specifically recognize the TMV RNA nucleation sequence but also how it can subsequently accommodate any sequence of RNA. It is proposed that the rigid parts of the subunit at low radius, comprising the proximal ends of two helices and the interconnecting hairpin bend, are responsible for the specificity while the flexible loop regions confer the ability to bind any RNA sequence once initiation has occurred.

Tomato Bushy Stunt Virus (TBSV).—The structure determination of TBSV has been extended to 2.9 Å resolution[125] and several structural features of great interest have appeared. The structure determination was by isomorphous replacement with two derivatives followed by icosahedral averaging.

The icosahedrally-ordered parts of the virus form a compact polyhedral shell about 30 Å thick. The 180 subunits, each of molecular weight 40 000, are arranged such that the surface shows 90 projections at crystallographic and local two-fold axis positions. The individual subunits are folded into three domains; an *N*-terminal arm, the surface domain (S), and the projection domain (P). The P and S domains are joined by a hinge region which can adopt

[124] A.C. Bloomer, J.N. Champness, G. Bricogne, R. Staden, and A. Klug, *Nature*, 1978, **276**, 362.
[125] S.C. Harrison, A.J. Olson, C.E. Schutt, F.K. Winkler, and G. Bricogne, *Nature*, 1978, **276**, 368.

one of two positions. Only 33 residues can be seen for the *N*-terminal arm but the molecular weight of the subunits suggests that this arm could be up to 90 residues long. The P domain consists of two antiparallel β-sheets, one of four and one of six strands, while the S domain consists of two four-stranded β-sheets and two small stretches of α-helix.

Of the 180 *N*-terminal arms, 60 are seen to be ordered, forming interdigitating groups of three at the three-fold axes. Three distinct regions of each arm are involved in hydrogen bonding to form a small β-sheet with both of the other two arms. These subunits for which the *N*-terminal arm can be seen are designated the C subunits. For the other 120 A and B subunits no density can be seen for the arm.

The individual S and P domains are identical for each of the 180 subunits. The differences between subunits are due to the conformation about the hinge region, the two states of the hinge differing by about 20°. No strong density can be ascribed to the RNA and the entire inner cavity, radius about 110 Å, is devoid of any interpretable electron density. It seems probable that the RNA is involved in interactions with *N*-terminal arms and there is evidence that protein does penetrate towards the particle interior.

A striking feature of the structure is the fact that all 60 C-type subunits are in contact with each other and in theory removal of all the other 120 subunits would leave an intact cage-like shell. One consequence of this is that no switch has to be postulated to control hinge configuration or particle size. Once C-subunit *N*-terminal arms are linked in the β-structure, only subunits in the A/B conformation with unlinked arms can occupy the intervening spaces. The binding of these A/B subunits gives the structure the rigidity and curvature of a complete T = 3 virus shell.

10 Transfer RNA and Amino Acyl tRNA Synthetases

Tyrosyl Transfer RNA Synthetase.—Monteilhet and Blow have reported substrate binding studies for tyrosyl tRNA synthetase from *Bacillus stearothermophilus*.[126] These studies, using [^{14}C]tyrosine, show that 1.5 moles of tyrosine binds to each dimeric synthetase molecule in the crystal. Binding of [^{14}C]ATP to the crystalline synthetase shows that the affinity of the enzyme for ATP is low and the dissociation constant measured, at least 10^{-3}M, is comparable to that measured in solution. In contrast, solution studies show only one tyrosine molecule is bound to the dimeric enzyme.

Crystallographic analysis at 2.7 Å resolution of the binding of AMP, ATP, AMP–PCP [adenylyl(α,β-methylene)diphosphate], tyrosine, and tyrosinyl adenylate shows that all five compounds bind at the same site. This site is located on one side of a β-sheet structure and consists of a cleft 10 Å deep which is accessible to solvent. No subsidiary binding-sites were seen for any of the ligands tested.

The tyrosine-binding site is a pocket surrounded on all sides by protein except for the narrow access channel. Such a site gives the maximum possible binding specificity in a simple one-step recognition process. The adenine

[126] C. Monteilhet and D.M. Blow, *J. Mol. Biol.*, 1978, **122**, 407.

moiety of AMP and ATP is also bound in this site with an affinity which is about 1000 times less, and this unexpected result is explained by comparing the two binding moieties in stereochemical terms. The binding-sites for the ribose-phosphate portion of the nucleotides cannot be identified with certainty.

The binding of tyrosinyl adenylate is clearly defined. The tyrosine side-chain binds in the site identified above, while the adenosine moiety lies in one side of a wide, cup-like depression and the adenine ring is in contact with side-chains at the *N*-terminal end of the helix C. There is no evidence that the adenine of ATP can bind in this position. The strong density of the phosphate group of the inhibitor can be seen and it may be that the binding of the tyrosinyl phosphate portion of the compound directs the binding of the adenosine moiety.

These binding studies show that the tyrosine pocket has no 'superspecificity' feature to account for the accuracy of tyrosine binding. The planar group of adenine, which is chemically quite different, is shown to bind in this same site but no reasons for the large difference in binding constants of tyrosine and phenylalanine to this enzyme can be seen. It seems that several independent specificities are involved and these may be mediated by conformational changes in the enzyme. Indeed, attempts to co-crystallize the enzyme with substrates and inhibitors have led to a new crystal form which could reflect a change in the protein conformation.

Yeast Phenylalanine tRNA.—Five papers[127—131] have been published in 1978 covering four independent structure determinations of yeast $tRNA^{Phe}$. Stout and co-workers[127] have published their work on the monoclinic crystal form and have reached an *R*-factor of 0.276 for an unconstrained model at 2.5 Å resolution. They have examined the binding-sites for osmium, gold, platinum, samarium, and gadolinium compounds used in the derivative studies and have identified possible binding-sites for magnesium ions, sodium ions, and spermine from difference electron-density maps.

The crystallographic refinement of yeast $tRNA^{Phe}$ in the orthorhombic crystal form has been reported.[128] This refinement has resulted in a final *R*-factor of 0.198 at nominal resolution of 2.7 Å. The refinement process, in which the bond lengths and angles within each phosphate and nucleoside group were constrained exactly to the standard values while the bond lengths and angles between phosphates and nucleosides were restrained close to their standard values, are reported in detail. In an accompanying paper the authors analyse the structure in detail[129] and the functional implications of these structural features are discussed.

In a paper[130] described as an addendum to their previous work Hingerty and co-workers report a refinement of the monoclinic crystal structure of yeast $tRNA^{Phe}$. They have used a combination of real-space refinement, energy

[127] C.D. Stout, H. Mizuno, S.T. Rao, P. Swaminathan, J. Rubin, T. Brennan, and M. Sundaralingam, *Acta Cryst.*, 1978, **B34**, 1529.
[128] J.L. Sussman, S.R. Holbrook, R.W. Warrant, G.M. Church, and S.-H. Kim, *J. Mol. Biol.*, 1978, **123**, 607.
[129] S.R. Holbrook, J.L. Sussman, R.W. Warrant, and S.-H. Kim, *J. Mol. Biol.*, 1978, **123**, 631.
[130] B. Hingerty, R.S. Brown, and A. Jack, *J. Mol. Biol.*, 1978, **124**, 523.

minimization and crystallographic least-squares on a set of *X*-ray data collected to 2.5 Å resolution at −5 °C. Their final *R*-factor was 0.21. Four magnesium-binding sites, a number of water molecules, and a possible spermine molecule have been identified.

The structure of yeast $tRNA^{Phe}$ in the orthorhombic crystal form has been refined at 2.5 Å resolution to a final *R*-factor of 0.20 when cations are included.[131] Two spermine molecules have been located, one of which is seen in other studies,[130] and four distinct magnesium ions have been identified, some of which have been reported in other work.[130] The spermine and magnesium ions appear to be important in the maintenance of the tRNA structure.

11 Other Globular Proteins

Prealbumin.—A Fourier refinement at 1.8 Å resolution for human plasma prealbumin has been reported.[132] The molecule is a tetramer of mol. wt. 55 000 and exhibits 222 symmetry. The tetrameric molecule shows remarkable resistance to denaturation, the internal organization appearing unchanged from pH 3.5—12. Strong acid or alkali, urea, and guanidinium chloride below 6M concentrations all fail to dissociate the tetrameric molecule.

The atomic model used as a starting point was that fitted to the 2.5 Å resolution electron density map. *X*-ray data to 1.8 Å resolution were collected on a multiple counter diffractometer, a single crystal being sufficient to collect the 33 800 relections. From a starting *R*-factor of 0.532 at 2.5 Å, refinement, using automatically interpreted difference Fouriers, has lowered the *R* factor to 0.289 for all reflections out to 1.8 Å resolution. The final model contains 1766 protein atoms, residues 1—9 and 124—127 not being clearly seen in the maps, and 102 solvent molecules.

The subunit secondary structure is composed of an extensive β-pleated sheet, 45 % of the amino-acid residues being present in the eight-strands. The strands are organized into two four-stranded sheets 10 Å apart and, since they form most of the surface of the molecule, make prealbumin a classic β-barrel protein. The sheets show the twisting usually observed in β-sheets, the twist per strand being about 20°. There is a single α-helix of nine residues in each subunit, the transition from sheet to helix leaving only one peptide bond in either secondary structure. The seven loops joining β-strands vary widely in both length and character.

The tertiary structure of the subunit, is largely determined by the two sheets, the interior of the subunit being composed of side-chains projecting from the sheets. These side-chains are predominantly aliphatic hydrophobic residues; Ala, Val, Leu, and Ile. A cluster of seven aromatic residues, four Phe and three Tyr, occlude the more open end of the barrel structure. Several water molecules are important in maintaining the tertiary structure, being hydrogen bonded to four buried residues (three Thr and one His). Stabilization of the structure by bonding between tyrosine side-chains and the backbone is also seen, in one case being mediated by an aspartic side-chain.

[131] G.J. Quigley, M.M. Teeter, and A. Rich, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 64.
[132] C.C.F. Blake, M.J. Geisow, S.J. Oatley, B. Rérat, and C. Rérat, *J. Mol. Biol.*, 1978, **121**, 339.

Quaternary interactions occur at two sets of interfaces closely organized around two of the molecular two-fold axes. The monomer–monomer interface is chiefly composed of antiparallel hydrogen-bond interactions which lead to the formation of eight-stranded β-sheets in the dimer. One of these interactions includes water molecules and tyrosine side-chains in the hydrogen-bonding system. The dimer–dimer interactions involve all four subunits and show equal contributions of hydrophilic and hydrophobic residues. Three symmetry related hydrogen-bond pairings are seen across the interface and fairly extensive hydrophobic interactions are seen.

The binding of thyroid hormone to prealbumin has been described.[133] The thyroxine-binding sites lie in the central channel of the molecule and the two binding-sites are crystallographically identical. Each binding-site itself has two-fold symmetry. Difference electron-density maps were calculated for crystals soaked in L-thyroxine and tri-iodo-L-thyronine and the hormone was model-built into the binding-site. The hormone binding allows each of the characteristic substituents to enjoy a favourable binding environment, the iodine atoms for instance are located in hydrophobic pockets while the 4^1-hydroxy-group is hydrogen bonded to a serine and a threonine residue *via* a water molecule. The hormones α-carboxylate and α-amino groups interact with a lysine side-chain and a glutamine side-chain respectively.

Using a computer graphics system DNA has been fitted to the outer cylindrical depressions on the prealbumin molecule. A DNA molecule can be fitted into these depressions with the DNA-helix axis parallel to the axis of the depression. The extended arms of the protein can fit closely into the wide groove of the DNA double helix and in such a way that they could act as a probe for a complementary base sequence. The authors suggest that prealbumin has evolved from an ancestral protein whose structure encompassed both hormone- and DNA-binding functions.

Rhodanese.—Two papers have been published describing the structure of bovine-liver rhodanese at 2.5 Å resolution.[134, 135] The molecule consists of a single polypeptide chain of 293 residues and is folded into an ellipsoid of 60 × 50 × 40 Å. The polypeptide chain is folded into two domains of nearly equal size with only a single piece of polypeptide crossing the interface. Domain I is composed of the *N*-terminal half of the chain, residues 1—142, while domain II is formed by residues 159—293. The active-site is located between the two domains and is made up of side-chains from both domains. The active-site also features a six residue salt-bridge network. The overall folding contains 37 % helix, 15 % parallel β-structures, and 24 % in β-turns. Each domain contains a central five-stranded parallel β-sheet and this sheet is flanked by two helices on one side and three on the other. Of the 19 β-bends, 17 occur at the surface of the molecule.

Domain–domain interactions are a mixture of hydrophobic interactions, hydrogen bonds, and salt bridges. The two domains show very close structural

[133] C.C.F. Blake, J.M. Burridge, and S.J. Oatley, *Biochem. Soc. Trans.*, 1978, **6**, 1114.
[134] J.H. Ploegman, G. Drent, K.H. Kalk, W.G.J. Hol, R.L. Heinrikson, P. Keim, L. Weng, and J. Russell, *Nature*, 1978, **273**, 124.
[135] J.H. Ploegman, G. Drent, K.H. Kalk, and W.G.J. Hol, *J. Mol. Biol.*, 1978, **123**, 557.

similarity but have virtually no similarity in sequence. This similarity was examined by calculating the r.m.s. deviations in the positions of the α-carbon atoms for the two domains after maximizing the overlap. The value obtained was 1.95 Å and the larger differences were all seen to occur on the surface of the molecule. An interesting fact is that the two domains are related to each other by an almost exact two-fold axis, the relationship being a rotation of 179° and a translation of less than 1 Å. Only 15 identical amino-acids are seen for the topologically equivalent residues, three of these being glycines. For the other positions the authors calculate a minimum base change per codon of 1.27.

In the active-site the bound substrate-sulphur atom forms a persulphide link to Cys-247 and its removal by cyanide is accompanied by a distinct negative feature in difference Fourier maps. The persulphide group is involved in four hydrogen bonds to peptide groups and one to a threonine side-chain. No metal ion has been located in the active-site despite evidence that a divalent ion is necessary for catalysis in solution. The authors discuss the implications of the structural homology within the domains and the topological similarity with other proteins.

Dihydrofolate Reductase.—The structure of dihydrofolate reductase from *Lactobacillus casei* has been reported.[136] The enzyme is present as a ternary complex with bound co-enzyme, NADPH, and an inhibitor, methotrexate. The electron-density map at 2.5Å resolution was interpreted on a computer graphics system and positions for all 162 α-carbon atoms were obtained.

The polypeptide chain of the protein is folded into an eight-stranded β-sheet with seven parallel strands and one antiparallel strand, this antiparallel strand being at the *C*-terminus. Four helices and six extended loops provide connectivity within the sheet. The methotrexate sits saddlelike over a helix in a deep cavity that cuts across the face of the molecule, one side of this cavity providing the binding-site for the nicotinamide ring of the NADPH. The remainder of the co-enzyme occupies a shallow groove that winds back over four β-strands. Co-enzyme and methotrexate binding involve only residues up to 102 in the sequence and these residues are seen to be conserved from species to species.

After alignment of 142 of the 159 α-carbons in the *E. coli* enzyme with structurally equivalent residues in the *L. casei* enzymes the r.m.s. positional deviation is 1.7 Å. The *E. coli* enzyme structure was determined in the absence of NADPH. The changes induced by co-enzyme binding appear to be very small and consist of movements of two flexible loop regions.

The methotrexate molecule is bound to the enzyme in an open conformation with its pteridine ring perpendicular to the *p*-aminobenzoyl group's aromatic ring. Sixteen protein–inhibitor interactions have been characterized. The NADPH molecule also assumes an open conformation with a distance of 17 Å separating the adenine and nicotinamide groups which are almost perpendicular to each other. The catalytic cycle involves hydride transfer from the NADPH to C-6 of dihydrofolate with the incorporation of a proton at N-5.

[136] D.A. Matthews, R.A. Alden, J.T. Bolin, D.J. Filman, S.T. Freer, R. Hamlin, W.G.J. Hol, R.L. Kisliuk, E.J. Pastore, L.T. Plante, N.-H. Xuong, and J. Kraut, *J. Biol. Chem.*, 1978, **253**, 6946.

The pteridine ring is inclined at about 45° to the nicotinamide ring and the C-6 atom of methotrexate is about 4.5 Å above the C-4 of the nicotinamide ring. The nicotinamide ring is so positioned that only the A-side hydrogen can be transferred.

The relevance of the structure of the inhibited complex to the productive ternary complex can only be inferred. Of the 21 strictly conserved residues 15 make direct interactions with the NADPH or methotrexate molecules while the remaining six are essential for maintaining the overall chain conformation. The proton donor on the enzyme does not seem to be a histidine side-chain and the only potential donors are Asp-26 and Thr-113, both of which are invariant in all known dihydrofolate reductase sequences. The surroundings of Asp-26 make it the more likely choice. The authors suggest that the differences seen by spectroscopic methods for the binding of dihyrofolate and methotrexate may be due to a 180° rotation about the C-6—C-9 bond of dihydrofolate. This rotation gives a closer approach of N-5 to the C-4 atom of the nicotinamide ring.

Glutathione Reductase.—The three-dimensional structure of the dimeric flavoenzyme, glutathione reductase, from human erythrocytes has been determined at 3 Å resolution.[137] The data were collected on a diffractometer for the native crystals and five heavy-atom derivatives. Refinement of the heavy-atom parameters gave an overall figure of merit of 0.85.

Although the amino-acid sequence is only partly known the polypeptide chain could be traced unambiguously. The flavin adenine dinucleotide (FAD) prosthetic group could be readily seen and the pyrophosphate group had the highest electron density in the map. The subunits contain 462 amino-acid residues and are folded into three structural domains. The FAD-binding domain is roughly globular and is made up of residues 1—139 and 273—344. The NADP-binding domain is also globular and consists of residues 140—272 while the interface domain is rather flat and is composed of residues 345—462. There is a large amount of secondary structure; 31 % of the residues are in helical conformations and 28 % in β-sheets. Many of the other residues are in reverse turns.

The FAD and NADP molecules are bound to their respective domains in extended conformations while the oxidized gluatathione is bound between the FAD-domain and the interface domain of the two-fold related subunit. The active-centre is clearly located between the two subunits. There is also an interaction between the flavin ring and the interface-domain of the related subunit, probably involving hydrogen bonds.

Both the FAD and NADP domains contain parallel β-sheet structures and the classic dinucleotide binding-folds are formed. The dinucleotides bind to the *C*-terminal ends of the parallel-sheet strands in the same manner as has been described for other nucleotide-binding proteins. There is also topological equivalence seen between the FAD- and NADP-binding domains.

Two redox systems, at least, are involved in transferring reducing

[137] G.E. Schulz, R.H. Schirmer, W. Sachsenheimer, and E.F. Pai, *Nature*, 1978, **273**, 120.

equivalents from NADPH to oxidized glutathione, these being the isoalloxazine ring of FAD and an internal disulphide bridge formed between residues 41 and 46 of the protein subunit. Direct transfer of a hydride ion from NADPH to the cysteines is not possible as the FAD ring lies in the way. The possible structures of the two states of the enzyme in the catalytic cycle have been inferred from the structure of the enzyme–NADP–oxidized glutathione complex and are described in detail by the authors.

Phospholipase A_2.—The three-dimensional structure of bovine pancreatic phospholipase A_2 has been determined at 2.4 Å resolution.[138] Three heavy-atom derivatives were used in the phase determination and the overall figure of merit is 0.802. The quality of the electron density map allowed easy tracing of the course of the polypeptide chain and the seven disulphide bridges showed up as the highest electron density features. The overall shape of the molecule is that of a flattened cylinder with dimensions of approximately 22 × 30 × 42 Å. There is a high degree of secondary structure; 50 % of the residues being in α-helices and 10 % in β-sheets. The molecule is composed of two long antiparallel helices both consisting of 19 residues and these two helices are connected by two disulphide bridges. Another short helix is sulphur-bridged to one of the long helices which is also sulphur-bridged to one of the two antiparallel β-strands. The fourth major helix is sulphur-bridged to the other β-strand.

The histidine residue thought to be involved in catalysis is in one of the long helices and its side-chain is located in a cavity on the surface of the molecule. The imidazole side-chain is pointed at by two aspartic acid side-chains, one of which also interacts with a calcium ion. The structure determination has corrected the sulphur bridges previously identified by chemical work and has also shown that the bovine-enzyme structure deviates substantially from the known structure of the porcine prophospholipase.

Elongation Factor Tu.—The crystal structure of elongation factor Tu(EF-Tu) has been determined at 2.6 Å resolution, the protein being slightly modified by proteolysis and bound to guanosine diphosphate.[139] The protein was isolated from *E. coli* B cells and crystallized by vapour diffusion. The crystals seem to contain a protein consisting of two polypeptides of molecular weights 39 000 and 6000 derived from the intact molecule by limited proteolysis. Diffraction data for the native crystals and three heavy-atom derivatives were collected on a rotation camera. The overall mean figure of merit is 0.57.

At 5 Å resolution the two molecules in the asymmetric unit were shown to be related by an almost exact two-fold axis. To improve the quality of the map, the density for these two molecules was averaged. The overall dimensions of the molecule are 75 × 50 × 45 Å and the chain is folded into two domains separated by a narrow waist region of about 20 Å in diameter and near the centre of the molecule. The so-called tight domain contains five α-helices and three β-strands and in this domain the chain could be traced with some certainty. The structure of the loose domain is harder to trace without sequence

[138] B.W. Dijkstra J. Drenth, K.H. Kalk, and P.J. Vandermaelen, *J. Mol. Biol.*, 1978, **124**, 53.
[139] K. Morikawa, T.F.M. La Cour, J. Nyborg, K.M. Rasmussen, D.L. Miller, and B.F.C. Clark, *J. Mol. Biol.*, 1978, **125**, 325.

information. The electron density of the guanosine diphosphate has been located in the map and shows that the GDP is bound to the tight domain with the base and ribose groups partly buried in the protein, though it is interesting to note that the GDP molecules on the two protein molecules in the asymmetric unit have slightly different conformations. The authors discuss the structure of the protein in relation to its range of functions in protein biosynthesis.

Aspartate Carbamoyltransferase.—The crystal structures of aspartate carbamoyltransferase, both unliganded and in the presence of cytidine triphosphate, have been reported at 2.8 Å resolution.[140] The protein is composed of six regulatory subunits, mol. wt. 17 000, and six catalytic subunits, mol. wt. 34 000. Each regulatory subunit is known to contain a zinc atom. The liganded and free forms of the enzyme crystallized in different space-groups and independent structure determinations were carried out for the two crystal forms.

The structure shows that the catalytic subunits (C) are closely associated to form C_3 trimers, two of these trimers being in close contact and nearly eclipsed in the active enzyme. Each C subunit is associated with a C subunit in the other C_3 trimer *via* a regulatory dimer (R_2). The two C subunits related through the R_2 dimer are 120° apart about the three-fold axis. The catalytic subunit consists of two domains connected by two helices. The polar domain contains four helices and a five-stranded parallel β-sheet while the equatorial domain contains three helices and a six-stranded parallel β-sheet. Chain tracing has been possible for nearly all of the C subunits and the C–C contacts have been identified. The regulatory subunits have been less easy to interpret due to intrinsic disorder in the electron density maps but the zinc-binding domain has been fitted with the known sequence. The R subunits has two four-stranded β-sheet structures with two helices separating them. The zinc ion is tetrahedrally bonded to four cysteine residues and the zinc-binding domain is in contact with the polar domain of its associated C subunit. The CTP-binding sites on both the R and C subunits have been identified and the active-site of the C subunit has been located in a pocket formed between two C subunits. There seems to be little change in the protein structure between the liganded and free forms of the protein. The authors also report the preparation of two crystal forms of the protein complexed with *N*-phosphonacetyl-L-aspartate, a putative transition state analogue.

Parvalbumin.—The replacement of the calcium ions in calcium-binding parvalbumin from calf muscle has been achieved by soaking crystals in mother liquor containing terbium chloride.[141] It was shown that at low terbium-ion concentrations the calcium ion in the EF site is displaced by terbium, while at higher terbium concentrations the calcium ion in the CD site is also displaced. A third site is seen in the difference Fourier map but not in the anomalous scattering difference Fourier. Since terbium is the only element in the crystals with significant anomalous diffraction with copper Kα radiation it can be

[140] H.L. Monaco, J.L. Crawford, and W.N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 5276.
[141] J. Sowadski, G. Cornick, and R.H. Kretsinger, *J. Mol. Biol.*, 1978, **124**, 123.

stated that this third peak is not a terbium ion. It may be due to a water molecule that was co-ordinated by the EF calcium-ion being replaced by a sulphate ion when terbium binds. No evidence of a third terbium ion binding to the protein can be seen despite the spectroscopic evidence which suggests the binding of three terbium ions.

L-Arabinose-binding Protein.—The diagrammatic representation of the structure of L-arabinose-binding protein has been corrected to show the two six-stranded β-sheets to be related by a two-fold axis.[142]

12 Protein Conformation and Structure Prediction

Analysis of Protein Conformations–Methods and Results.—A wide range of methods for analysing individual protein structures and for comparing two or more structures have been reported in 1978.

Remington and Matthews[143] have proposed a method to compare any pair of proteins in a completely general manner. The method compares all possible structural segments of a given length in one protein with all possible structural segments of the same length in the second protein. These comparisons reveal any structural similarities between the two proteins and also provide a basis for estimating the probability of a given degree of similarity occurring by chance. The method has shown significant correspondence between phage T4 and hen-egg-white lysozyme but has shown that the similarity between phage T4 lysozyme and calcium-binding parvalbumin is no better than would be expected by chance.

Argos and co-workers[144] have compared the active-site geometries of four zinc-containing enzymes; carbonic anhydrase, liver alcohol-dehydrogenase, thermolysin, and carboxypeptidase. The zinc ions were seen to be co-ordinated by three protein groups and, additionally, a water molecule and a substrate carbonyl may assume a fourth or fifth position. A group whose function is to abstract a proton from water during catalysis was found to have a constant spatial arrangement with respect to the zinc atom. The analysis shows many interesting similarities between the zinc-containing enzymes and enzymes of related function.

An automatic procedure for analysing protein–protein interactions has been reported and used to study the interaction of bovine pancreatic trypsin inhibitor with trypsin.[145] The method allows a large number of configurations to be scanned by eliminating consideration of side-chain detail. The van der Waals criterion for hydrogen bonding has been examined in some detail using neutron diffraction determined structures[146] and the investigation and parametrization of a molecular dielectric constant has been published.[147] Wolfenden has examined the interaction of proteins with solvent water and proposes that bound water in proteins will often be bound to the peptide bonds,

[142] F.A. Quiocho, G.L. Gilliland, and G.N. Phillips jun., *J. Biol. Chem.*, 1978, **253**, 2496.
[143] S.J. Remington and B.W. Matthews, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2180.
[144] P. Argos, R.M. Garavito, W. Eventoff, and M.G. Rossmann, *J. Mol. Biol.*, 1978, **126**, 141.
[145] S.J. Wodak and J. Janin, *J. Mol. Biol.*, 1978, **124**, 323.
[146] Z. Rahim and B.N. Barman, *Acta Cryst.*, 1978, **A34**, 761.
[147] D.A. Greenberg, C.D. Barry, and G.R. Marshall, *J. Amer Chem. Soc.*, 1978, **100**, 4020.

preferentially interacting with the carbonyl groups.[148] Hagler and Moult have used the Monte Carlo method to perform a computer simulation of the structure and energetics of the ordered and disordered solvent in crystals of a small peptide and of lysozyme.[149] Stellwagen and Wilgus[150] have analysed the relationship of protein thermostability to accessible surface area of the protein and shown that such a correlation appears to exist. A function useful in visualizing the surface topography, polarity, and charge of a protein molecule has been presented.[151] The function can represent the structure as a three-dimensional net or by mapping onto a plane and can be used to calculate surface areas or to delimit complementary sites in contacts between subunits. Finney has described a method for examining the molecular area and internal environment of protein molecules[152] and has presented results when the method was applied to ribonuclease-S. Significant numbers of internal polar–apolar group interactions are implied.

The packing of the α-helices in sperm whale myoglobin has been examined.[153] It has shown that a helix of close-packed spheres forms a useful approximation to actual peptide helices and the packing of such model helices corresponds to the three general classes of interactions actually observed. Using the geometry implied by this model the authors propose an algorithm for predicting strong helix–helix interaction sites in peptide chains of known sequence. The binding of phosphate moieties at the *N*-termini of helices in proteins has been shown to correspond to an optimal interaction of the helix dipole with the charged phosphate.[154] It also seems that this favourable arrangement has been discovered several times during evolution and that, in some enzymes, the helix dipole might be involved in catalysis.

A structural role for valine and isoleucine residues has been proposed[155] suggesting that steric effects favour the generation of parallel β-sheet strands when these amino-acids are involved. Go and Taketomi[156] have presented a lattice model of protein folding from which they deduce that specific long-range interactions are essential for highly co-operative stabilization of the native conformation while the short range interactions accelerate the folding and unfolding transitions.

Janin and Chothia have examined the role of hydrophobicity in the binding of co-enzymes.[157] They have shown that the binding of flavin mononucleotide to flavodoxin, and NAD^+ to lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase leads to the co-enzymes becoming almost totally buried in the protein. The 600 $Å^2$ surface area of the co-enzyme and a similar area of the protein become inacessible to solvent. This loss of surface area is the

[148] R. Wolfenden, *Biochemistry*, 1978, **17**, 201.
[149] A.T. Hagler and J. Moult, *Nature*, 1978, **272**, 222.
[150] E. Stellwagen and H. Wilgus, *Nature*, 1978, **275**, 342.
[151] J. Greer and B.L. Bush, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 303.
[152] J.L. Finney, *J. Mol. Biol.*, 1978, **119**, 415.
[153] T.J. Richmond and F.M. Richards, *J. Mol. Biol.*, 1978, **119**, 537.
[154] W.G.J. Hol, P.T. Van Duijnen, and H.J.C. Berendsen, *Nature*, 1978, **273**, 443.
[155] C. Toniolo, *Macromolecules*, 1978, **11**, 437.
[156] N. Go and H. Taketani, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 559.
[157] J. Janin and C. Chothia, *Biochemistry*, 1978, **17**, 2943.

major source of stabilization energy for the three enzymes examined and contributes 25–30 kcal mol^{-1} to the free energy of dissociation. Computer model-building has been used to examine drug–nucleic acid interactions.[158] Geisow has analysed the role played by the secondary structure of pro-hormones in the conversion by proteolysis.[159] His results suggest that the activation cleavages occur at unstructured regions of the polypeptide chain, often near reverse turns – these two factors indicating that the sites have surface locations. Potential cleavage sites which are not processed seem to be confined to regions of secondary structure and even if accessible their rigid conformations make them poor substrates for the activating proteases.

Crippen has offered an objective definition of the domains of a protein given its α-carbon co-ordinates.[160] An algorithm is used which groups segments of the chain together when there are a relatively large number of contacts between the two segments. The result is a tree structured organization with the highest level clusters representing recognizable domains and the lowest level clusters representing pieces of secondary structure. von Heijne and Blomberg[161] have analysed 72 β-sheets containing 2610 amino-acid residues and determined the preferred locations of hydrophobic, neutral, and polar groups within the strands. Richardson and co-workers[162] have identified a secondary structure which they have named the β-bulge. The β-bulge is a region between two consecutive β-type hydrogen bonds and involves two consecutive residues in one strand with a single residue in the other strand. Compared to regular β-structure the β-bulge puts the usual alternation of side-chain direction out of register, introduces a slight bend in the sheet, and locally accentuates the usual right-handed twist. Almost all of the 91 β-bulges observed are between anti-parallel strands and are usually between a narrow rather than a wide pair of hydrogen bonds. The functional roles of the β-bulge probably include compensating for the effect of a single insertion or deletion within β-structure and providing the strong local twist required to form closed β-barrel structures.

Warme and Morgan[163, 164] have surveyed long-range atomic interactions in 21 proteins and have compared the observed distributions of atomic interactions with the distributions expected for random interactions. They find[163] that sulphur atoms are attracted to other sulphur atoms but avoid negatively charged oxygen atoms, while the negatively charged oxygen atoms tend to interact with charged amino groups, peptide —NH groups, hydroxy-groups, and polar methylene groups. Carbonyl oxygen atoms frequently associate with polar and neutral methylene groups while the carbonyl carbon atoms are often found near charged amino groups. In a second paper[164] the authors consider the atoms of a given amino-acid side-chain as a group and examine the interactions of that group with particular types of atoms and with other amino-acid side-chains. They find that 35 residue–atom pairs exhibit frequencies of interaction

[158] H.M. Berman, S. Neidle, and R.K. Stodola, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 828.
[159] M.J. Geisow, *F.E.B.S. Letters*, 1978, **87**, 111.
[160] G.M. Crippen, *J. Mol. Biol.*, 1978, **126**, 315.
[161] G. von Heijne and C. Blomberg, *Biopolymers*, 1978, **17**, 2033.
[162] J.S. Richardson, E.D. Getzoff, and D.C. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2574.
[163] P.K. Warme and R.S. Morgan, *J. Mol. Biol.*, 1978, **118**, 273.
[164] P.K. Warme and R.S. Morgan, *J. Mol. Biol.*, 1978, **118**, 289.

that differ by at least 50 % from the expected values for random interactions. Of these 35 pairs, 21 show interaction frequencies greater than expected while the other 14 show frequencies lower than expected.

Janin and co-workers have analysed the side-chain dihedral angles of 2536 residues in 19 protein structures.[165] The distribution of torsion angles around the C—C bond is found to be trimodal, one conformation being rare except for serine residues. The torsion angles for the C—C bond show characteristics distributions for residues with a tetrahedral γ-carbon, for aromatic residues, and for aspartic acid or asparagine residues. The side-chain conformations show torsion angles within 15—18° from the conformations with the lowest calculated energies. Crippen and Kuntz[166] have surveyed atom packing in 21 proteins and have found that the atom density around a given central atom is determined primarily by its covalently bonded neighbours and its proximity to the protein's surface.

Levitt has examined the preferences of amino-acid residues for different conformations.[167] He has found that 19 of the 20 amino-acids have a weak but statistically significant preference for only one type of secondary structure. The preferences suggest that bulky amino-acids, that is those branched at the β-carbon or having large aromatic side-chains, prefer β-sheet, while the shorter polar side-chains together with glycine and proline prefer reverse turns. All other side-chains prefer α-helix except arginine which shows no preference. Polar side-chains which contain hydroxy-groups disrupt α-helix, while the other polar side-chains disrupt β-sheet. Hydrophobic side-chains appear to disrupt reverse turns.

Bello[168] has examined the tight atom-packing in the cores of proteins while Manavalan and Ponnuswamy[169] have calculated a new set of hydrophobicities based on the surrounding hydrophobic environment of amino-acid residues in 14 protein molecules. Lumley Jones[170] has considered regular and non-regular secondary structure in proteins in terms of the dynamics of the peptide bond. Crampin and co-workers[171] have attempted to quantify the internal structural heterogeneity of proteins and have attempted to predict the core-forming residues in protein sequences.

Salemme has described details of the construction and use of molecular models designed to investigate the coupled conformational properties of polypeptide structures.[172]

Structure Prediction.—Although several papers in the previous part of this section have dealt partly with the prediction of protein structures the papers covered here deal exclusively with predictive methods and their accuracy. Argos and co-workers[173] have applied the Chou-Fasman method with an

[165] J. Janin, S. Wodak, M. Levitt, and B. Maigret, *J. Mol. Biol.*, 1978, **125**, 357.
[166] G.M. Crippen and I.D. Kuntz, *Internat. J. Peptide Protein Res.*, 1978, **12**, 47.
[167] M. Levitt, *Biochemistry*, 1978, **17**, 4277.
[168] J. Bello, *Internat. J. Peptide Protein Res.*, 1978, **12**, 38.
[169] P. Manavalan and P.K. Ponnuswamy, *Nature*, 1978, **275**, 673.
[170] R. Lumley Jones, *Nature*, 1978, **272**, 185.
[171] J. Crampin, B.H. Nicholson, and B. Robson, *Nature*, 1978, **272**, 558.
[172] F.R. Salemme, *J. Mol. Biol.*, 1978, **126**, 591.
[173] P. Argos, M. Hanei, and R.M. Garavito, *F.E.B.S. Letters*, 1978, **93**, 19.

extended data base to predict the structures of 24 proteins of known tertiary structure. The increased data base does not appear to improve the quality of the prediction despite the considerable shifts of some of the amino-acid in the hierarchy of ability to form or break secondary structures.

Rose[174] has described methods for locating peptide chain turns on the basis of the hydrophobicity of the residues concerned. It is proposed that turns occur at those sites in the polypeptide chain where the hydrophobicity is at a local minimum and this is really a chemically rationalized statement of the predictive methods which search for turn-forming residues, all of which are non-hydrophobic. Matheson and Scheraga[175] have described a method, based on hydrophobic bonding, for predicting the nucleation sites for protein folding. The method involves searching the sequence for pockets of non-polar residues whose free energy of interaction when they came together compensates for the increase in free energy that is required to bring them into contact. Wu and co-workers[176] have compared various methods of structure prediction for the β-sheet regions of immunoglobulin chains. They have also used their own method to evaluate the effects of nearest-neighbours on the conformations of amino-acids in proteins. They find that wrong predictions of β-sheet out-number correct predictions but better predictions are obtained when two or more of the methods agree. The consistent overprediction of β-structure can be improved by using the data for known β-sheet-breaking tripeptides.

Sternberg and Thornton[177] have reviewed the predictive methods available and have listed the necessary stages in predicting a protein structure from its sequence. The conformations of peptide and glycopeptide anti-freezes from fish blood have been predicted[178] and seem to contain several β-turns which would allow the polar residues to interact with the ice structure.

Garnier and co-workers have reported a set of predictive methods and analysed their accuracy when used to predict the structures of 26 proteins.[179] The method gave 49 % accurate prediction for the sample proteins when the overall helix and extended-chain content was not taken into account. This rose to 63 % when certain optimization techniques were applied. Ways of further increasing the accuracy of the predictions using information on hydrophobic triplets and homologous sequences were also explored.

13 Muscle

The *X*-ray diffraction patterns from lobster and crayfish muscles have been examined and show very clear layer lines attributable to the thin filaments.[180] These layer lines are well separated from the myosin layer lines. The patterns obtained from relaxed muscles show an important contribution from the regulatory proteins and have allowed the arrangement of the troponin complexes to be deduced. The troponin diffraction has also allowed an accurate

[174] G.D. Rose, *Nature*, 1978, **272**, 586.
[175] R.R. Matheson jun. and H.A. Scheraga, *Macromolecules*, 1978, **11**, 819.
[176] T.T. Wu, S.C. Szu, R.L. Jernigan, H. Bilofsky, and E.A. Kabat, *Biopolymers*, 1978, **17**, 555.
[177] M.J.E. Sternberg and J.M. Thornton, *Biochem. Soc. Trans.*, 1978, **6**, 1119.
[178] M.-H. Loucheux-Lefebvre, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 1352.
[179] J. Garnier, D.J. Osguthorpe, and B. Robson, *J. Mol. Biol.*, 1978, **120**, 97.
[180] J. Wray, P. Vibert, and C. Cohen, *J. Mol. Biol.*, 1978, **124**, 501.

determination of the pitch of the actin helix in relaxed muscle. In rigor, the intensities of the diffraction pattern are altered due to the attachment of cross-bridges. The patterns support previous proposals that in rigor the cross-bridges can only attach to certain 'target areas' on the actin filaments.

Lamvik[181] has employed an electron scattering technique to determine the mass per unit length of rabbit-striated-muscle thick-filament and the results are consistent with models containing three myosin molecules per 143 Å repeat. Using electron microscopy and optical diffraction methods Luther and Squire[182] have examined the three-dimensional structure of frog-muscle M-band and M-region. Their results support the idea that the myosin cross-bridges are arranged on a three-stranded helix.

14 Membranes and Membrane Proteins

A direct method for determining membrane electron-density profiles on an absolute scale has been described[183] The method is based on the isomorphous exchange of cholesterol in the membrane with a halogenated analogue of cholesterol.

Nelander and Blaurock[184] have continued their work on the stacking of the membranes in nerve myelin and have extended the phasing to include reflections 7—15. The four possible phase combinations have been used to calculate electron density profiles and these do not differ greatly from one another. All four profiles show a higher electron density in the extracellular half of the membrane bilayer than in the cytoplasmic half. This is interpreted as showing that an appreciable part of the myelin protein is in the extracellular half of the membrane.

The reversible structure modification of frog-sciatic-nerve myelin caused by treatment with dimethyl sulphoxide has been studied by low-angle *X*-ray diffraction methods.[185] These experiments have shown that the bilayer profile undergoes a specific asymmetric change prior to the phase transformation.

The incorporation of bacteriorhodopsin into unilamellar lipid vesicles and its temperature-dependent aggregation has been examined by a variety of methods including *X*-ray diffraction.[186] At temperatures below the lipid phase transition, bacteriorhodopsin crystallizes into patches showing the same hexagonal lattice observed in the purple membrane. Above the phase transition, and with a sufficiently high lipid to protein ratio, these aggregates break down to give monomeric protein molecules. Hiraki and co-workers[187] have demonstrated that reconstituted bacteriorhodopsin molecules will form a hexagonal lattice spontaneously whereas bacterio-opsin molecules do not.

Electron microscopy and optical diffraction have been used to examine the orientation of cytochrome *c* oxidase in crystalline membranes.[188] Using antibodies against specific cytochrome *c* oxidase subunits it was shown that all

[181] M.K. Lamvik, *J. Mol. Biol.*, 1978, **122**, 55.
[182] P. Luther and J. Squire, *J. Mol. Biol.*, 1978, **125**, 313.
[183] N.P. Franks, T. Arunachalam, and E. Caspi, *Nature*, 1978, **276**, 530.
[184] J.C. Nelander and A.E. Blaurock, *J. Mol. Biol.*, 1978, **118**, 497.
[185] H. Hashizume and N. Masaki, *Arch. Biochem. Biophys.*, 1978, **186**, 275.
[186] R.J. Cherry, U. Müller, R. Henderson, and M.P. Heyn, *J. Mol. Biol.*, 1978, **121**, 283.
[187] K. Hiraki, T. Hamanaka, T. Mitsui, and Y. Kito, *Biochim. Biophys. Acta*, 1978, **536**, 318.
[188] T.G. Frey, S.H.P. Chan, and G. Schatz, *J. Biol. Chem.*, 1978, **253**, 4389.

cytochrome *c* oxidase molecules in the crystal were arranged such that subunit IV was facing outwards and subunits II and III were facing into the closed vesicles. This orientation is, interestingly, the opposite to that found for intact mitochondria.

15 Other Biological Structures

Small Molecule Structures of Biological Interest.—Sakaki and co-workers have reported the crystal structure of 1-methyl-3-carbamoyl-pyridinium complexed with indole-3-acetic acid.[189] Stacking of the indole and pyridinium rings is observed and may provide a model for the stacking interaction between the nicotinamide ring of NAD^+ and tryptophan residues in proteins.

The three-dimensional structures of two inhibitors of dihydrofolate reductase have been determined[190] and their structures compared with the natural substrates.

The crystal structures of two oxidation states of a flavin–nicotinamide complex have been determined[191] and the three-dimensional structures of two compounds related to catalytic flavoenzyme intermediates have been elucidated.[192] These compounds may give some information on the reaction mechanism of flavoenzymes.

Three polypeptides of biological interest have been reported in 1978. *X*-ray diffraction studies on gramicidin A, both native and complexed with caesium ions, have shown that the molecules form cylindrical helical channels.[193] In the native crystals these channels are 32 Å long with a diameter of about 5 Å. Caesium ions bind in this channel causing it to shorten to 26 Å while increasing in diameter to 6.8 Å.

The toxic bicyclic octapeptide β-amanitin from the 'death-cap' fungus, *Amanita phalloides,* has had its three-dimensional structure determined.[194] The structure is seen to be T shaped, the cross bar of the T containing seven groups capable of forming hydrogen bonds – the stem of the T by comparison containing only hydrophobic residues. These two regions may be important in the toxin's reaction with DNA-dependent RNA polymerase.

The conformation of the endogenous morphine-like substance, [Leu^5]enkephalin, has been reported.[195] The conformation is produced by a Tyr-Gly-Gly-Phe β-bend and stabilized by antiparallel hydrogen-bonding between Tyr and Phe. Comparison of the structure with that of known opiate agonists has allowed three hydrophilic and two hydrophobic regions to be identified as contributing to the recognition of the molecule at the opiate receptor site.

Ribosomes.—Two neutron scattering studies of *E. coli* ribosomes have been published. Stuhrmann and co-workers have examined the distribution of

[189] T. Sakaki, M. Inoue, S. Senda, and K. Tomita, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 21.
[190] A. Camerman, N.W. Smith, and N. Camerman, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 87.
[191] D.J.T. Porter and D. Voet, *Acta Cryst.*, 1978, **B34**, 598.
[192] M. Bolognesi, S. Ghisla, and L. Incoccia, *Acta Cryst.*, 1978, **B34**, 821.
[193] R.E. Koeppe II, K.O. Hodgson, and L. Stryer, *J. Mol. Biol.*, 1978, **121**, 41.
[194] E.C. Kostansek, W.N. Lipscomb, R.R. Yocum, and W.E. Thiessen, *Biochemistry*, 1978, **17**, 3790.
[195] G.D. Smith and J.F. Griffin, *Science*, 1978, **199**, 1214.

protein and nucleic acid in 70*S* ribosomes and the 30*S* subunits.[196] The 30*S* subunits appear more homogeneous than the 70*S* ribosomes. This is interpreted chemically as showing that the rRNA is located mainly in the central regions with the proteins distributed concentrically on the outer surface. Langer and co-workers[197] have used neutron scattering methods to map the distances between seven pairs of proteins in the 30*S* subunit. They discuss their results in relationship to other information on the structure of the 30*S* subunit.

DNA-helix Destabilizing Protein.—A large two-dimensional crystal of this protein from bacteriophage T4 has been examined by electron diffraction.[198] The crystals diffract to about 3.7 Å and attempts are in hand to determine the three-dimensional structure of the protein.

16 Fibrous Proteins and Polysaccharides

Collagen.—Small-angle *X*-ray and neutron diffraction patterns have been obtained from porcine intervertebral disks.[199] The patterns observed are different from those obtained from rat tendon and can either reflect a difference in molecular length or indicate that non-collagenous materials are attached to the collagen in the disks.

A theoretical approach to diffraction by the collagen fibril has been published by Woodhead-Galloway and Young.[200] Low-angle *X*-ray scattering studies on rat-tail tendons with added heavy-metal stains have been reported.[201] Fixing and staining of the tendon fibres gives an intensified 38 Å row line and the preservation of most equatorial features seen in the native pattern. Staining without fixation led to the disappearance of the native equatorial features.

Gathercole and Keller[202] have presented a number of unusual *X*-ray diffraction effects concerning the low-angle meridional reflections of collagen which they claim can be explained on the basis of the planar crimp structure of the tendon units. Bordas and co-workers have examined the diffraction patterns from normal costal cartilage.[203] The low concentration and the lack of order of the collagen fibrils in the cartilage means that they cannot be examined using conventional *X*-ray sources and the work reported used synchrotron radiation.

The low-angle diffraction pattern of corneal stroma has been reported[204] and shown to arise both from the arrangement of the collagen fibrils and from the packing of the tropocollagen molecules along the axis of the fibrils. The effects of swelling on the diffraction pattern were observed.

[196] H.B. Stuhrmann, M.H.J. Koch, R. Parfait, J. Haas, K. Ibel, and R.R. Crichton, *J. Mol. Biol.*, 1978, **119**, 203.
[197] J.A. Langer, D.M. Engelman, and P.B. Moore, *J. Mol. Biol.*, 1978, **119**, 463.
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[199] C. Berthet, D.J.S. Hulmes, A. Miller, and P.A. Timmins, *Science*, 1978, **199**, 547.
[200] J. Woodhead-Galloway and W.H. Young, *Acta Cryst.*, 1978, **A34**, 12.
[201] B. Brodsky, D.W.L. Hukins, D.J.S. Hulmes, A. Miller, S. White, and J. Woodhead-Galloway, *Biochim. Biophys. Acta*, 1978, **535**, 25.
[202] L.J. Gathercole and A. Keller, *Biochim. Biophys. Acta*, 1978, **535**, 253.
[203] J. Bordas, J. Woodhead-Galloway, and D.W.L. Hukins, *Biochem. Biophys. Res. Comm.*, 1978, **84**, 627.
[204] J.M. Goodfellow, G.F. Elliott, and A.E. Woolgar, *J. Mol. Biol.*, 1978, **119**, 237.

Chondroitin Sulphate.—Chondroitin-4-sulphate, a sulphated glycosaminoglycan, is of interest because of its structural role in proteoglycans. Winter and co-workers[205] have determined the molecular conformation of a regular 3-fold helical form of chondroitin-4-sulphate. The diffraction patterns obtained from the sodium and the calcium salt of a proteoglycan rich in chondroitin-4-sulphate have been reported[206] and models proposed for proteoglycan organization in connective tissue.

Spectrin.—Spectrin from human red blood cells has been examined by diffraction methods both in films and in fibres.[207] The patterns obtained from fibres are similar to those obtained from keratin, myosin, and fibrinogen suggesting that the spectrin secondary structure is largely composed of α-helices.

α-Chitin.—The structure of α-chitin has been determined by *X*-ray diffraction methods.[208] The α-chitin used was obtained from deproteinized lobster-tendon and the polysaccharide chains exhibit extensive inter- and intra-molecular hydrogen-bonding.

17 Synthetic Polypeptides

The structures of several synthetic polypeptides have been reported during 1978. The conformation of poly(N^{ε}-carbobenzoxy-L-lysine) has been determined, in four helicogenic solvents, by small-angle *X*-ray scattering methods.[209] As expected the main polypeptide chain adopts an α-helical conformation but the side-chain conformations were dependent on the solvent. Films and fibres of several monodisperse homo-L-oligopeptides have been examined[210] and the structures of their component peptides determined. A model which accounts for the observed temperature dependence of the diffraction patterns of poly(γ-methyl-L-glutamate) and poly(γ-benzyl-L-glutamate) has been proposed[211] and the structure and mechanical properties of films of poly(γ-methyl-D-glutamate) have also been reported.[212] St. Pierre and co-workers[213] have determined the structures of poly(L-tyrosyl-L-lysine), poly(L-tyrosyl-L-lysyl-L-lysine) and poly(L-tyrosyl-L-lysyl-L-lysyl-L-lysine) at various pH's and in a range of solvents. *X*-ray diffraction studies on films and fibres of poly[N^{5}-(2-hydroxyethyl)-L-glutamine] have also been published.[214]

[205] W.T. Winter, S. Arnott, D.H. Isaac, and E.D.T. Atkins, *J. Mol. Biol.*, 1978, **125**, 1.
[206] J.J. Cael, W.T. Winter, and S. Arnott, *J. Mol. Biol.*, 1978, **125**, 21.
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[208] R. Minke and J. Blackwell, *J. Mol. Biol.*, 1978, **120**, 167.
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[211] N. Matsushima and K. Hikichi, *Polymer J.*, 1978, **10**, 437.
[212] J. Watanabe, M. Naka, J. Watanabe, K. Watanabe, and I. Uematsu, *Polymer J.*, 1978, **10**, 569.
[213] S. St. Pierre, R.T. Ingwall, M.S. Verlander, and M. Goodman, *Biopolymers*, 1978, **17**, 1837.
[214] P. Spadon, A.S. Verdini, and A Del Pra, *Biopolymers*, 1978, **17**, 2029.

PART III: Conformation and Interaction of Peptides and Proteins in Solution *edited by R.H. Pain, with contributions by T. Brittain, F.E. Cohen, D.P.E. Dickson, H.W.E. Rattle, M.J.E. Sternberg, R.M. Stephens, R.M. Thomas, and E.J. Wood.*

1 Theoretical Aspects of Protein Conformation

Contributed by F.E. Cohen and M.J.E. Sternberg

This report describes the current status of theoretical studies on polypeptides and proteins. We aim to highlight the changes that have occurred since the field was last reviewed.[1—3]

Energy Functions.—In the search for an appropriate potential function, several procedures involving *ab initio* quantum mechanical calculations, semi-empirical models, empirical formulae, and various blends of the three have been developed.[4—15] The method of choice often rests upon the scope of the problem, the number of degrees of freedom in the system, and the accuracy of the solution desired. Hillier and Robson[4,5] have conducted a detailed examination of the relative merits of *ab initio* and empirical calculations.

The hydrogen bond, particularly amide hydrogen-bonding, has been studied extensively by several investigators.[6—11] Del Bene[6,7] has examined this phenomenon through molecular orbital theory using *ab initio* self consistent field calculations. Del Bene and Cohen[8] have extended this work to consider the hydrogen bonding properties of heterocyclic aromatic compounds. Pullman *et al.*[9] have used similar potential functions to study the structure of liquid and aqueous formamide and the models developed are consistent with ESCA, n.m.r., and dielectric constant data. Scheiner and Kern have used similar calculations on peptides and conclude that interpeptide hydrogen-bonding has much the same effect as hydration, and also that the α-helical conformation of a single strand of polyglycine is most stable *in vacuo*.

In spite of these successes, several studies have pointed out problems with existing potential functions.[12—14] Hagler and Lapiccirella[12] have shown that the steric effect, which is of prime importance in determining conformations is under represented. Greenberg *et al.*[13] conclude that a dielectric constant inadequately reflects the dielectric permeability of the medium and hence the nature of charge–charge interactions. The need for a dielectric function was

[1] G. Nemethy and H.A. Scheraga, *Quart. Rev. Biophysics*, 1977, **10**, 239.
[2] M.J.E. Sternberg and J.M. Thornton, *Nature*, 1978, **271**, 15.
[3] D.J. Osguthorpe and B. Robson, in *'Amino-acids, Peptides, and Proteins'*, ed. R.C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1979, Vol. 10, p. 208.
[4] I.H. Hillier and B. Robson, *J. Theor. Biol.*, 1979, **76**, 83.
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[6] J.E. Del Bene, *J. Amer. Chem. Soc.*, 1978, **100**, 1387.
[7] J.E. Del Bene, *J. Amer. Chem. Soc.*, 1978, **100**, 1395.
[8] J.E. Del Bene and I. Cohen, *J. Amer. Chem. Soc.*, 1978, **100**, 5285.
[9] A. Pullman, H. Berthod, C. Giessner-Prette, J.F. Hinton, and D. Harpool, *J. Amer. Chem. Soc.*, 1978, **100**, 3991.
[10] S. Scheiner and C.W. Kern, *J. Amer. Chem. Soc.*, 1977, **99**, 7042.
[11] S. Scheiner and C.W. Kern, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2071.
[12] A.T. Hagler and A. Lapiccirella, *J. Amer. Chem. Soc.*, 1978, **100**, 4026.
[13] D.A. Greenberg, C.D. Barry, and G.R. Marshall, *J. Amer. Chem. Soc.*, 1978, **100**, 4020.

examined. Even the semi-empirical backbone torsional potential has been questioned in the light of certain n.m.r. and i.r. spectra data. Mitra[14] believes a two-fold barrier is superior to the conventional three-fold potential. Clearly, a better representation of intramolecular forces is desirable.

An accurate representation of the solvent structure or the protein–solvent interface must be considered equally important.[15,16] The Monte Carlo calculations of Hagler and Moult[15] provide a sound basis for this study. The solvent structures around a hexapeptide and hen-egg-white triclinic lysozyme were determined. Although the results were preliminary, this powerful technique may yield new information on the energetics of hydration. Gelles and Klapper[16] have used similar calculations to establish a linear correlation between the surface areas of side-chains and their solute transfer energies.

Energy Calculations on Polypeptides.—The vast complexities of an accurate energy-surface for a polypeptide make computations on all but the smallest systems unfeasible. While no open-chain folding of an all atom representation of a protein has been successful, the conformations of several small peptides, including the enkephalins, have been examined through energy calculations.[17—25]

Several studies have concentrated on the structure of cyclohexapeptides.[17—19] Go and Scheraga[17] have re-examined the conformations of *cyclo*-hexaglycyl using a Monte Carlo method and find good agreement between the minimum energy conformations and those observed in the hemihydrate crystal. Kopple *et al.*[18] have examined another hexapeptide, *cyclo*-(Gly-D-XXX-L-YYY)$_2$ (where XXX and YYY are any of the twenty amino-acids) and find the backbone to be extended in the region of the D-residues and turn about L-YYY-Gly. The enforced curvature of these cyclic structures makes them reasonable models for the reverse turns frequently observed in protein structures.

The enkephalins, endogenous peptides with morphine-like activity found in mammals, are well suited to conformational analysis by energy calculations.[20—23] The work of Loew and Burt[20] on the Met-enkephalin indicates that the biologically active conformation where the pentapeptide resembled morphine is a high-energy conformer. The minimum-energy conformation results when a hydrogen bond is formed between the hydroxy-group of the Tyr-1 side-chain and the carboxy-oxygen between Gly-3 and Phe-4.[21] Balodis *et al.*[22] have concluded that an induced fit between the enkephalin and the morphine receptor is required.

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[24] Y. Patterson and S.J. Leach, *J. Amer. Chem. Soc.*, 1978, **100**, 49.
[25] M. Bansal and G.N. Ramachandran, *Internat. J. Peptide Protein Res.*, 1978, **11**, 73.

Another interesting application of energy calculations has been to explain certain types of crystal packing.[26—28] Bernstein and Hagler[26] employed periodic boundary-conditions together with *ab initio* calculations to examine the crystal packing of a Schiff base. The triclinic crystal was found to have a lower energy than the orthorhombic form. Hagler and Leiserowitz[27] have shown that adipamide rarely packs in orthorhombic or higher symmetries. The implication of symmetry constraints on packing models is also examined.

Analysis and Prediction of Protein Secondary Structure.—The partial success of methods to predict the secondary structure of proteins suggests that the formation of α-helices, β-strands, and reverse turns is, to some extent, determined by the local sequence. Work has continued on explaining the preference of certain amino-acid types for different secondary structures by use of explicit-energy calculation, solvent accessibility, and statistical analysis.

Considerable attention has been paid to the understanding of the local conformation of the main chain that has a non-repetitive structure, *i.e.* regions that are neither α-helices nor β-strands.[29—38] Zimmerman and Scheraga[29—31] have published a series of papers on the preference of various dipeptides for forming bends. Conformational energy calculations based on empirical parameters were performed on various dipeptides. Some of the minimum-energy conformations correspond to bend structures. Discrepancies between these findings for dipeptides containing serine and those observed in protein structures have been attributed to solvent effects which are not accounted for in the computations. Rose and Seltzer[32] have devised an algorithm to identify chain reversals (*i.e.* turns) as the local minima of the radius of curvature of main chain. As these turns nearly always occur on the surface of the protein, the residues involved will tend to have hydrophilic side-chains. Accordingly, a plot along the sequence of the free energies of transferring each amino-acid side-chain from water into a non-polar environment shows that the turns almost invariably occur at the local minima of the energy.[32] Surprisingly, the number of turns in a protein was found to increase linearly with molecular weight, and not by a $\frac{2}{3}$ power which would be expected by a surface to volume ratio.[34] A new, commonly occurring, unit of non-repetitive structure has been described by Richardson *et al.*[35] – the β-bulge. This occurs as a distortion in β-pleated sheets, generally the antiparallel structure, when the usual alternation of side-chain directions is put out of register in one of the β-strands by a break in the regular hydrogen-bonding arrangement. The β-bulge, of which there are different types, accentuates the usual twist of the β-strand, and therefore assists a β-sheet to form a closed β-barrel structure.

[26] J. Bernstein and A.T. Hagler, *J. Amer. Chem. Soc.*, 1978, **100**, 673
[27] A.T. Hagler and L. Leiserowitz, *J. Amer. Chem. Soc.*, 1978, **100**, 5879.
[28] J. Caillet, P. Claverie, and B. Pullman, *Theor. Chim. Acta*, 1978, **47**, 17.
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[30] S.S. Zimmerman and H.A. Scheraga, *Biopolymers*, 1978, **17**, 1871.
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[32] G.D. Rose and J.P. Seltzer, *J. Mol. Biol.*, 1977, **113**, 153.
[33] G.D. Rose, *Nature*, 1978, **272**, 586.
[34] G.D. Rose and D. Wetlaufer, *Nature*, 1977, **268**, 769.
[35] J.S. Richardson, E.D. Getzoff, and D.C. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2574.

There have been further analyses of the preferences of residue types to adopt the α-helical, β-strand, turn and coil (α, β, t, and c) conformations.[39—47] This work aims to improve empirical methods of secondary-structure prediction by including all the data from the ever increasing number of proteins of known sequence and structure. A major problem in this work has been the assignment of the secondary structure. Levitt and Greer[39] have devised an automatic procedure to make these assignments (α, β, t, and c) based on local conformation and hydrogen-bonding patterns. Analysis of their assignments in more than sixty proteins, some of which are homologous, shows that nearly all the residues have a weak, but significant, preference for only one type of secondary structure.[40] Garnier *et al.*[41] have devised a new four-state (α, β, t, and c) predictive scheme which considers the probable conformation of residue i from the residue types at positions i − 8 to i + 8 in the sequence. It is shown that more successful predictions (57 % accuracy) are made if one incorporates a rough estimate of the α-helix and β-structure content of the protein. A novel approach to understanding the stereochemical basis for the conformation preference of residues has been made by Richards and Richmond.[42] They demonstrated that the accessible surface-area of a side-chain type when in an α-helix and in a β-sheet, correlates well the respective empirical parameters of Chou and Fasman.[48]

It is unlikely that the incorporation of data from a few more protein structures will markedly improve any of the current empirical methods of predicting secondary structure. Thus a different approach to the problem is required, possibly one based on hydrophobicity as suggested from the work on turns of Rose and Seltzer,[32—34] and from the work of Richards and Richmond[42] on α-helices and β-sheets.

Analysis of Tertiary and Quaternary Structure of Proteins.—The emphasis of recent examinations of tertiary and quaternary structure has been on hydrophobicity and close packing. This contrasts with the work of a few years ago when the topology of the chain fold was considered extensively.

One aspect of tertiary structure formation is the packing of α-helices and β-sheets. Chothia *et al.*[49] have proposed simple models to explain the observed range of packing angles for the commonly observed structural units: two

[36] M.H. Loucheux-Lefebvre, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 1352.
[37] R. Lumley Jones, *Nature*, 1978, **272**, 185.
[38] Y. Nakata, T. Akaike, and S. Inoue, *Biopolymers*, 1978, **17**, 937.
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[43] T.T. Wu, S.C. Szu, R.L. Jernigan, H. Bilofsky, and E.A. Kabat, *Biopolymers*, 1978, **17**, 555.
[44] P. Argos, M. Hanei, and R.M. Garavito, *F.E.B.S. Letters*, 1978, **93**, 19.
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[49] C. Chothia, M. Levitt, and D. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4130.

α-helices; an α-helix against two parallel β-strands; and a stacked pair of β-pleated sheets. The α-helix is represented as a cylinder with ridges formed by the side-chains and the β-sheet as a twisted, but smooth, surface. The structures have to pack tightly to exclude water from a hydrophobic core. Helix–helix packing in myoglobin has been analysed in detail by Richmond and Richards.[50] Each interaction was quantified by the change in the surface area accessible to solvent that resulted from packing the two α-helices. Different packing types with specific interhelical separation and orientation were observed. Probable helix–helix interaction sites for each type were predicted from the sequence by finding hydrophobic patches that include a specific type of residue. The role played by hydrophobicity in packing also has been shown by Sternberg and Thornton:[51] often β-sheets are formed so that the most hydrophobic β-strand is buried in the centre of the β-sheet and the other β-strands are arranged outwards in both directions in order of decreasing hydrophobicity. In a novel calculation involving the effects of the helix dipole, Weill and Andre[52] have shown that a head-to-tail association of helical polypeptides can be favoured over the side-by-side antiparallel arrangement in certain environments. Thus the helix dipole may be implicated in protein structure as well as protein function (see below).

Features of the entire protein structure, not just the α-helices and β-sheets, have been analysed. There has been particular emphasis on quantifying which side-chain and atom types tend to be exposed to solvent.[53—57] The results are broadly similar to earlier work on solvent accessibility.[58, 59] However, analysis of a larger number of different proteins has lead to empirical measures of the free energy of transfer of a residue from inside to the surface of a protein. The values are similar to those determined experimentally by Nozaki and Tanford.[60] Related work considered the observed side-chain conformations[61] and also side-chain–side-chain and atom–atom interactions.[62—66]

In view of the interest shown by the above analyses, it is important that different methods are being developed to quantify aspects of tertiary and quaternary structure.[67—69] Greer and Bush[67] have described a method to represent the molecular surface of a protein which will assist in studies on shape-

[50] T.J. Richmond and F.M. Richards, *J. Mol. Biol.*, 1978, **119**, 537.
[51] M.J.E. Sternberg and J.M. Thornton, *J. Mol. Biol.*, 1977, **115**, 1.
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[55] P. Manavalan and P.K. Ponnuswamy, *Nature*, 1978, **275**, 673.
[56] C. Tanford, *Science*, 1978, **200**, 1012.
[57] J.L. Finney, *J. Mol. Biol.*, 1978, **119**, 415.
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[59] C. Chothia, *Nature*, 1975, **254**, 304.
[60] Y. Nozaki and C. Tanford, *J. Biol. Chem.*, 1971, **246**, 2211.
[61] J. Janin, S. Wodak, M. Levitt, and B. Maigret, *J. Mol. Biol.*, 1978, **125**, 357.
[62] P.K. Warme and R.S. Morgan, *J. Mol. Biol.*, 1978, **118**, 273.
[63] P.K. Warme and R.S. Morgan, *J. Mol. Biol.*, 1978, **118**, 289.
[64] R.S. Morgan, C.E. Tatsch, R.H. Gushard, J.M. McAdon, and P.K. Warme, *Internat. J. Peptide Protein Res.*, 1978, **11**, 209.
[65] H.A. Pohl, *J. Biol. Phys.*, 1978, **4**, 144.
[66] P.A. Westhaus and H.A. Pohl, *J. Theor. Biol.*, 1978, **70**, 157.
[67] J. Greer and B.L. Bush, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 303.

specific recognition. Folding domains have been identified by Crippen[68] using a tree structure to represent the interactions of protein segments.

The evolutionary significance of structural similarities between or within proteins has been discussed. Levine *et al.*[70] have suggested that divergent evolution could explain the structural similarity between pyruvate kinase and triose phosphate isomerase. Both enzymes contain a β-barrel formed from eight parallel β-strands, adjacent β-strands being connected by α-helices that form an outer cylinder roughly co-axial with the barrel axis. Tang *et al.*[71] found two structurally similar domains in the acid proteases although only about 20 % of the topologically equivalent residues are conserved. Thus it is difficult to decide whether the structural similarity arose by gene duplication, fusion and divergence, or by convergent evolution.

Prediction of Tertiary and Quaternary Structure of Proteins.—In the last few years, attempts to predict the tertiary structure of a protein have relied on simplified models for the protein conformation and rough energy functions.[72, 73] Pancreatic trypsin inhibitor was often studied and some of the methods predicted a structure that seemed fairly similar to the crystallographic model. However, the success of these predictions has been questioned by Hagler and Honig.[74] They represented the inhibitor by a chain of alanine residues with glycines located where the bends were observed in the native structure. They obtained a minimum energy conformation whose r.m.s. deviation from the crystal structure was comparable to those obtained in the simulations with the correct sequence. Furthermore, they pointed out that neither in their simulation nor in the other studies was the correct fold reproduced.

Other workers have studied the problems associated with the prediction of the tertiary and quaternary structure of proteins.[75—80] Monte Carlo simulations of folding on a lattice model for the protein are popular. Havel *et al.*[75] have considered the effects of various distance constraints on the prediction of a structure. A set of random conformations that obeyed the constraints were generated and then compared to the crystallographically determined structure. For example, information on the distance of each residue to the molecular centroid is insufficient to predict a structure similar to the native. Rackovsky and Scheraga[76] studied the folding of α-helix-coil–α-helix and extended-coil-extended structures using empirical free-energies to determine residue–residue interactions. Wodak and Janin[77] considered whether the interaction mode of trypsin with its inhibitor can be predicted. About nine structures were found that had non-bonded interactions and buried-surface areas similar to those of

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[73] I.D. Kuntz, G.M. Crippen, P.A. Kollman, and D. Kimelman, *J. Mol. Biol.*, 1976, **106**, 983.
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[75] T.F. Havel, G.M. Crippen, and I.D. Kuntz, *Biopolymers*, 1979, **18**, 71.
[76] S. Rackovsky and H.A. Scheraga, *Macromolecules*, 1978, **11**, 1.
[77] S.J. Wodak and J. Janin, *J. Mol. Biol.*, 1978, **124**, 323.

the native. All the studies highlight the difficulty in deducing a correct protein structure by a consideration of residue–residue interactions.

Several workers are following an alternative approach to the prediction of tertiary structure by model-building studies to pack α-helices and β-strands.[81—92] For example, the tertiary and quaternary fold of the ribosomal protein L7 was predicted, based on solution data, suggesting an elongated structure with a high α-helical content and stereochemical arguments about the location and packing of α-helices.[81] However, many of the proposed methods suffer from the drawback that, at present, they are non-automated and have not been applied to several proteins so that the objective nature of the algorithm is hard to assess. Model-building studies have also been used to predict the tertiary structure of relaxin[82,83] based on its sequence similarity to insulin and several α-helical coiled-coil structures.[84—87]

Protein Function.—The intimate relationship between structure and function in proteins has been demonstrated by several approaches. Hol *et al.*[93] have shown that the α-helix has an electric dipole of about one-half elementary charge at each end. Thus the positive *N*-terminus of an α-helix would interact favourably with negatively charged groups. Helix-dipole effect may be important in both the structure and function of proteins. Indeed, the commonly observed binding of charged phosphate moieties at the *N*-terminus of α-helices and the *C*-terminus of parallel β-pleated sheets may be the result of convergent evolution to a functional favourable conformation. Argos *et al.*[94] have observed structural similarities in the active-centre geometries of zinc-containing proteins. A different approach toward the understanding of function is from a consideration of solvent accessibility.[95—97] For example, the oxidation and reduction potentials of haem proteins has been related to the extent of haem exposure to solvent.[95]

Protein Dynamics.—Energy calculations[98—101] and analysis of experimental

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[82] T.L. Blundell, S. Bedarkar, E. Rinderknecht, and R.E. Humbel, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 180.
[83] N. Isaacs, R. James, H. Niall, G. Bryant-Greenwood, G. Dodson, A. Evans, and A.C.T. North, *Nature*, 1978, **271**, 278.
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[86] A.D. McLachlan, *J. Mol. Biol.*, 1978, **122**, 493.
[87] A.D. McLachlan, *J. Mol. Biol.*, 1978, **124**, 297.
[88] V.I. Lim, *F.E.B.S. Letters*, 1978, **89**, 10.
[89] O.B. Ptitsyn and A.V. Finkelstein, *Bioorg. Khim.*, 1978, **4**, 349.
[90] A.L. Mazanov and V.I. Lim, *Mol. Biol.*, 1978, **12**, 219.
[91] V.I. Lim, *Mol. Biol.*, 1978, **12**, 214.
[92] A.V. Finkelstein, *Mol. Biol.*, 1978, **4**, 340.
[93] W.G.J. Hol, P.T. van Duijnen, and J.C. Berendsen, *Nature*, 1978, **273**, 443.
[94] P. Argos, R.M. Gravito, W. Eventoff, and M.G. Rossman, *J. Mol. Biol.*, 1978, **126**, 141.
[95] E. Stellwagen, *Nature*, 1978, **275**, 75.
[96] E. Stellwagen and H. Wilgus, *Nature*, 1978, **275**, 342.
[97] J. Janin and C. Chothia, *Biochemistry*, 1978, **17**, 2943.
[98] M. Karplus and J.A. McCammon, *Nature*, 1979, **277**, 578.

results[102—104] have been used to detail the thermal motion of globular proteins. Karplus and McCammon[98] have used molecular dynamics calculations to investigate structural variations in pancreatic trypsin-inhibitor over a longer time scale than in the earlier work. Highly anisotropic motion is observed with the r.m.s. positional variation of all atoms being 0.75 Å. The orientation of buried tyrosine rings fluctuated by $\pm 30°$. Detailed experimental data are now being derived from analysis of the thermal parameters (*i.e.* *B*-values) obtained in refinement of crystal structures.[102—103] Blake *et al.*[102] have shown that in the 2.5 Å structure of hen-egg-white lysozyme side-chains that are exposed generally have higher thermal parameters, *i.e.* greater conformational variability, than those side-chains that are buried.

Conclusion.—We consider that there have been two dominant features in this year's work. The success of protein-folding studies has been re-evaluated by the findings of Hagler and Honig.[74] They demonstrated that previous studies neither reproduced the correct topological fold nor a significantly small r.m.s. deviation from the native structure. It is encouraging, therefore, that different approaches to the analysis and prediction of protein structure are being developed. Many investigators have used solvent accessibility in novel ways to quantify aspects of protein structure. These include the prediction of reverse turns,[32—34] the packing of α-helices,[50] and the oxidation potential of haem proteins.[95] We expect that there will be further developments along these lines.

2 Folding of Globular Proteins

Contributed by R.M. Thomas

Theoretical approaches to the formation of secondary structure and folding are considered elsewhere in this volume.[105] An introduction to protein stability and conformation has been published.[106] Folding and unfolding reactions are discussed at length with particular reference to bovine pancreatic trypsin inhibitor, a small protein with an established folding pathway.[107]

Solution Conformation.—N.m.r. experiments on the internal mobility of trypsin inhibitor have provided further evidence for a dynamic model of globular proteins[108,109] and it is inferred that hydrophobic domains formed early in the folding process might act as nucleating centres. The measurement of thermal-quenching curves has been suggested as a novel approach to the

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[100] N. Go, *Biopolymers*, 1978, **17**, 1373.
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[103] W. Bode, *J. Mol. Biol.*, 1979, **127**, 357.
[104] G. Wagner and K. Wüthrich, *Nature*, 1978, **275**, 247.
[105] F.E. Cohen and M.J.E. Sternberg, this volume, p. 233.
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investigation of structural mobility.[110] A theoretical investigation of the flexibility of trypsin inhibitor concludes that the flexibility of orientation of peptide groups and that of the backbone and side-chains are co-operatively linked.[111] The contribution of non-regular structures and bends to overall conformational mobility are also discussed.[112] Comparison of a scattering curve calculated from *X*-ray atomic co-ordinates with that obtained from large angle diffuse *X*-ray scattering measurements on ribonuclease indicates that the solution structure does not differ from the crystalline structure.[113]

The conformation and secondary structure of polypeptides from snake[114] and diptheria[115] toxins, and hormone zymogens[116] have been considered from a biological activity standpoint.

Stability.—*Thermodynamics.* Several experimental techniques reveal a layer of water surrounding macromolecules and this is confirmed by simulation of the structure and energetics of solvent in crystals of lysozyme and a small peptide.[117] The effect of pressure on the swelling of the non-polar protein elastin indicates that hydrophobic interaction becomes weaker as pressure increases.[118] Rate constants of the hydrogen-exchange reactions of proteins subjected to pressure indicate either increased solvent penetration or the inducement of slight unfolding.[119] Hydrophobic free-energies used in the calculation of conformational stabilities and association constants of protein–protein interactions must be supplemented by a term for the enthalpy of fusion.[120] The denaturation of the thermostable cytochrome *c* from *Thermus thermophilus* occurs at a higher temperature and the free energy is greater than for mesophilic homologues.[121] The correlation of accessible surface area and thermostability suggests that the size of domains and polymerization are critical to thermostability.[122] Thermodynamic parameters and entropies of interaction for the unfolding of human immunoglobulin G by guanidinium chloride (GuCl) are found to depend on the extent of denaturant binding.[123]

The thermodynamic processes involved in folding and unfolding transitions are given detailed consideration in the individual studies.

Stability of Proteins towards Denaturation. Lipophilin, a hydrophobic myelin basic protein, forms an irreversibly denatured state which retains at least 60 % organized secondary structure in 8M-urea or 6M-GuCl even after eight days. Transfer of the protein from 2-chloroethanol to GuSCN results in the reburial of tryptophan residues exposed in the organic solvent. It is concluded that the

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[114] A. Menez, G. Langlet, N. Tamiya, and P. Fromageot, *Biochimie*, 1978, **60**, 505.
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[122] E. Stellwagen and H. Wilgus, *Nature*, 1978, **275**, 343.
[123] S. Lapanje, B. Crešnar, V. Vlachy, and J. Škerjanc, *Biochim. Biophys. Acta*, 1978, **537**, 380.

resistance of the protein to denaturants reflects the presence of an impervious hydrophobic core.[124] Crosslinking studies on myelin basic protein reveal that the conformation in sodium dodecyl sulphate (SDS) is similar to that in the membrane.[125]

The lipoprotein apo(E-A-II) is stable to 4M-GuCl, 8M-urea, and 2 % SDS but dissociates into two subunits after reduction.[126] Hydrogen-exchange measurements on the highly hydrophobic rhodopsin in the presence of the detergents Cemulsol LA90 and Ammonyx LO indicate that its structure is unperturbed.[127] Bleached rhodopsin is stabilized by membrane but loses its native conformation in the detergents. It is suggested that long-range physical properties of the phospholipid bilayer stabilize the native protein conformation.[128] Optical measurements on tryptophan residues and viscosity data indicate no major conformational change when sesame α-globulin is treated with the non-ionic detergents Triton X100 or Brij 36T.[129]

Comparison of n.m.r. spectra for the isoinhibitor from *Helix pomatia* with that from homologous inhibitors indicates that conformation flexibility is lowered but that stability to thermal denaturation is raised when the content of amino-acids with charged side-chains is increased.[130] A salt bridge between the *N*- and *C*-termini of trypsin inhibitor may be abolished by transamination of Arg-1. The modified protein has a lower thermal denaturation temperature and higher interior proton-exchange-rate. It is estimated that the stabilizing influence of the salt bridge in the native protein is 4.2 $kJmol^{-1}$.[131]

Removal of the haem group from cytochrome *c* peroxidase from *Saccharomyces cerevisiae* causes no great secondary structure change,[132] whereas e.p.r. studies of the thermal inactivation of iron-superoxide dismutase show that the reconstituted protein is slightly less stable than the native enzyme.[133] The biphasic, acid-induced transition of RNAase A is completely reversed by substrate analogues 2′- or 3′-CMP, as indicated by tyrosine exposure[134] and the stabilization of carp parvalbumin IV by tightly bound calcium-ion is of an enthalpic nature.[135] The stabilities of various proteases in the presence of SDS and urea, but in the absence of substrate, have been compared.[136] Dehydrofolate reductase from *Escherichia coli* is greatly stabilized to heat denaturation by NADPH or methotrexate but not by trimethoprim or pyrimethamine.[137] 1.2M-NaCl or 0.75M-$(NH_4)_2SO_4$ stabilize IgG fragments to thermal pertur-

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bation, the results being interpreted in terms of a dynamic model of protein behaviour in aqueous solutions.[138]

Effect of Solvent.—The influence of solvent on conformation and activity has been reviewed.[139] A monograph on denaturation from a physico-chemical and experimental point of view has been published.[140] Ionic effects on protein denaturation have also been considered.[141]

Denaturation and Partially Denatured States. C.d. and fluorescence measurements indicate an irreversible unfolding transition for the soluble adenosine triphosphatase (ATPase) from *Micrococcus lysodeikticus* on heating, which is not reversed by ATP or $CaCl_2$. The denatured state retains appreciable secondary structure, and in the presence of GuCl the transition occurred at a lower temperature.[142] The ATPase from sarcoplasmic reticulum was resistant to complete denaturation in GuCl and its behaviour suggests that it has several domains of varying resistance to unfolding induced by this solvent.[143] The hydrophobic coat-protein of fd bacteriophage is only converted into a random coil by very high GuCl concentrations and dimerizes in anionic detergents or phospholipid.[144] Conformational changes in β-lactoglobulin have been monitored as a function of pH, temperature, and protein concentration. The conformational change at pH 7.5 involves exposure of apolar residues to the solvent and an increased affinity for hydrocarbons. The results suggest that an aqueous two-phase partition system may be used to characterize hydrophobic surface-properties of proteins.[145] The hydrophobic regions of bovine serum albumn (BSA) differ with regard to their thermal stability. Heating from 17—47 °C results in increased exposure of hydrophobic segments to the solvent, culminating in a predenaturation rearrangement.[146] Calorimetric data from RNAase indicate that a pretransition which occurs in 1.25M-GuCl occurs without gross unfolding of the protein but does not represent simple denaturant-binding.[147] It is also found that e.p.r. is a sensitive probe of conformational change in RNAase.[148] Small reversible thermally induced changes in F-actin monitored by e.p.r. reflect the thermal stability of the protein below an irreversible transition temperature of 65 °C.[149] Fluorescence studies on the effect of temperature on fibrinogen and its derivatives are reported.[150]

The variation of surface tension of aqueous solutions of fibrinogen, transferrin, γ-globulin, and albumin with temperature has been used as an index of

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conformational change.[151] α-Tropomyosin undergoes a transition at 31 °C when the molecule loses the ability to form supramolecular structures.[152] The denaturation of ribosomal proteins has been investigated with c.d. and scanning microcalorimetry.[153] The c.d. results indicate that these proteins have a different conformation in solution to that when bound to the ribosome.[154] Irreversible thermal denaturation of lysozyme is dependent on ionization of Glu-35.[155] A comparison of the denaturation of triclinic lysozyme by SDS and bromoethanol shows that although the two compounds differ chemically, their mode of action is similar, providing evidence for a general mechanism of binding for hydrophobic–hydrophilic denaturants.[156] However, a comparative *X*-ray diffraction study of α-chymotrypsin denaturation by urea and by GuCl demonstrates that the two denaturants act in completely different ways. GuCl binds exclusively to the surface of the protein causing changes in the hydrophobic interior, whereas urea is capable of penetrating the molecule.[157] An estimate of tryptophan and tyrosine exposure as lysozyme is unfolded by dioxan reveals that the two amino-acids behave differently; tyrosyl exposure increases with denaturation but tryptophan, exposed at low dioxan concentration, is reburied as denaturant concentration increases.[158] Solvent perturbation studies on rabbit Clq reveal a pH-induced increase in tyrosine and tryptophan exposure at low pH.[159] Gel filtration has been used to follow the GuCl induced expansion of myelin basic protein, suggesting a lack of intramolecular stabilization in this molecule.[160] Cytochrome *c*-552 is remarkably acid stable, and is unfolded at lower GuCl concentrations at high pH. It is implied that the denaturation does not represent a true two-state equilibrium.[161] The endothermic unfolding of cytochrome *c* by SDS has been compared with the binding of the detergent to erythrocyte glycoproteins.[162] Observations on the structural transitions of immobilized chymotrypsinogen A have been reported.[163]

The denaturation of swine pepsinogen by urea has been studied and found to be fully reversible.[164] The effect of interactions in the zymogen molecule may determine the activity of pepsin. Denaturation of pepsin at pH 6 results in an expanded molecule with some loss of secondary structure. Both native and alkali denatured forms unfold co-operatively in the presence of GuCl.[165] Unlike other alkaline proteases mesentericopeptidase is unfolded by urea at

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[164] F. Ahmad and P. McPhie, *Biochemistry*, 1978, **17**, 241.
[165] F. Ahmad and P. McPhie, *Internat. J. Peptide Protein Res.*, 1978, **12**, 155.

neutral pH following first order kinetics.[166] The denaturation of ferri- and apomyoglobin from *Thunnus thynnus* by GuCl has been compared to that of beef, buffalo, and sperm whale myoglobins.[167] The GuCl induced denaturation of *Lumbricus terrestris* paramyosin indicates that it does not possess a region of special stability, as seen in its molluscan analogue.[168] The heat denatured form of a Bence-Jones type cryoprotein is not fully unfolded and retains some antigenic sites, but loses its cryoprecipitation properties. It is suggested that heat denaturation affects only the variable region.[169] The effects of GuCl, pH, and temperature on calf-brain tubulin are also reported.[170]

Enhancement of Structure. The effect of 25—75 volume % ethanol on the conformations of various proteins and glycoproteins has been probed with c.d.[171,172] Higher alcohol concentrations organize the main chains into higher helix and β-sheet content, the degree of reorganization being dependent on disulphide bridge cross-linking of the main chain. Similar events occur when phytohaemagluttinins are treated with SDS. Following initial disruption, the main chain is reconstructed with a higher helix content. Hydrogen-exchange studies on corticotrophin show that the partially organized structures present in aqueous solution are replaced by a strongly stabilized α-helical structure in trifluorethanol.[173] Physical and chemical methods reveal a coil-to-helix change when a cyanogen bromide fragment of troponin C with an intact binding-site binds calcium ion.[174]

Fragments and Complementation.—Considerable attention has been paid to complementing fragments of cytochrome *c* and their derivatives. Complementation as measured by several physical techniques occurs between the major non-haem peptide 1 — 104 and the *N*-terminal haem peptides 1 — 38, 1 — 65, and 1—80.[175] Limited tryptic digestion of the ferrous form of the complex 1—38: 1—104 produces two derivatives 1—25: 23—104 and 1—38: 56—104 in a ratio of 2 : 1 which both possess considerable native properties. While there is strong interaction between 1—25 and 39—104 no biological activity is seen and it is concluded that while the sequence 39—55 is not required for the formation of ordered structure, residues 26—38 provide an important stabilization of the native structure.[176] Fragment complementation has also been used in a study of the dynamics of cytochrome *c* which are found to be strongly influenced by oxidation and reduction of the iron atom.[177] RNAase (1—118) is similar hydrodynamically, and in its spectral characteristics, to intact RNAase.

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[177] R.R. Hantgan and H. Taniuchi, *J. Biol. Chem.*, 1978, **253**, 5373.

Reduction and re-oxidation results in an expanded form which is unable to bind the complementing fragment 105—124 significantly, even when incubated with it during re-oxidation. RNAase (1—118) incubated with microsomal disulphide interchange enzyme and peptide 105—124 regains 30% native activity, and fragment 105—124 prevents inactivation of RNAase (1—118) by the interchange enzyme. It is suggested that the free energy of RNAase (1—118) is not lower than that of its isomers, while interaction with RNAase (105—124) favours the correct formation of RNAase (1—118) over its isomers.[178] A peptide fragment, mol. wt. = 5000, dissociates from human-leukocyte interferon during reduction and denaturation, probably as a result of proteolysis. The resultant fragment, mol. wt. = 21 000, still retains its antiviral activity.[179] The major peptide produced by the cyanogen bromide (CNBr) cleavage of trypsin inhibitor which lacks six *C*-terminal residues forms random disulphide bridges and is unable to refold under conditions where it is well established that the native protein does so (see ref. 107). Replacement of Met-52 with Hser-52 produces a species that refolds slightly less readily than the native protein and has an altered spectrum of single disulphide intermediates.[180] CNBr cleavage of soybean trypsin inhibitor produces three peptides. Using structure enhancing solvents it is possible to induce significant α-helical structure, while reduction and *S*-carboxymethylation produces β-structure in two of the fragments. The results support the idea that partial cleavage and reduction allow formation of more regular structures than are seen in the native protein.[181] Cleavage of the haem domain of sulphite oxidase with CNBr produces two peptides which are essentially structureless. Addition of haemin to either peptide enhanced the c.d. spectrum and promoted native-like antigenicity in the *N*-terminal fragment and helix formation in the *C*-terminal fragment. When haem was added to a mixture of the two peptides, the folding of the intact domain was reattained.[182]

Folding and Folding Pathways.—*Theoretical.* Folding pathways and intermediates have been briefly reviewed.[183] A cluster model of protein folding has been used in an examination of multiphasic folding and unfolding mechanisms.[184] The role of non-polar clusters, which introduce heterogeneity in the interior of globular proteins, in the folding process is discussed.[185] A simple model for thermal denaturation has been proposed.[186]

Experimental. The kinetics and aromatic difference spectra of the thermally induced unfolding of α-lactalbumin have been analysed.[187] The partially unfolded state, *H*, produced by temperature jump has an appreciable amount of

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secondary structure and is similar to, although not identical with, the acid unfolded form, *A*, previously reported.[188] The GuCl and acid-induced unfolding of human α-lactalbumin have been compared with the results for bovine α-lactalbumin.[189]

The thermal unfolding of RNAase A has been examined with ^{13}C-n.m.r. The results indicate a multistage process; initial unfolding of some less rigid regions of the backbone (including residues 17—24) produces a local mobile state. This process destabilizes the entire protein so that on further heating it unfolds to a loose structure. However, unfolding is incomplete at pH 1.1 and 70 °C.[190] A low level of activity, sensitive to *N*-ethyl maleimide, is seen in reduced RNAase early in the refolding process. An amino-acid sequence adopts a preferred non-random conformation in a given solvent reflected in an increased probability of forming the native conformation. Formation of such molecules, even with low probability, will act as a nucleation step, increase thermodynamic stability, and drive the process in favour of native state.[191] N.m.r. evidence has been presented for an intermediate formed early in the refolding of RNAase A and indicates that an *N*-terminal helix forms rapidly when the protein is subjected to refolding conditions.[192] The expanded *x*-helical intermediate (*H*) stable in 0.8M-GuCl reported on the folding pathway of *Staphylococcus aureus* Penicillinase has been well characterized. A multi-domain model corresponding to a kinetic pathway of folding is proposed in which the domains can separate without appreciable loss of secondary structure.[193] Fluorescence quantum-yield measurements on the folding processes of bovine carbonic anhydrase are consistent with a stepwise acquisition of structure. A four state model is suggested.[194] The anomalous re-folding of the protein has been interpreted in terms of the formation of an off-pathway, incorrectly folded species.[195] Cytochrome *c* from species follow a common folding mechanism in methanol and acid.

$$N \rightleftharpoons I \rightleftharpoons II \rightleftharpoons III$$

I is a loosened form of the native protein with both methionine and histidine ligands attached to the haem iron, II the co-operatively unfolded protein with the methionine ligand displaced, and III the unfolded protein with both ligands displaced.[196] Equilibrium unfolding studies show that stabilities of many types of cytochrome *c* fall in a narrow range and that reduction stabilizes the tertiary structure by $\simeq$ 1 kcal mol^{-1}. The kinetics of reduced and oxidized cytochrome-*c* are consistent with sequential model for the protein.[197] Immunochemical analysis of trapped intermediates in the refolding of trypsin inhibitor has been used to determine the probability of formation of segments antigenically similar to either the native or denatured protein. It is shown that no

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segment adopts a native-like conformation until the entire polypeptide is able to do so simultaneously. The folding process does not appear to consist of addition of structures to a native-like nucleation centre.[198]

Domains. Antibodies have been produced to the three domains and several subdomains, as well as to the intact molecule of BSA. The domains are antigenically different and each possesses a minimum of two antigenic sites.[199] Refolding of reduced BSA is enhanced by the addition of antibodies, but only in the domain to which the antibody bears specificity. These studies also indicate that the *C*-terminal refolds more rapidly than the *N*-terminal of each domain, implying a *C*- to *N*-terminal folding pathway. This is consistent with the existence of a nucleating centre in the *C*-terminal of each domain.[200] The three dimensional structure of rhodanese reveals two domains of remarkably similar conformation.[201] Slight homology between the domains is noted but the structural similarity is reflected far more in the tertiary structure than in the primary sequence.[202] Proteolytic cleavage of bovine rhodopsin produces three fragments. The c.d. of the solubilized proteolyzed protein is identical to that of the native state. The helical nature of the native protein is retained even when the fragments are dissociated, indicating independently stabilized domains within rhodopsin.[203] The effect of denaturants on cell-surface protein have been investigated, and the results obtained suggest the presence of structural domains along the flexible polypeptide chain.[204, 205] Limited tryptic digestion of sarcoplasmic reticulum ATPase produces two fragments which cannot be separated without denaturation. It is found that the two fragments are mutually structurally dependent and retain activity in the cleaved form.[206] Fluorescence studies on the binding of the hydrophobic *C*-terminal peptide of cytochrome *b*-5, which represents the membrane-binding domain of the intact protein, to lecithin vesicles are reported.[207] Summation of c.d. spectra of the two isolated, independently folding domains of rat-liver sulphite oxidase produces the native spectrum, most of the secondary structure residing in the haem domain. Differences between the size of domains produced by trypsin, chymotrypsin, and papain suggest that the interdomain hinge region is at least 30 residues long.[208] Studies on the domains and their interactions are also reported.[209] The trypsin and chymotrypsin inhibitory properties of soybean trypsin inhibitor reside in two separate domains into which the protein may be separated after CNBr and pepsin treatment. The isolated domains retain their inhibitory

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properties but are less stable than the native protein.[210] Egg-white ovomucoid, which consists of three domains, may be reversibly unfolded by GuCl. Difference spectroscopy and viscosity measurements reveal a multiphasic transition reflecting the independent unfolding of separate domains at different denaturant concentrations.[211] One of the two domains of pancreatic elastase is able to fold independently, and complementation of the two domains produces a complex of native conformation but only weak activity.[212] Digestion of chicken-erythrocyte histone H5 is initially rapid and results in the formation of a globular folded resistant peptide, which retains all the α-helical content of the intact histone. It is suggested that the protein consists of three domains; disordered segments at both *N*- and *C*-terminals with a central globular portion.[213] The importance of domains and hinges to the assembly of viral proteins is discussed.[214]

Disulphide Bonding. A peptide 13—105 of hen-egg-white lysozyme (HEWL) with five thiol groups reforms two native-like disulphide bonds. The reoxidized peptide possesses native-like activity towards antisera raised against the intact protein and to chitin affinity chromatography. The results support a modular view of folding.[215] Studies on an enzymatically active form of HEWL which lacks one native disulphide bond suggest that forces stabilizing the native protein may still operate, although perturbed, in forms with modified disulphide bridges.[216] Reoxidation of reduced denatured bovine seminal RNAase in the presence of glutathione produced a monomeric form which differed in its c.d. spectrum from the native dimeric species. The activity of the monomer was approximately twice that of the dimer, and amino-acid analysis reveal two bound glutathione molecules which may block the two cysteine residues responsible for intermolecular disulphide bonding.[217] As reduced chymotrypsinogen forms aggregates in aqueous solutions, studies on refolding the protein have been made in low concentrations of GuCl, which favours native-like refolding. Some protein species isolated during folding had incorrect disulphide pairing which could be rearranged by thiol catalysed interchange to give the fully renatured species.[218] Incorrect disulphide pairing was also detected in the reoxidation of a taiwan cobra reduced cardiotoxin.[219] Studies on the denaturation of interchain disulphide cross-linked rabbit-skeletal tropomyosin suggest an inherently unstable region of the molecule at which chains segreate before dissociation.[220] It is concluded that disulphide bond formation in pepsin is determined by peptide fragments located outside

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[220] S.S. Lehrer, *J. Mol. Biol.*, 1978, **118**, 209.

disulphide bridge loops as well as by the size and conformation of the loops.[221] The denaturation of cytochrome-*c* D450 by metabolites of cyclophosphamide can be blocked by cysteine. The denaturation of the enzyme may therefore result from interaction of the metabolites with sulphydryl groups in the protein.[222] In conclusion, it is worth noting that 'there should be no pride or shame for a protein to have or not to have disulphide bonds, since it folds up by the same mechanism in either case'. (see ref. 191) The formation of disulphide bonds in pancreatic trypsin inhibitor is also discussed in ref. 107.

Proline Isomerization. The implications of *cis–trans* isomerization of proline for the rate of folding of globular proteins has been discussed. Half-times for refolding are related quantitatively to proline content. Unphysiological half-times are explained in terms of independent folding in domains.[223]

The refolding of the homologous proteins, tuna-heart and horse-heart ferricytochrome *c*, is stated to be incompatible with the proline isomerization model.[224] Analysis of the folding transition of RNAase and measurement of the slow refolding step in a variety of conditions does not allow the conclusion that the slow step is due to proline isomerization, and isomerization of disulphide bonded loops is suggested as the alternative.[225] However, measurement of the formation of the slow refolding species of RNAase during acid catalysis reveal two reactions, suggesting that at least two 'essential' proline residues produce a slow refolding species by isomerization after unfolding.[226] Kinetic analysis of the folding of carp parvalbumins is reported. The form with one proline residue consistently exhibited a slow refolding phase not seen in the other two which contain no proline.[227] ^{13}C and ^{1}H n.m.r. studies of proline isomerization in hormones and peptides are also reported.[228—230] The involvement or otherwise of proline isomerization as a rate-limiting step in the folding of proteins seems still to be an open question.

3 Circular Dichroism

Contributed by T. Brittain

C.d. maintains its place as perhaps the prime method of secondary-structure determination in proteins. The technique is still widely used for studies on small peptides and ligand-binding systems. The introduction, over the past year, of a stopped-flow c.d. apparatus will undoubtedly lead to an upsurge in the use of this form of detection for the previously 'indirect' observations of rapid changes in the secondary structures of proteins.

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General.—*Reviews.* A number of general reviews have appeared covering the whole range of application of c.d., together with special references to biopolymers.[231—235] More specific reviews have covered the areas of c.d. in the vacuum u.v. region[236] and the c.d. behaviour of proteins in the presence of electric fields,[237] together with a review of the uses of spectropolarimetry to study complex equilibria in solution, including stereoselective effects.[238]

Instrumental. A number of instruments have been described for use in the measurement of transient changes in c.d.[239] The use of c.d. detection in stopped-flow reaction systems will undoubtedly open up a wide area hitherto accessible only indirectly.[240, 241] A stopped-flow apparatus with an accessible wavelength range of 200—750 nm has been constructed. This new machine with its far-u.v. c.d. capability allows the observation of rapid alterations in protein backbone conformation associated with folding and unfolding reactions. Results have been obtained from two such reactions followed at 222 nm, representing direct monitoring by c.d. of rapid secondary structure changes in proteins.[242]

For observation in the vacuum u.v. region, an instrument using a reflecting biotite plate for a linear polarizer has been constructed, which allows extended c.d. measurements down to 1300 Å?[243] A new near-i.r. c.d. spectrophotometer has been described.[244]

The problem of using c.d. for dispersing samples has been overcome to some extent by the use of an optical attachment designed for use with the customary mass-produced dual beam spectrophotometers.[245]

Recent advances in vibrational c.d. instrumentation have led to the incorporation of a new CaF_2 photoelastic modulator and a Xe lamp into the spectrometer. These changes have substantially increased sensitivity and extended the long wavelength range of the instrument.[246]

Theory. A theory of c.d. for the amide transition of a polypeptide in the random-coil conformation has been developed on the basis of the exciton theory of optical activity and the Monte Carlo approach to the treatment of statistical systems. This theory has been further extended to be applicable to electronic transitions of chromophores bound to the side-chains of random-coil polypeptides. The c.d. has been calculated for the specific case of Acridine

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[233] T. Samejima, *Kogaku Kyoiku,* 1977, **25,** 358.
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[246] R. Clark and P.J. Stephens, *Proc. Soc. Photo-Opt. Instrum. Eng.,* 1977, **112,** 127.

Orange–random-coil polypeptide complexes and compared with experimental observations. It is found that the longer axes of the bound dye-molecules are almost parallel or perpendicular to the polypeptide chain depending on whether the polypeptide is highly ionized or not.[247]

Both classical theory and linear response formulation treatments have been used in the problem of c.d. of helical polymer systems.[248, 249]

For conformational calculations on *cyclo*-(Val-Gly-Pro-Asn-Gly-Met)$_2$ (I) a model *cyclo*-(Ala-Gly-Pro-Ala-Gly-Ala)$_2$ (II) was used in order to avoid the possibility of multiple side-chain conformers. Calculations indicated that II gave 81 unique minimum energy conformers. These were used to compute the theoretical c.d. The theoretical c.d. and solvent exposure of amide groups of only four of the conformers resembled the observed experimental data.[250]

The long wavelength chiroptic properties of oligopeptides in the parallel-chain pleated sheet (PCPS) and polyproline I and II conformations have been calculated using the Frozen Core MO procedure. For PCPS conformations the π–π^* transition with the highest positive rotatory strength appears at a shorter wavelength than the transition with the highest negative strength and its electric dipole transition moment is polarized mainly perpendicularly to the chain axis. The computed c.d. spectrum of a hexamer in the PCPS conformation is analogous to that observed for H-Ala-Gly-Gly-Ala-Gly-Gly-OH which may have a conformation similar to the pleated sheet, stabilized by intramolecular hydrogen-bonding. The computed π–π^* transition of highest positive rotatory strength is the longest wavelength π–π^* transition in the polyproline system and is polarized along the helix axis. This is analogous to that calculated for *cyclo*-(Pro-Pro-Pro) of symmetry C_3. The type II c.d. spectrum may only be qualitatively reproduced if very strong n–π interactions are invoked.[251]

A theory of vibrational c.d. has been developed, based upon the theory of intensity borrowing by forbidden transitions. The vibrational transition exhibiting c.d. is a one quantum jump in a mode capable of mixing different electronic states. The theory was developed on the basis of the adiabatic and crude adiabatic approximations. Within the crude adiabatic approximations, effects due to any change in the equilibrium configuration on electronic excitation are examined. Vibrational effects in electronic c.d. have also been analysed.[252]

Anharmonic effects in vibrational c.d. have been treated using a formalism developed for the evaluation of the rotational strength of a vibrational transition using first-order anharmonic wave functions admitting c.d. in overtone and combination transitions. Explicit expressions have been derived for the rotational strengths of two quantum vibrational-transitions. It has also been shown that anharmonic contributions to the c.d. of vibrational fundamentals

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[252] D.P. Craig and T. Thirunamachandran, *Mol. Phys.*, 1978, **35**, 825.

may arise through anharmonic resonance between a fundamental and an overtone or combination, or through second order effects.[253]

A sector rule has been developed for optically active lactones and lactams through the application of perturbation theory. The observed difference in sign of c.d. between similar lactams and lactones is significant in the theoretical assignment of the long wavelength n–π^* transitions in peptides. The peptide transition resembles most nearly the $^1A_2 \leftarrow\!\!- {}^1A_1$ (n–π^*) transition of the carboxylate ion, whilst a transition resembling the $^1B_1 \leftarrow\!\!- {}^1A_1$ (n–π^*) in carboxylate ion is expected at only slightly shorter wavelength.[254]

The role of c.d. in spectroscopy has been elucidated partly by sum rules. By summing the rotational-strength moments over all the transitions it has been possible to obtain expressions explicitly dependent upon nuclear geometry, atomic charges, and local atomic anisotropy. In the framework of SCF MO theory coupled oscillator, local orbital distortion and inherent dissymmetry contributions have been identified and their relative magnitudes established. From this work a significant conclusion reached is that if the SCF orbitals can be localized then the traditional correlation expressions are qualitatively correct for the dispersion, while both SCF and CI terms are often of equal importance for individual transitions.[255]

A scattering formalism treatment of optical activity in absorption bands has allowed the c.d. band shapes in the whole spectrum to be accounted for and related to the lifetimes of the molecular states involved.[256]

The chiroptic properties of the chromophoric groups of lysozyme in the near u.v. have been predicted, based on expressions for the momentum moment associated with the electronic transitions. The chiral strengths calculated *via* a Hamiltonian matrix, yield information regarding the relative contributions and interactions of the specific chromophores of the protein. The near-u.v. c.d. of lysozyme was predicted to arise primarily from chiral strengths associated with the tryptophan ^1L_a and ^1L_b transitions. The positive maxima at 288.5 nm arises from chiral strengths associated with the O–O component of tryptophan ^1L_b bands. The tryptophan ^1L_atransition making contributions of mixed sign leads to the negative ellipticity at 305 nm and 275 nm and positive ellipticity at 294 nm. Changes of c.d. on binding tri-*N*-acetylglucosamine are accounted for in terms of specific electronic interactions between ^1L_a transitions of tryptophan and π–π^* transitions of the inhibitor.[257]

The problem of calculating c.d. for aggregated chromophores with non-overlapping wavefunctions has been formulated in a general way with regard to band shape. A consequence of this formulation is that a series of exact and approximate results previously derived for absorption spectra apply to c.d. as well. The contribution to aggregate c.d. from magnetic transition dipoles of the individual chromophores can always be written as a function of the intrinsic c.d. of the non-interacting chromophores. Using a specific example there are

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[255] D. Caldwell, *Proc. Soc. Photo-Opt. Instrum. Eng.*, 1977, **112**, 138.
[256] A. Traspaderne and F. Castano, *Chem. Phys. Letters*, 1978, **56**, 318.
[257] T.M. Hooker and W.J. Goux, *Jerusalem Symp. Quantum Chem. Biochem.*, 1977, **10**, 123.

whole classes of aggregate c.d. spectra, which arise solely as a consequence of finite band-widths.[258]

Small Molecules, Model Compounds, and Synthetic Polymers.—*Amino-acids and Derivatives.* A number of papers have appeared concerned with metal–amino-acid complexes. Three geometrical isomers of bis(β-alaninato)(oxalato)-cobaltate(III) and (β-alaninato)(glycinato)(oxalato)cobaltate(III) have been resolved into their optical isomers and their c.d. spectra compared with those of bis(glycinato)(oxalato)cobaltate(III).[259] A similar study of the three isomers of L-histidinatoiminodiacetatocobaltate(III) has led to their characterization by c.d. methods.[260] The four isomers of tris(*S*)-asparaginato-*ON*(-1) cobalt(III) have been prepared and their absolute configuration determined by means of c.d. It has been shown that the Λ-*mer* isomer is thermodynamically preferred and is the sole isomer formed in the presence of activated charcoal.[261]

Cu(acac)L (acac = acetylacetone, L = L- and D-Phe, D-Tyr, D-Try) and CuL_2 have been characterized by the use of c.d. The dichroism of the *d–d* and charge-transfer transitions of the acetylacetonato ligand in the Cu(acac)L was observed. According to the size of the c.d. bands of acetylacetonato for the *d–d* transitions at 450—650 nm the mixed ligand chelates have been arranged in the series D-Trp > D-Tyr > D-Phe.[262]

The optical activity of copper complexes with proline and proline derivatives has also been reported. The *vicinal* contribution of the asymmetric N-atom to the optical activity of Cu^{II} complexes with proline, hydroxyproline, and allohydroxyproline is not predominant and it does not significantly exceed the *vicinal* contribution of the asymmetric γ C-atom. The presence or absence of an axial ligand is indicated only by the position of the low intensity $^2B_{1g} \rightarrow {}^2A_{1g}$ transition. The sign and amplitude of the basic Cotton effect which corresponds to the $^2B_{1g} \rightarrow {}^2B_{2g}$ transition are determined by the chirality of the nonplanar chelate rings.[263]

The c.d. of Ala and Ile and their ethyl esters and *N*-acetyl derivatives in water, 50 % EtOH, or dioxane has allowed a correlation between their conformations and the pH of solution.[264,265]

N-Acetoacetyl derivatives of aliphatic and aromatic (*S*)-α-amino-acids show enantiomorphic c.d. spectra in ethanolic KOH but similar spectra in MeCN, EtOH, and CF_3CH_2OH.[266]

The vibrational c.d. of D- and L-Ala in D_2O have been presented and an interpretation of the vibrational c.d. of alanine proposed.[267]

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The c.d. spectra of *trans*-(*N*-ethylene)chloro-L-aminocarboxylato-n^2-ethylene platinum(II) at 33 000 cm^{-1} and 37 000 cm^{-1} are characteristic of complexes with an asymmetric N-atom. The c.d. sign depends on the substituents on the N-atom and the additivity law holds for methyl and benzyl derivatives of L-prolinato, L-hydroxyprolinato, and L-valinato complexes. The quadrant rule is applicable to the contribution of the substituents on the N-atom.[268]

C.d. and proteolytic enzyme susceptibility of the tetrazole analogues of amino-acids and peptides have been studied. It was found that leucine aminopeptidase splits the peptide bond between amino-acids and tetrazole. Most of the derivatives carrying the tetrazolyl group linked to the chirality centre possess a negative Cotton effect in the c.d. spectrum in the range 196—219 nm.[269]

It has been shown that dicyclohexylamine salts of nine *N*-lithioethoxycarbonyl L-β-amino-acids give a negative Cotton effect at 330—350 nm in $CHCl_3$ and C_6H_6.[270]

2,4-Dinitrophenyl amino-acids have been shown to have wide application in the determination of the absolute configuration of amino-acids.[271]

Dipeptides and Oligopeptides. A number of papers have appeared in the last year concerning the characteristics of complexes between Cu^{II} and oligo- and polypeptides. The c.d. spectra of the Cu^{II} and Ni^{II} complexes (1 : 1) of Ala-Arg, Arg-Ala, Arg-D-Ala, D-Ala-Arg, *N*-Gly-Lys, Phe-Leu, D-Phe-Leu, Phe-Leu-Phe, Phe-Leu-D-Phe, D-Phe-Leu-Phe, and D-Phe-Leu-D-Phe have been measured and it has proved impossible to predict the signs of the Cotton effects in the region 200—700 nm.[272]

Poly(L-Lys-L-Tyr) (I) in the presence of Cu^{II} at a molar ratio of I:Cu = 4—6 forms a complex at pH > 6.0, as indicated by c.d. observations, as does poly(L-Lys). The c.d. spectrum of Cu^{II}-poly(L-Lys) indicates that four N-atoms lie at the corners of the co-ordination square, the four N-atoms are from two amino groups of lateral chains and two peptide groups. The similarity of the spectra suggests that this is also the case for the Cu^{II}–I complex. The presence of a band at 390 nm in the spectrum of Cu^{II}–I also suggests the involvement of the phenolate *O*-atom in apical co-ordination.[273]

A detailed investigation of the formation of the Cu^{II} complex with $(L\text{-Lys})_n$ $n = 4,25$, using c.d. shows that complex formation occurs as a two step process. The first step involves the co-ordination of two N-atoms, one from the α-amino-terminus and the other from the adjacent deprotonated amide. In the second step the additional N-atoms of side-chains are ligated. A comprehensive investigation of spectral changes has allowed the assignment of three bands to each type of N co-ordination.[274] Parallel studies on Cu^{II}–poly(L-Orn)

[268] Y. Terai, H. Kido, and K. Saito, *Bull. Chem. Soc. Japan*, 1977, **50**, 3265.
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[270] T. Yamada, S. Kuwata, and H. Watanabe, *Tetrahedron Letters*, 1978, **21**, 1813.
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[272] J. Nahlik-Wojcik, B. Tomicka, M. Kauzmark, F. Karczynshi, and G. Kupryszewski, *Roczniki Chem.*, 1977, **51**, 1095.
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[274] A. Garnier and L. Tosi, *Bioinorg. Chem.*, 1978, **8**, 493.

complexes have shown the same reaction sequence to be operative as in the poly(L-Lys) and poly(L-Arg) cases. Initial co-ordination of two amine nitrogens, followed by two amide nitrogens, leads to optical activity in the Cu *d–d* transitions and characteristic charge-transfer transitions in the 250—320 nm region.[275]

Analogous studies have also appeared using substituted poly(amino-acids). The cupric complexes of poly(N^{ε}-acetoacetyl-L-Lys), $[Lys(Acac)]_n$, poly(N^{δ}-acetoacetyl-L-Orn), $[Orn(Acac)]_n$ and poly(N-γ-acetoacetyl-L-diaminobutyric acid)$[A_2bu(Acac)]_n$ and the model compound n-hexyl acetoamide have been studied. While in the complex of the model compound one chelating group is bound to one Cu^{II}, in the polymeric complexes two β-ketoamide groups are bound. Studies on the chiroptic properties of the three polymeric complexes show that the peptide backbone does not undergo conformational transitions and remains in the α-helical form even when 20 % of the side-chains bind Cu^{II}. However, the optical activity of the β-ketoamide is substantially altered by complex formation and this is discussed in terms of asymmetric induction from the chiral backbone.[276]

Two studies of Cu^{II} binding to the cyclic antimicrobial agent gramicidin S analogues showed intense chiroptic effects, the sign, form, and amplitude of the curves in the 450—650 nm region depending on the chemical structure of the analogues.[277] The c.d. of Cu^{II} complexes of γ,γ'-dihydroxy- and di-*O*-trityl, $N\gamma N\gamma'$-diphthaloyl-, -dibenzyl-, -dibenzylidene- and -disalicylidene- and $N\gamma N\gamma N\gamma' N\gamma'$-tetrabenzylgramicidin S were reported.[278] The conformations of gramicidin S analogues, 5-1′,5′-1-bis(δ-aminovaleric acid) and 5-1′-δ-aminovaleric acid gramicidin S have been determined by c.d.[279]

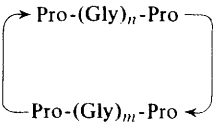
cis-Pt(4-Amino-2,2,6,6-tetramthylpiperidinyl-1-oxy)$_2$$(NO_3)_2$ binds to poly(L-Glu)(I), poly(L-Asp)(II), and poly(L-Lys)(III). On binding, induced c.d. appears for an α-helix in the region of the chromophore π–π^* bands; any transition to a random coil drastically reduces this c.d. Using extensively labelled polymers it was found that the helix-to-coil transition varied according to whether the transition was monitored in the peptide backbone or chromophore region. This study showed that the chromophore stabilizes the local helical structure. It was suggested that the induced c.d. arose from the coupling of the carboxylate π–π^* and the bound $^1B_1$ transition moments.[280]

A study of the complexes between Ni^{II} and Co^{II} with poly(L-Lys), poly(L-Orn), and poly(L-diaminobutyric acid) has been reported, together with a general study of the c.d. and kinetic properties of metal–oligopeptide complexes.[281, 282]

[275] C.V. Phan, L. Tosi, and A. Garnier, *Bioinorg. Chem.*, 1978, **8**, 21.
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[282] J.J. Czarnecki, *Diss. Abs.*, 1977, **38**, 629.

Again, over the past year, a large number of studies has been made on peptides containing the cyclic imino-acid proline, mainly as models for the naturally occurring cation-binding peptides.

It has been shown that *cyclo*-(Pro-Gly)$_4$ (I) binds Li^+, Na^+, K^+, Cs^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} to give 1 : 2 and 1 : 1 complexes, the binding constants for Cs^+ and Ba^{2+} being comparable to those of natural cyclic peptides. The conformations of the complexes have been analysed by means of c.d.[283] C.d. and n.m.r. have shown that *cyclo*-(Pro-Gly-Gly-Pro)$_2$ (I) has two major C_2 symmetric conformations. One of the conformers, an all *trans* X-Pro with two 1 ⟶4 intramolecular hydrogen bonds, exists to > 95 % in D_2O or CD_3CN.[284] It was found



(I)

that in analogous antamanide-like cyclopeptides (I) (I: $n = 1$, $m = 1,2$; $n = 2$, $m = 2,3$; $n = m = 3$); (II, $n = m = 1$) I formed 2 : 1 peptide sandwich complexes and 1 : 1 complexes with Mg^{2+} and Ca^{2+}, whilst II formed 1 : 2 sandwich complexes with Mg^{2+}: II was found to have a high selectivity for Mg^{2+} and Ca^{2+} over alkali ions, whereas I binds selectively the alkali ions with an ionic radius ~1 Å. In all cases three types of c.d. spectra were observed.[285] The cyclic dodecapeptide *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ has also been studied by means of c.d. in aprotic solvents. Titration curves of Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} in MeCN showed 1 : 1, 2 : 1, and 1 : 2 sandwich complexes were formed. The stability for divalent cations increased with the ionic radius of the cation, with the stability of the peptide–K^+ complex being similar to that of valinomycin.[286]

Conformational studies of the pentapeptide (Gly-Pro)$_4$ and the two polypeptides H-[Gly-(Pro)$_3$]$_n$-OH and H-[Gly-(Pro)$_4$]$_n$-OH have been carried out as model studies of a repeating peptide of a protein in human-parotid saliva. In the zwitterion form of Gly-Pro$_4$ all the Pro adopts a *trans* conformation whereas the Pro residues 3 and 4 show both *cis* and *trans* isomers as the pH is reduced. The c.d. spectra show a characteristic strong negative band at 202 nm, reminiscent of a poly(Pro)(II) helical structure; these spectra have been compared to those of the natural protein.[287, 288]

It has been shown that in low polarity solvents *N*-butyloxycarbonyl-L-Pro-Gly may have a *trans* II β-turn conformation as attested by the strong negative Cotton effect at 225 nm. However, this does not seem to be the case in polar

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[288] N. Helbecque and M.H. Loucheux-lefebvre, *Internat J. Peptide Protein Res.*, 1978, **11**, 353.

solvents.[289] C.d., together with other techniques, has shown that the Ac-Pro bond of Ac-Pro-NHEt has both *cis* and *trans* forms, whereas Ac-Gly-NHMe is flexible. The Gly-Pro bond in Ac-Gly-Pro-NHMe has the fully extended C_5 structure, whilst the predominant conformation of Ac-Pro-Gly-NHMe contains 4 ⟶ 1 hydrogen-bonding.[290] H-(Pro)$_n$-OH ($n \geqslant 3$) in H_2O or aqueous PrOH has typical c.d. features of polyproline (I) and (II) with the intensities of the Cotton bands increasing with chain-length and reaching the values characteristic of poly(Pro) at $n \sim 20$.[291]

H-(Leu-Glu-X-Leu-Glu-X-Lys)$_n$-OH[I, X = Ser, Ser(Ac), Ala], and H–(X′-Glu-Ser-X^2-Glu-Ser-Lys)$_n$–OH(II,X′ = Ala, X^2 = Ala, Leu; X′ = X^2 = Leu) have been prepared as models for tropomyosin. In II the ellipticities increase as the hydrophobic character of the side-chains increases. In I [X = Ser(Ac)] the α-helix content increases with chain length, and with X = Ser, Ala the ellipticities increase in 80 % CF_3CH_2OH or $(CF_3)_2CHOH$.[292]

The c.d. of H-(Lys)$_3$-(Ala)$_3$-Tyr-(Ala)$_3$-(Lys)$_3$-NHEt.7HCl (I) and H-(Lys)$_6$-(Ala)$_3$ Tyr-(Ala)$_3$-(Lys)$_6$-NHEt.13HCl (II) in aqueous or aqueous alcoholic solution shows that both have a partial α-helical structure depending on pH, ionic strength, solvent, and temperature. Both I and II have random-coil conformations at acidic or neutral pH. No β-structure was observed for either I or II. The addition of MeOH or NaOH increases the helical content of the peptide.[293]

It has been proposed that polyoxyethylene supports may be used in conformational studies, as binding of oligopeptides to the support did not appear to alter their c.d.[294]

The induced c.d. of poly[γ-2-(9-carbazolyl)ethyl-L-Glu](I) in the solid state and in films has been measured. It appears as if, at low concentrations in CH_2Cl_2, I has a right-handed α-helical conformation.[295]

Me_3CP_2C-D-(Alle)$_m$-(i-Leu-D-Alle)$_n$-OMe (Alle = alloisoleucine) has a preferred β-helical structure in CF_3CH_2OH for $m = 1$, $n = 2,3$; $m = 0$, $n = 3$ and a left-handed α-helix for $m = 0$, $n = 4$.[296]

Schiff's base formation involving polypeptides has been probed using c.d. The Schiff's base formation between *o*-, *m*-, *p*-HO-C_6H_4CHO and poly(L-Lys) has been followed using c.d. and a model proposed to explain the stereospecific binding and consequent dramatic changes of equilibria in hydrolysis.[297] The

[289] E. Benedetti, V. Pavone, C. Toniolo, G.M. Bonora, and M. Palumbo, *Macromolecules*, 1977, **10**, 1350.

[290] E.R. Stimson, S.S. Zimmermann, and H.A. Scheraga, *Macromolecules*, 1977, **10**, 1049.

[291] M. Rothe, H. Rott, and J. Mazanek, *Peptides Proceedings European Peptide Symposium*, 1976, 309.

[292] R.S. Hodges, S.A. St.Pierre, and F.S. Tjoeng, *Peptides, Proceedings American Peptide Symposium*, 1977, 411.

[293] M. Mayer, P. Gauduchon, G. Spach, and P. Wahl, *Biopolymers*, 1978, **17**, 337.

[294] M. Multer, H. Multer, and E. Bayer, *Peptides, Proceedings American Peptide Symposium*, 1977, 403.

[295] M. Hatano, H. Nomori, and M. Yoshikawa, *Peptides, Proceedings American Peptide Symposium*, 1977, 390.

[296] T. Paganetti, V. Rizzo, A. Baici, P. Neuenschwander, and G.P. Lorenzi, *Peptides, Proceedings American Peptide Symposium*, 1977, 395.

[297] M. Dentini, P. De Santis, M. Sarino, M.P. Sforza, and A.S. Verdini, *Makromol. Chem*, 1978, **179**, 1041.

pyridoxal Schiff's base formation with poly(L-Lys), poly(L-Orn) and poly(L-α,γ-diaminobutyric acid) has shown that only the butyric acid derivative is characterized by optical activity similar to that in the pyridoxal enzymes.[298]

Ac[NHC(CHPh)CO]$_2$ groups have been attached to the ε-amino groups of the lysine residues in poly(Glu^{92}Lys^{8}) and induced c.d. observed between 250 and 330 nm, due to the dissymmetric disposition of the two AcNH(CHPh)CO groups under the chiral field of the polypeptide chain. The pH dependence of the induced c.d. observed for the co-polymer but absent in the low molecular weight structural model is related to the α-helical and random-coiled conformations of the polypeptide chain.[299]

In dilute cyclohexane solution it has been shown that Z-Gly-Ser(CMe_3)-Ser-Gly-O$(CH_2)_{17}$Me (I, Z = $PhCH_2O_2C$) has a type I or II β-turn structure. It appears that it may now be possible to calculate the conformation of proteins in solution with greater accuracy utilizing the c.d. data, if the β-turn spectrum is used in combination with those of other conformations.[300] The identification of β-turns has been further extended to studies in the vacuum u.v. region. β-Sheet structure is characterized in a broad u.v. region down to 165 nm, with a short wavelength, intense band at 169 nm. The β-turn measured using poly(Ala_2-Gly_2) also shows features down to 165 nm. The similarity between theoretical and experimental c.d. suggests a dominance of β-turn in the model compound. The β-turn is characterized by a very intense band at 207.5 nm and strong negative bands at 191 nm and 169 nm. C.d. also shows that poly(Ala_2-Gly_3) and poly(Ala_3-Gly_3) possess large amounts of β-turn structure.[301]

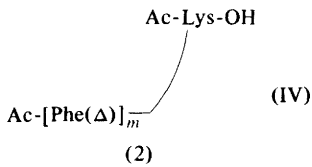
The signs of the dichroism in three wavelength-regions in the vacuum u.v. of poly(Pro) I and II, as well as the α-helix, antiparallel β-sheet, and parallel β-sheet have also been published.[302]

The chirality of cholesteric liquid crystals of concentrated solutions of poly(γ-alkyl-L-Glu)[alkyl = benzyl (I), Bu (II), hexyl(III), octyl (IV)] have been determined by measuring the sign of the induced c.d. of Acridine Orange, dissolved in the mesophase. The chirality depends on temperature, concentration, solvent, and alkyl chain-length. Thus, I and II in solutions of $ClCH_2CH_2Cl$ or CH_2Cl_2 form left-handed cholesteric mesophases at room temperature when the volume fraction of solute is > 0.164. In solutions of I in *m*-cresol and III in $CHCl_3$ the chirality of cholesteric structure changes from right to left through the nematic structure as temperature is increased. Solutions of IV in $CHCl_3$ form a left-handed cholesteric mesophase.[303]

The c.d. properties of the water soluble block co-polymers $(A)_m$–$(B)_n$–$(A)_p$[$A_{m,p}$ = poly(D,L-Lys d_7) or poly(D,L-Lys); $(B)_n$ = poly(L-Ala) or poly(L-Phe)] have been reported.[304]

Poly(L-Glu) containing glutamic acid γ-(*p*-phenylazoanilide) undergoes *trans–cis* isomerization, when irradiated at 369 nm and *cis–trans* isomeriz-

[298] M. Dentini, G. Perretti, M. Sarino, P. De Tantis, and A.S. Verdini, *Biopolymers*, 1978, **17**, 897.
[299] O. Pieroni, A. Fissi, G. Montagnoli, and F. Gardelli, *Biopolymers*, 1977, **16**, 1677.
[300] M. Kawai and G. Fasman, *J. Amer. Chem. Soc.*, 1978, **100**, 3630.
[301] S. Brahms, J. Brahms, G. Spach, and A. Brack, *Proc. Nat. Acad. Sci., U.S.A.*, 1977, **74**, 3208.
[302] E.S. Pysh, *Jerusalem Symp. Quantum Chem. Biochem.*, 1977, **10**, 409.
[303] N. Mori, *Nippon Kagaku Kaishi*, 1978, **6**, 864.
[304] J.C. Howard, F. Cardinaux, and H.A. Scheraga, *Biopolymers*, 1977, **16**, 2029.



ation, when irradiated at 448 nm. The *trans* isomer shows a positive c.d. band at 370 nm and a negative band at 330 nm due to the π–π^* transition.[305]

The c.d. of Ac-[Phe(Δ)]$_n$-Lys(CO_2CH_2Ph)-OR [I: Phe(Δ) = NHC(CHPh)CO; n = 1,2 R = H, Me] Ac-Phe(Δ)-Tyr(Δ)-Gly-OH [II: Tyr(Δ) = NHC(CHC_6H_4OH-*p*)CO], Ac-Phe(Δ)-Phe(Δ)-Phe(Δ)-Val-OH (III), and the branched dehydropeptides (IV) (m = 1,2) (Structure 2) showed that only one dehydro residue had a very weak c.d. band, whereas the peptide containing two adjacent dehydrophenylalanine residues has similar shaped c.d., that were independent of the *C*-terminal residue. The influence of urea upon the c.d. of Ac-Phe(Δ)-Phe(Δ)-Val-OH is discussed.[306]

A number of papers appeared concerned with the structure of biologically active peptides, the endorphins and enkephalins. The c.d. of methionine enkephalin and α-, β-, and γ-endorphin has been recorded; the far-u.v. c.d. suggests that the three endorphins possess only limited secondary structure in aqueous solution, while the α-helix potential is quite high for β-endorphin. The near-u.v. c.d. of the four peptides, however, shows significant differences with perhaps some chain folding occurring in β-endorphin.[307] The c.d. of methionine enkephalin has been determined between 190—330 nm, from 5—50 °C at pH 1—12 in 2,2,2-trifluoroethanol. The data have been interpreted without assuming that the enkephalin preferentially occupies a single conformation in dilute solution.[308]

Proteins.—*Non-chromophoric Proteins.* It has been shown that yeast phosphofructokinase has 34 % α-helical structure and 17—22 % β-structure.[309] The phosphofructokinase from rabbit muscle shows a marked ellipticity at 270 nm. In the presence of AMP, and ATP, marked conformational changes occur at concentrations corresponding to dissociation constants $K_{AMP} = 10\mu M$, $K_{ATP} = 30\mu M$, whereas ITP showed no effect at 100μM. The active form of the enzyme is characterized by a low α-content and high β-content. Addition of activators such as NH_4^+, AMP, and fructose-6-phosphate lead to formation of the active form, whilst free ATP added to the active form caused no visible conversion to the inactive form in the 210—240 nm region.[310]

[305] O. Pieroni, A. Fissi, J.L. Houben, C. Domenici, and E. De Gubernatis, *Chimica e Industria*, 1977, **59**, 590.
[306] O. Pieroni, A. Fissi, S. Merlino, and F. Ciardelli, *Israel J. Chem.*, 1977, **15**, 22.
[307] R.G. Hammond, D. Puett, and N. Ling, *Peptides, Proceedings American Peptide Symposium*, 1977, 100.
[308] M.A. Spirtes, R.W. Schwartz, and W.L. Mattice, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 602.
[309] G. Damaschun, H. Damaschun, R. Kroeber, P. Plietz, M. Becker, D. Zirwer, and G. Kopperschlaeger, *Ergeb. Exp. Med.*, 1977, **24**, 61.
[310] R. Grosse, K. Eckert, M. Otto, R. Hemrich, and H. Jacobasch, *Ergeb. Exp. Med.*, 1977, **24**, 73.

Porcine, bovine, and equine pancreatic phospholipases A_2 all show c.d. curves in the far- and near-u.v., suggestive of a moderate α-helical content, in slight disagreement with *X*-ray results. It is therefore concluded that the positive band centred at 190 nm and the negative bands at 208 and 223 nm are caused not only by α-helices but by asymmetric structures, such as rings containing disulphide bonds. The main-chain conformations of these molecules are very resistant to acid but sensitive to SDS and alkali, the stability being determined chiefly by disulphide bonds and hydrophobic interactions. The tertiary structure of the enzyme from equine sources is reported to be different from the porcine and bovine enzymes.[311]

CNBr cleavage fragment 1—120 of thermolysin shows a disordered conformation in solution at pH 9.6. The secondary structure of fragment 121—205, containing the Zn^{2+} binding-site, is markedly affected by Ca^{2+} binding. The addition of Ca^{2+} causes a decrease in ellipticity to yield a spectrum typical of a peptide having a large amount of α-helical structure. Fragment 206—316 undergoes a large conformational transition at temperatures of 60—70 °C; at lower temperatures this fragment contains between 33—45 % α-helix, compared to the situation in which the native enzyme fragment 206—316 contains 50 % α-helix.[312]

Native Kunitz soybean trypsin inhibitor does not appear to possess any regular structure, whereas reduction of disulphide bridges and *S*-carboxymethylation leads to the appearance of some significant regular conformation. CNBr Fragments 1—84 and 1—114 assume a β-conformation; however, the 85—114 and 115—181 fragments are structureless. On addition of SDS it is possible to induce structure in all the fractions studied.[313]

Limulus polyphemus lectins I and III show large differences in their c.d. spectra in the 200—600 nm range. Limulin III appears to have no ordered conformation, but a limited conformational change can be induced on addition of Ca^{2+}.[314]

The phytohaemagglutinins of lentil, peanut, soybean, and castor bean all possess a high degree of β-structure. The tertiary structure and main-chain conformation of the lectins is disorganized by low concentrations of SDS, but at higher concentrations new orders of higher helical content are formed, as shown by changes in the c.d. associated with the tyrosine and tryptophan chromophores.[315]

Low concentrations of Ca^{2+} have been shown to affect the structure of pig-serum factor X, altering the amounts of α-helix and β-structure from 10 and 60 to 35 and 18 % respectively.[316]

A biphasic increase in c.d. ellipticity at 222 nm of troponin C (TnC) is observed on addition of Ca^{2+}. Approximately 62 % of the total helical change occurs with Ca^{2+} binding to a class of sites with a K_{Ca} or $\sim 2.7 \times 10^7$ M^{-1}. The

[311] B. Jirgensons and G.H. De Haas, *Biochim. Biophys. Acta*, 1977, **494**, 285.
[312] A. Fontana and C. Vita, *Peptides, Proceedings American Peptide Symposium*, 1977, 432.
[313] C. Toniolo, G.M. Bonora, C. Vita, and A. Fontana, *Biochim. Biophys. Acta*, 1978, **532**, 327.
[314] A.C. Roche, J.C. Maurizot, and M. Monsigny, *F.E.B.S. Letters*, 1978, **91**, 233.
[315] B. Jirgensons, *Biochim. Biophys. Acta*, 1978, **536**, 205.
[316] R.M. Howell and S.L. Deacon, *Biochem. Soc. Trans.*, 1977, **5**, 1554.

remaining spectral changes occur with Ca^{2+} binding to a class of sites with K_{Ca} of $\sim 3.1 \times 10^5$ M^{-1}. The biphasic responses are interpreted in terms of distinct structural changes occurring in TnC with Ca^{2+} binding to the high affinity Ca^{2+} sites and to the low affinity Ca^{2+} regulatory sites.[317]

Ca^{2+} binding to the single high-affinity site on porcine-intestinal calcium-binding protein (I) leads to a great increase in the aromatic residue optical activity. Analysis of the changes in c.d. on Ca^{2+} binding suggests that one of the five Phe present in the molecule is also perturbed as well as the sole Tyr. A series of di- and trivalent cations all produce the same changes in the optical activity between 300 and 250 nm. However, in the peptide region, I showed a c.d. spectrum typical of an α-helical protein and was not altered by any of the cations. The affinity of the protein for the various cations is related to their ionic radii.[318]

The tryptophan residues in carboxypeptidase Y, from *Saccharomyces cerevisiae,* are largely buried in an apolar asymmetric environment in a protein with 40 % β-structure.[319]

Phase transfer techniques have allowed the c.d. spectra of chymotrypsin to be obtained in cyclohexane solution. The enzyme appears to maintain the native tertiary structure with possible local conformation and chromophoric perturbations.[320]

Threonine deaminase from *Salmonella typhimurium* displays a positive c.d. with a maximum at 410 nm owing to the aldimine bonded co-factor, pyridoxal phosphate. The substrates L-Thr, L-alloThr, and L-Ser and the competitive inhibitors D-Thr, D-alloThr, and D-Ser cause a loss of c.d., presumably because of transfer of the aldimine bond from the protein to the ligands. The allosteric effectors L-Ile and L-Val have no effect on the protein optical activity. L-Ile prevents loss of optical activity induced by L-Thr and L-alloThr but not by D-Thr, D-alloThr, D-Ser, or L-Ser. The dependence on substrate of L-Ile inhibition is explicable in terms of pertubation of either initial substrate-binding or the catalytic mechanism *per se.* The results definitively demonstrate the separation of inhibitor and active-sites.[321]

The c.d. of hypocalcaemic protein from bovine-parotid gland, dialysed from 6.6M-urea, is very similar to that of the native protein, as is that of the protein dialysed from 6M-guanidine.HCl. The thermal transition, followed at 222 nm, of the urea-treated protein resembles that of the native protein, whilst that of the guanidine-treated protein is different.[322]

The effects of Phe, NaCl, and pH on the conformation of chorismate mutase–prephenate dehydratase (I) have been investigated by far- and near-u.v. c.d. At pH 8.2 I shows 10—20 % helix and 40—50 % β-structure. Addition of

[317] J.D. Johnson and J.D. Potter, *J. Biol. Chem.*, 1978, **253**, 3775.
[318] K.J. Dorrington, D.I. Kells, A.J. Hitchman, J.E. Harrison, and T. Hoffmann, *Canad. J. Biochem.*, 1978, **56**, 492
[319] M. Deconinck, B. Couteaux, Y. Looze, L. Gillet, E. Polastro, and J. Leonis, *Experimentia,* 1978, **34**, 569.
[320] P.L. Luisi, F. Bonner, and C. Walsoe, *Peptides, Proceedings American Peptide Symposium,* 1977, 591.
[321] J.G. Hofler and R.O. Burns., *J. Biol. Chem.*, 1978, **253**, 1245.
[322] T. Mizutani, P. Kuo, and A. Mizutani, *Chem. Pharm. Bull.*, 1977, **25**, 2821.

the allosteric effector, ImM Phe, or 0.4M-NaCl, or lowering the pH to 7.4 produces little or no change in the secondary structure.[323]

The c.d. spectrum of the apoprotein form of pig-brain thromboplastin shows two negative bands at 208 and 218 nm and has a low helical content of ~ 15 %, but may contain ~ 40 % β-structure. SDS does not increase the α-helical content, but slightly decreases the 218 nm band associated with the β-structure. Sodium taurodeoxycholate produces a transition from α-helix and β-structure to random-coil. However, the α-helical conformation required for lipid binding, as well as any hydrophobic sites for Factor VII in the β-region, must pre-exist in thromboplastin apoprotein.[324]

C.d. spectra indicate that the glycoprotein antifreezes from *Trematomus borchgrevinki* and *Eleginus gracilis* have an unordered conformation in solution, whereas the protein antifreezes of *Pseudopleuronectes americamus* and *Myoxocephalus verrucosus* have a right-handed α-helical structure, with an unusually high helix content. These differences indicate that the freezing-point depressing activity of these substances does not depend on their having a particular secondary structure.[325]

The native fatty-acid synthetase complex from the insect *Ceratitis capitata* contains 43 % α-helix and 23 % β-structure. Lipidation and cholate treatment do not modify the structure of the enzyme complex, whereas treatment with SDS alters the structure to 43 % α-helix and 8 % β-sheet.[326]

Secondary-structure predictions, using the Chou and Fasman method, for human-plasma prealbumin have been modified to obtain predicted contents in agreement with experimental c.d. data. The number of correctly predicted amino-acid conformations then increases from 36 to 50 %.[327]

Corynebacterium diphtheria toxin has been shown to contain 20 % α-helix and 25—30 % β-structure, whilst the fragment A, obtained by limited trypsin hydrolysis, has only about 10 % α-helix and a somewhat elevated β-content. The c.d. spectra also indicate that the local environments around Trp and Phe differ between fragment A and the toxin.[328]

C.d spectra have shown that in bovine-heart mitochondrial ATPase ~ 80 % of the amino-acid residues form α-helices and the amount of β-structure is small.[329]

NADPH, trimethoprim, pyrimethamine, or methotrexate binding to dihydrofolate reductase causes small changes in the enzyme far-u.v. c.d., which may arise from alterations in the polypeptide backbone or else results from ligand interactions with the enzyme aromatic residues. At temperatures near 45 °C the enzyme undergoes irreversible denaturation. Binary complexes with

[323] M.J. Gething and B.E. Davidson, *European J. Biochem.*, 1978, **86**, 159.
[324] R.M. Howell and H. Rezvan, *Biochem. Soc. Trans.*, 1977, **5**, 1552.
[325] J.A. Raymond, W. Radding, and A.L. De Vries, *Biopolymers*, 1977, **16**, 2575.
[326] J.G. Gavilanes, M.L. Lizarbe, A.M. Municio, and M. Onaderra, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 998.
[327] B. Rerat and C. Rerat, *J. Chim. Phys. Phys.-Chim. Biol.*, 1977, **74**, 1211.
[328] D. Puett, J.H. Hash, and J.P. Robinson, *F.E.B.S. Letters*, 1978, **89**, 59.
[329] A.G. Sukhomudrenko, A.A. Kiladze, V.N. Shchipakin, and V. Evtodienko, *Bioorg. Khim.* 1977, **3**, 1078.

NADPH or methotrexate stabilize the enzyme towards heat denaturation, whereas trimethoprim and pyrimethamine do not.[330]

It has been found, using c.d., that 25 vol% EtOH has only a slight effect on the conformations of human-blood serum α_1 acid glycoprotein, human-serum α_1 antitrypsin, pancreatic DNase I, porcine pepsinogen, Kunitz soybean trypsin inhibitor, and oxidized and reduced *S*-carboxymethylated RNase, whilst 50—75 vol% EtOH significantly altered the conformations. The tertiary structure was perturbed and the polypeptide main-chain reorganized into new conformations of higher helix and β-structure contents, the degree of reorganization chiefly depending on the disulphide cross-links within the molecules. Whereas unfolded RNases are refolded by 25 vol% EtOH, native RNase is resistant even to 50—66 vol% EtOH. The conformation of DNase and α_1 antitrypsin is more sensitive to 25 vol% EtOH than α_1 acid glycoprotein, pepsinogen, and soybean trypsin inhibitor. Almost complete restoration of native conformation could be achieved by dilution or dialysis against H_2O.[331]

Small, negative c.d. bands at 297 nm as exhibited by isolated 30*S* and 50*S* ribosomal subunits from three bacteria, *Bacillus stearothermophilus, B. subtilis,* and *E. Coli* Q13. The c.d. of the 30*S* subunits are more intense than those of the 50*S* subunit. The dissociation of 70*S* ribosomes causes an enhancement of the intensity at 297 nm. The subunits of *B. stearothermophilus* are found to be more heat-stable than those of *E. Coli.* Treatment with 5M-urea and 0.5% SDS causes considerable reduction of the negative c.d. around 297 nm of the 30*S* subunits, but no significant change for the 50*S* subunits.[332]

Chromophoric Proteins. Horseradish peroxidases, containing hematins substituted 2,4- with vinyl, enyl-1-pro, enyl-1-but, ethyl, hydroxyethyl, and acetyl groups, have been studied using c.d. In the Soret region the vinyl-, propenyl-, and butenyl- peroxidases give intense, positive Cotton effects, together with strong negative bands at 280—290 nm. This suggests that the side-chain double bonds and aromatic amino-acids interact in a specific manner. The alkenylhematin peroxidases, which show the unique Cotton effects in the Soret region, also have a higher rate of formation of Compound I with H_2O_2. This effect is attributed to conformational factors.[333]

Isoenzymes P_1 and P_7 of turnip peroxidase (I) c.d. spectra show different ellipticites at the same wavelength in the Soret region. These results have been compared to those for Japanese radish I and horseradish I. It appears to be a common property of plant peroxidases that the negative c.d. present in the Soret region for the reduced forms inverts on addition of cyanide. The c.d. in the far u.v. indicate an appreciable helical content, together with a very high unordered content, > 50 % for P_1 and P_7.[334]

[330] B.B. Kitchell and R.W. Henkens, *Biochim. Biophys. Acta,* 1978, **534,** 89.
[331] B. Jirgensons, *Biochim. Biophys. Acta,* 1978, **534,** 123.
[332] T. Uchiumi, A. Hachimori, A. Takeda, and T. Samejuna, *Biochim. Biophys. Acta,* 1978, **519,** 513.
[333] P.I. Ohlsson, K.G. Paul, and I. Sjoholm, *J. Biol. Chem.* 1977, **252,** 8222.
[334] H.B. Dunford, *Canad. J. Biochem.,* 1977, **55,** 834.

A modified analytical method has been developed, which makes it possible to calculate the secondary structure of hemopexin (8 % α-helix, 10 % β) and the actual wavelength (229 nm) and intensity of the 231 nm positive c.d. band. Measurement and analysis of the spectra in alkaline solution gives information ascribing a major part of the intensity at 229 nm to the B_{1u} transition of Tyr, together with some other electronic transition. An assignment of the individual extremes of the c.d. spectrum in the near u.v. to individual electronic transitions has been made. The dichroic bands of Trp in this region, together with their temperature dependence, corroborates the idea of a very compact hydrophobic core in hemopexin.[335]

UDP-galactose-4-epimerase (I) and its native (I-NAD) and reduced (I-NADH-UMP) forms have been studied by c.d. The reduced form exhibits large positive c.d. at 340 nm attributable to the bound NADH, which is 50 times larger than that of free NADH. This enhancement reflects dissymmetric interactions involving the 340 nm transition and is most consistent with the dihydropyridine ring of NADH being highly immobilized in the reduced complex. In the 200—230 nm region both enzyme forms exhibit negative bands at 220 nm and shoulders at 208 nm. The ellipticities of the reduced form are 7 % greater and this is interpreted as reduction, leading to an increase in α-helix at the cost of unordered structures.[336]

The c.d. spectrum of ceruloplasmin has been decomposed into six bands in the 300—900 nm region, attributable to type I copper. It has been shown that an additional band at 320 nm arises from type III copper. Incubation with NO produces a slight decrease in ellipticity around 600 nm and a marked decrease at 456 nm, indicating the non-equivalence of the type I coppers. Prolonged exposure to NO leads to reduction of type I copper. The protein contains 46 % β-structure and 54 % unordered form.[337, 338]

Type I substrates, cyclohexane, and benzo[*a*]pyrene, when added to microsomal P_{450}, induce a marked decrease in the negative ellipticity at 418 nm with a concomitant increase in the negative ellipticity at 395 nm. These substrates produce a low- to-high spin transition. Incubation of type I substrates with P_{450} in the presence of NADPH results in changes attributable to oxygenation. Reverse type I substrates, such as alcohols, when added to type I substrate complexes reverse the effects of the type I substrate. The maximum chain-length for alcohol effectiveness is C_4.[339]

Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* exhibits positive ellipticity at 250, 283, and 292 nm and two negative bands at 327 and 480 nm, with a low intensity positive band at 390 nm. The enzyme contains 8 % α, 38 % β, and 54 % unordered structures. Significant changes in the c.d. spectra are associated with thermal inactivation of the enzyme at 47 °C.[340]

The carbomonoxy derivatives of haemoglobin A and S have different c.d. in the Soret region, as do their β-subunits. However, the deoxy derivatives only

[335] M. Kodicek, Z. Hrkal, and Z. Vodrazka, *Biochim. Biophys. Acta*, 1977, **495**, 268.
[336] S.S. Wong, J.Y. Cassim, and P.A. Frey, *Biochemistry*, 1978, **17**, 516.
[337] S. Freeman and E. Daniel, *Biochim. Biophys. Acta*, 1978, **534**, 132.
[338] F. Van Leeuwen and B.F. Van Gelder, *European J. Biochem.*, 1978, **87**, 305.
[339] H. Shichi, K. Kumaki, D.W. Nebert, *Chem.-Biol. Interact.*, 1978, **20**, 133.
[340] C.T. Hou, R.N. Patel, M.O. Lillard, A. Felix, and J. Florance, *Bioorg. Chem.* 1978, **7**, 115.

show differences in the presence of inositol hexaphosphate. These differences seem to be a consequence of altered tertiary structure.[341] The $R \longrightarrow T$ transition in chemically modified human haemoglobins and deer haemoglobins have been studied by c.d.[342] In the range 350—650 nm, oxygenated abnormal haemoglobins show little difference in their c.d. from normal haemoglobin A. In the deoxy form, Hb Chesapeake and Hb Capetown are similar to HbA, whilst Hb Yakima and Hb Kempsey are markedly different. In the oxy form, Hb Capetown and HbA are markedly altered by addition of inositol hexaphosphate, whilst the other haemoglobins are not. Correlations between spectra and functions are proposed.[343] The c.d. spectrum of the CO derivative of HbA, in the Soret region, is sensitive to the addition of benzene hexacarboxylate (I) and inositol hexaphosphate (II), whereas the same derivative of Hb Deer Lodge and Hb Providence-Asp are not. In contrast, in the deoxy forms none of the haemoglobins are sensitive to I or II.[344]

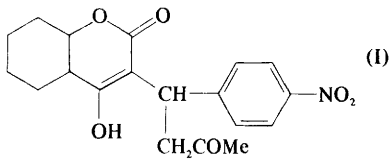
The oxidized form of bovine-heart cytochrome-*c* oxidase and its cyanide complex exhibit broad negative extrema at 570 and 620 nm. A single Cotton effect is resolved at each extremum, except in the case of the CN^- complex where two Cotton effects at 558.9 and 572.4 nm are located. The reduced enzyme displays a major positive band at 608 nm and minor ones at 570 and 518 nm. CO and CN^- cause the band at 608 nm to move to 604 and 602 nm respectively. Computer resolution suggests a common profile, whereby two major transitions of about equal strength constitute the main band between 602 and 608 nm. The results of the shifts obtained with CO and CN allow assignment of the two Cotton effects to cytochrome a_3 and cytochrome *a* respectively. The dependency of the ellipticity of the band at 520 nm upon added ligand is consistent with the charge-transfer nature of this band.[345]

The α-band c.d. of bovine rhodopsin decreases and the γ-band increases on treatment with detergents; the β-band is only slightly influenced. The γ-band is three times larger than the α. Both in rhodopsin and isorhodopsin the γ-band, probably due to retinal and aromatic amino-acids, is photo bleached. The α-band is also reversibly reduced by the addition of $(NH_4)_2SO_4$.[346] Some controversy has arisen over the measurement by c.d. of phototransients in bacteriorhodopsin.[347, 348]

Extrinsic Chromophores. The interactions of acenocoumarin (3), coumachlor, phenprocoumon, and warfarin with human and bovine serum albumin all generate extrinsic Cotton effects.

However, the differences in the signs and intensities of the observed Cotton effects suggests differential molecular binding mechanisms are operative.[349]

[341] C. Fronticelli, *J. Biol. Chem.*, 1978, **253**, 2288.
[342] C.F. Plese, *Diss. Abs.*, 1978, **38**, 4212.
[343] M. Nishibu, *Juzen Igakkai Zasshi*, 1977, **86**, 379.
[344] E. Bucci, A. Salahuddin, J. Bonaventura, and C. Bonaventura, *J. Biol. Chem.*, 1978, **253**, 821.
[345] F.C. Yong, *Canad. J. Biochem.*, 1978, **56**, 176.
[346] T. Suzuki, M. Makino, and M. Azuma, *Biochim. Biophys. Acta*, 1977, **491**, 115.
[347] P.M. Bayley and M. Anson, *J. Phys. E.*, 1977, **10**, 1299.
[348] M.A. Slifkin and T.I. Allos, *J. Phys. E.*, 1977, **10**, 1300.
[349] N.A. Brown and W.E. Mueller, *Pharmacology*, 1978, **17**, 233.



(I)

(3)

The c.d. induced in Cibacron Blue (I) and Congo Red (II) upon binding to several dehydrogenases has been studied as a probe of the conformation of the bound dyes. The c.d. of II are quite similar when bound to lactate, glyceraldehyde-3-phosphate, and alcohol dehydrogenases, but the bands are of opposite sign when bound to cytoplasmic malate dehydrogenase. In the case of bound I all the dehydrogenase spectra are quite different. These differences argue for at least local changes in dye conformation or environment when bound to the dehydrogenase.[350]

A discussion of induced c.d. in enzyme–dye complexes has appeared.[351]

From the pH dependency of the extrinsic Cotton effects, due to the metal-thiolate chromophores in metallothioneins it appears the stability of the metal clusters decreases in the order Cu > Cd > Zn. The extrinsic Cotton effects of Cd, Zn-thioneins of varying Cd to Zn ratio can be simulated using the difference c.d. of Cd-thionein, Zn-thionein and cysteine-thionein. In the near-u.v. the metal-free protein shows only Cotton bands due to the S–S chromophore at 282.5 and 260 nm. From the intrinsic c.d. of Cd and Zn-thionein it follows that the protein conformation consists of < 5 % helical or pleated sheet.[352]

Hormones.—Calculations using crystal structure co-ordinates have predicted that ~40 % of the calculated c.d. from Tyr of hexameric insulin is due to one of the four Tyr residues *e.g.* A14 interacting with the nearby B1-Phe. This was tested by measuring the Tyr c.d. of an isomorphous insulin analogue, des-B1-Phe. Contrary to prediction the c.d. is the same as that of insulin, the B1-Phe thus not making a large contribution to the c.d. of A14-Tyr. This result is probably due to thermal motion of the B1 and A14 rings; improvements which take such motion into account are discussed.[353] In insulin–transferrin complexes prepared from the serum of patients with diabetes mellitus, the proportion of α-helix in the insulin molecule (4 %) is reported to be less than that from normal subjects (8 %). No change is found in the aromatic amino-acid spectra; thus the observed changes are due to differences in the tertiary structures of the proteins.[354]

The c.d. spectra of bradykinin (I) and eleven analogues, containing β-cyclohexyl-L-Ala, Gly, glycollic acid, β-Phe-L-lactic acid and

[350] R.A. Edwards and R.W. Woody, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 470.
[351] J.F. Towell, *Diss. Abs.*, 1978, **38**, 3185.
[352] H. Rupp and U. Weser, *Biochim. Biophys. Acta*, 1978, **533**, 209.
[353] A. Vollmer, J. Fleischhauer, W. Strassburger, H. Thiele, D. Brandenburg, G. Dodson, and D. Mercola, *Biophys. J.*, 1977, **20**, 233.
[354] N.K. Gracheva and I.G. Kharitonenkov, *Probl. Endokrinol.*, 1978, **24**, 8.

2,2,6,6-tetramethyl-1-oxyl-4-piperidylacetic acid substitutions, have been analysed in solutions of various polarity, temperature, pH, concentration, and ionic strength. The positions, signs, and intensities of the Cotton effects of I at 20 °C in aqueous solution are only very weakly dependent on concentration (10^{-2}—10^{-4}M), pH (1.2–8.2), or ionic strength. The c.d. is more sensitive to pH and ionic strength in EtOH than H_2O. In H_2O, EtOH, or heptane–EtOH solution the intensity of the c.d. band at 220 nm sharply decreases with increasing temperature (–100 °C to +60 °C); the effect is smaller in trifluoroethanol and non-existent in hexafluoroacetate. These results indicate that I exists in two or more closely related conformational states in equilibrium. The majority of amino-acid residues exist in a β-conformation.[355] A c.d. study of the aromatic side chains of bradykinin has also appeared. Comparison of the c.d. of [Phe(H_4)^5]-, [Phe(H_4)^8]-, [Phe(H_4)^{5,8}]-, [TyrOMe^5]-, [TyrOME^8]-, and [TyrOME^{5,8}]- bradykinin under a variety of conditions allows the monitoring of the behaviour of the aromatic side-chains and an estimation of their respective contributions in the spectral regions 320—210 nm. The conformational non-equivalence of the residues Phe-5 and Phe-8 together with some overall conformational features have thus been established.[356]

Substitutions at positions 7(D-Phe) and 8(Gly, Ala, D-Ala, and D-Trp) markedly affect somatostatin conformation, whereas substitution at position 6 (D-Phe) and 12 (Leu) have no effect and substitution with D-Phe at position 11 has only a small effect. A β-bend involving residues 6—13 explains the effects of substituting D-Phe for L- residues. The established contribution of the single Trp, a single Phe, and all the aromatic residues to the c.d. in 6M-guanidine.HCl is +27 000 deg cm^2 d mol^{-1} at 219 nm and +70 000 deg cm^2 d mol^{-1} at 225 nm.[357]

The glycoprotein hormone, follitropin, displays bands in the near u.v. which are absent in homologous tryptophan-free hormone. In the far u.v. the dichroism is very similar to other glycoprotein hormones with little or no indication of α-helix. The single tryptophan is in a domain of the β-subunit, in or near a region of subunit–subunit contact in the glycoprotein hormone.[358]

Above 260 nm the near-u.v. c.d. of lutroprin α under denaturing conditions with the disulphides intact, reduced, and reduced and *S*-carboxymethylated are similar, showing that the disulphide chromophores do not contribute appreciably to the near-u.v. c.d. However, the spectra differ considerably from that of the native lutropin α demonstrating that the tertiary structure of lutorpin α has a major influence on the near-u.v. c.d. The magnitude of the 232.5 nm positive band increases with increasing guanidine.HCl concentration and with decreasing temperature. The results indicate some conformational flexibility.[359]

Immunoproteins.—The c.d. of two IgD myeloma proteins have been shown to

[355] V.T. Ivanov, M.P. Filatova, S. Reissmann, T.O. Rentova, and N.M. Chekhlyaeva, *Bioorg. Khim.*, 1977, **3**, 1157.
[356] K. Lintner, S. Fermandjian, D. Regoli, and J. Barabe, *European J. Biochem.*, 1977, **81**, 395.
[357] L.A. Holladay, J. Rivier, and D. Puett, *Biochemistry*, 1977, **16**, 4895.
[358] L.C. Guidice, J.G. Pierce, K.W. Cheng, R. Whitley, and R.J. Ryan, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 725.
[359] D. Puett and L.A. Holladay, *Internat. J. Peptide Protein Res.*, 1977, **10**, 102.

differ in the presence of a strong positive band at 235 nm. The structural feature responsible for this difference is localized in the Fab region.[360]

The topology of variable-region tryptophan side-chains and their interaction with bound dinitro- and trinitrophenyl haptens in MOPC-315 IgA myeloma protein has been studied by means of c.d.[361]

In the pH region 5.5—7.5 the c.d. spectra of type λ Bence-Jones protein (Tod) with intact interchain disulphide bond [L(SS)] and the constant fragment C_L do not change with pH, while the spectra of Tod protein in which the interchain disulphide bond is reduced and alkylated[L(RA)] and of the variable fragment (V_L) do. The c.d. spectrum of C_L does not change with concentration. The dimerization constant for L(RA) is 4×10^4 M^{-1}, which is smaller than that of V_{8L} (1×10^5 M^{-1}). The ellipticity at 278 nm for L(RA) dimer is different from that for L(SS) dimer and is pH dependent. The L(RA) and L(SS) thus have different conformations, which are discussed on the basis of the conformations of V_L and C_L and the interactions between the paired domains.[362]

α_1-Antitrypsin from human-blood plasma contains ~15—20 % α-helix the remainder of the protein being β-sheet and aperiodic structure. The conformation of the protein is stable up to pH 8.8, but undergoes reversible changes at pH 10.3 and drastic denaturation at pH 11.6. The environment of the side-chain chromophores is strongly affected at pH 2.5, whereas the main chain is changed only slightly. Denaturation occurs in 3.5M-guanidine.HCl, whilst SDS leads to disorganization and enhancement of α-helical content. The Phe-band fine-structure is observed in the native protein and also after denaturation with acid, guanidine.HCl, or SDS.[363]

Comparison of the c.d. of cloacin–immunity protein complex with that of cloacin and the ability of a cloacin-lacking mutant to bind the immunity protein shows that the binding of immunity protein imposes a definite structure on the cloacin molecule. This structure is probably a prerequisite for an effective killing activity of the bacteriocin. There is evidence that cloacin consists of a structural part with appreciable helicity, and a part with little periodic structure which is more susceptible to proteolysis.[364]

Spectroscopic comparison of several 6-hydroxyxanthen-3-ones with various substitution at position-9 indicates that the induced c.d. of fluorescein bound by rabbit and sheep antifluorescein antibodies reflects primarily the microenvironment surrounding the hydroxyxantheanone moiety of the hapten. The optical activity may be due to dynamic coupling between one or more aromatic chromophores in the combining site and transitions in the hapten. Hyperimmunized sheep produce two of the three types of antifluorescein antibodies observed in rabbits. Tlhe different types exhibit different induced c.d.[365]

Membrane Proteins.—Analysis of the conformation of erythrocyte-membrane

[360] R. Jefferis, J.B. Matthews, and P.M. Bayley, *Immunochemistry*, 1977, **14**, 393.
[361] M.R. Freed, *Diss. Abs.*, 1977, **38**, 1005.
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[363] B. Jirgensons, *Biochim. Biophys. Acta*, 1977, **493**, 352.
[364] W. Gaastra, G. Koopmans, and F.K. De Graaf. *Biochem. Biophys. Res. Comm.*, 1978, **80**, 97.
[365] T.W. Athey and R.E. Cathou, *Immunochemistry*, 1977, **14**, 397.

proteins, as derived from c.d. studies, shows that differences in conformation correlate with the severity of disease in sickle-cell patients. The differences observed include a decrease in ordered secondary structure and the apparent absence of some peptides in some particularly severe cases.[366] Four structural transitions have been detected in erythrocyte ghosts (*A, B, C,* and *D*) at temperatures of 50 °, 57 °, 63 °, and 77 °C. The largest c.d. changes are associated with the *A* and *D* transitions and indicate a significant amount of protein unfolding. It appears the *A* transition may involve spectrin, while the *B* transition may be localized on the cytoplasmic side of the membrane and is very sensitive to small changes in ionic strength, pH, and Ca^{2+} concentration.[367]

C.d. studies on filamentous virus coat protein in lipid vesicles show that distinctive spectra result which depend on the history of the protein and method of preparation.[368]

A review and discussion of the c.d. of bulk pigments, chlorophylls, proteins, complexes, chloroplast particles, *etc.* of photosynthetic membranes has appeared.[369] Wild type barley and spinach chloroplast fragments at −196 °C show c.d troughs at 640, 653, 676, and 695 nm and a maximum at 667 nm. A barley mutant shows a large trough at 684 nm and a small trough at 695 nm and a positive peak at 670 nm; a feature of this system is the absence of the 640 nm band, which is attributable to the light harvesting chlorophyll *a/b* protein. The 695 nm band observed in spinach, barley, and barley mutant appears to be detergent-sensitive and may be due to a labile chlorophyll *a*–protein complex.[370]

C.d. has been used to investigate fluidity and chromophore interactions in purple membranes.[371]

Protein–Nucleic Acid Complexes.—The addition of poly(L-Lys) to calf-thymus DNA causes a marked change in the c.d., with a shift in wavelength maxima for the positive band, a decrease in the intensity of this band and an increase in the intensity of the negative band. Similar, but less substantial changes are found when poly(L-Lys) is added to T2 DNA. Addition of poly(L-Lys) to *E. coli* DNA does not shift the positive band but slightly decreases the intensities of both the positive and negative bands.[372] The complexes produced from poly(L-Lys) and DNA from calf thymus, phage T2, and *E. coli,* when heated from 30—85 °C, show approximately constant wavelength maxima but decreased intensity with increasing temperature.[373]

Complexes of fragments of *E. coli* DNA and $(Lys\text{-}Ala)_{50}$, $(Lys\text{-}Ala_2)_{37}$, $(Lys\text{-}Ala_2)_{26}$, $(Lys\text{-}Ala_3)_{18}$, $(Lys_3\text{-}Pro)_{29}$, and $(Orn_3\text{-}Gly)_{28}$ show extreme sensitivity towards changes in temperature and ionic strength in their c.d. spectra. The

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[368] R.W. Williams and A.K. Dunker, *J. Biol. Chem.*, 1977, **252**, 6253,
[369] R.P.F. Gregory, *Top. Photosynth.*, 1977, **2**, 465.
[370] O.D. Camaani and K. Sauer, *Biochim. Biophys. Acta*, 1978, **501**, 545.
[371] S.S. Gupte, *Diss. Abs.*, 1978, **38**, 1099.
[372] M.A. Novoseler, *Doklady Akad. Nauk Arm. S.S.R.*, 1978, **66**, 44.
[373] M.A. Novoseler, *Doklady Akad. Nauk Arm. S.S.R.*, 1978, **66**, 108.

spectral changes have been explained in terms of structural relaxation and by liquid-crystal domain formation in solution.[374]

C.d. spectra of model nucleoproteins (Lys-X-Ala)$_n$-DNA have been obtained and shown to differ according to whether X = Ala, norVal, or X = Val, Leu.[375]

C.d. studies of actinomycin complexes with natural and synthetic DNAs have allowed the identification of G_pC and G_pG as the most favourable binding-sites for the intercalation of actinomycin.[376,377]

Rat-liver nuclei have been used to prepare two types of chromatin, by exposure to micrococcal nuclease and by shearing, which are identical in their protein-DNA content and in the presence of five histones. Sheared chromatin shows an increased c.d. signal at 280 nm, which is not related to loss of superstructure, but to structural changes of DNA. These observations offer a new and sensitive probe of chromatin structure.[378]

The c.d. spectra have been reported for the complexes of bacteriophage T4 gene 32 protein (gp 32) and poly(dA), poly(dT), poly(dA)–poly(T) and double stranded (ds) poly d(A–T), and for the interaction of a proteolytic fragment of gp 32 (gp 32*-I) with T4 DNA and ds poly d(A–T). Comparison of the observed and theoretical spectra indicate that gp 32 keeps the single stranded (ss) polynucleotides in a *C*-DNA type conformation. The conformation of poly d(A–T) in the gp 32*-I complex is closer to *C*-DNA than the complex with gp 32 and the ss T4 DNA in complex with gp 32*-I is also in *C*-DNA conformation.[379]

Ribosomal proteins free in solution possess unique conformations, with significant amounts of α-helix and β-structure, which are extensively disrupted by guanidinium chloride. By subtracting RNA contributions from the c.d. spectra of ribosomal particles it is possible to show that conformations of the proteins are different from those in free solution.[380]

4 Magnetic Circular Dichroism

Contributed by T. Brittain

Over the past year the volume of the m.c.d. literature has continued to grow. Once again the bulk of the protein literature has been concerned with studies on haem proteins, for reasons of sensitivity and the possibility of paramagnetism, but papers have also appeared concerned with the m.c.d. of aromatic amino-acids, iron–sulphur proteins, and lanthanide-ion binding.

General and Theory.—A rotatable-magnet system for m.c.d. studies employing permanent magnets and capable of producing fields of 0.5—1.3 T has been

[374] A.S. Krylov, G.V. Gurskii, N.O. Kondrateva, L.I. Maryash, A.I. Poletaev, and V.A. Shibnev, *Mol. Biol.*, 1978, **12**, 297.
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[378] G. De Murcia, G.C. Das, M. Erord, and M. Daune, *Nucleic Acids Res.*, 1978, **5**, 523.
[379] M.F. Maestre, J. Greve, and J. Hasoda, *Jerusalem Symp. Quantum Chem. Biochem.*, 1977, **10**, 99.
[380] S.H. Allen and K.P. Wong, *Biochemistry*, 1978, **17**, 3971.

designed. The magnet system has performance characteristics comparable to superconducting systems and the leak of field perturbations on photomultipliers are negligible.[381]

Recent developments in the field of magnetic c.d. spectroscopy have been reviewed.[382] Applications of the Faraday effect and m.c.d. have been reviewed and related to applications in optical instrumentation and the detection of atomic and molecular structure.[383]

The transition energies, oscillator strengths, and the A/D values of porphine, protoporphyrin, and porphyrin have been calculated within the framework of the Pariser–Parr–Pople approximation and are in reasonable agreement with experimental data. The m.c.d. of the Q and Soret bands in heme a and photoheme have been analysed in terms of an apparent A term, originating from the magnetic mixing between the components of the nearly degenerate excited state.[384]

Fully retarded ellipticity expressions for m.c.d. of polymeric systems have been derived *via* a canonical transformation of the Hamiltonian in the Frenkel exciton approximation. The modifications required for application to small molecular systems are also outlined.[385]

Group theory has been used in the calculation of m.c.d. coefficients.[386]

Recent progress in m.c.d. has made it possible to add efficient criteria for spectra assignments, to distinguish between vibrational structure and distinct electronic transitions, and to separate close-lying transitions of different polarization.[387]

A review of the general theory of m.c.d. and simple electron theory, which allows formulation of general rules of m.c.d. signs of low energy transitions in cyclic conjugated molecules, has appeared.[388]

Proteins and Models.—M.c.d. spectra have been obtained for various Fe–S complexes, with and without inorganic sulphur, as models for rubredoxin and 2-Fe ferredoxin. The m.c.d. band shapes ascribe the bands around 390 nm and 490 nm to Faraday A terms for mononuclear Fe–S. The bands arise from charge-transfer from SH to Fet_2 and $e3d$ orbitals respectively. The m.c.d. magnitudes decrease by more than half for binuclear Fe–S with inorganic sulphur. The low m.c.d. magnitude, as well as the possible core symmetry as low as D_{2d}, attributed the bands to Faraday B terms. Amongst the complexes studied, bis(*o*-xylyldithiolato)ferrate(III)monoanion gives an m.c.d. spectrum closest to rubredoxin. However, the binuclear complex bis(*o*-xylyldithiolato)-μ_2-sulphidoferrate(III) dianion does not have an m.c.d. similar to spinach ferredoxin.[389]

[381] B. Norden, R. Hakansson, and S. Danielsson, *Chem. Ser.*, 1977, **11**, 52.
[382] M. Hateno, *J.A.S.C.O. Appl. Notes*, 1975, **8**, 6.
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[387] E.W. Thulstrup, *J. Mol. Struct.*, 1978, **47**, 359.
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[389] T. Muraoka, T. Nozawa, and M. Hatano, *Bioinorg. Chem.*, 1978, **8**, 45.

The m.c.d. and c.d. of 2-Fe ferredoxins from *Pseudomonas putida, Spiruline maxima,* chromatium HIPIP, and 4-Fe ferredoxins from *Clostridium pasteurianium* have been measured in the range from near i.r. to near u.v. In the 2-Fe proteins measured at a field of five tesla the m.c.d. signals are smaller than the corresponding c.d., whereas in the 4-Fe and 8-Fe cases the signals are of comparable magnitude.[390]

The low temperature m.c.d. of haem *a* and some chemical derivatives have been recorded and the data used partially to assign the spectrum of mammalian cytochrome *c* oxidase.[391]

M.c.d. has been used to detect the chemical modification of a single tryptophan residue in dihydrofolate reductase, the destruction of which completely inhibits the ability of the enzymes to bind either dihydrofolate or NADPH.[392]

Lanthanide binding to concanavalin A has been followed using m.c.d. The m.c.d. spectrum of Pr^{3+} bound to concanavalin A is not affected by excess Ca^{2+} or the addition of specific carbohydrate effectors. Also there is no evidence for lanthanide-ion binding to site S_2.[393]

The m.c.d. of aromatic amino-acids and their derivatives have been presented.[394]

Complexes belonging to a given type, in the series, $[Co(NH_3)_5(OCOCHRNH_3)]^{3+}$, $Co[(NH_2CHRCO_2)(NH_3)_4]^{2+}$, and $[Co(NH_2CHRCO_2)(en)_2]^{2+}$ show nearly identical m.c.d. spectra irrespective of variations in their natural c.d., whilst the complexes of D-, L-, and DL-Ala show identical spectra.[395]

M.c.d. spectra for horse-radish peroxidase compounds I and II have been recorded and analysis suggests that the chromophores in both intermediates are electronically similar, yielding a $\pi \longrightarrow \pi^*$ spectrum of the same band energies.[396]

The m.c.d. of the Q_{oo}- and Q_v-bands of myoglobin (Mb), Hb, Hb dimers, and isolated chains, as well as horse-radish peroxidase, are sensitive to structural differences in the heme environment. Room-temperature visible m.c.d. of native Hb differs from that of Mb, Hb dimers, and isolated chains as well as that of model pentaco-ordinate complexes, being characterized by a greater value of the intensity ratio of the *A*-term in the Q_{oo}-band and the *A*-term in the Q_v band. Evidence is given for two forms of ferroperoxidase, a neutral form showing Hb-like m.c.d. and an alkaline form with Mb-like m.c.d. Differences observed between the m.c.d. of the hemeproteins became more pronounced at liquid nitrogen temperatures. With the exception of the 580 nm band of deoxy Mb and reduced peroxidase, the visible m.c.d. did not show any appreciably temperature-dependent *C*-terms. The temperature dependence of the Soret

[390] P.J. Stephens, A.J. Thomson, J.B. Dunn, J.A. Keiderling, J. Rawlings, K. Rao, and D.O. Hall, *Biochemistry,* 1978, **17,** 4770.
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[393] C.E. Richardson and W.D. Behnke, *Biochim. Biophys. Acta,* 1978, **534,** 267.
[394] M. Miwa and T. Komiyama, *Asahi Garasu Kogyo Gijutsu Shoreikai Kenkyu Hokoku,* 1977, **31,** 295.
[395] E. Ueda, N. Matsuoka, and Y. Shimura, *Bull. Chem. Soc. Japan,* 1978, **51,** 1895.
[396] M.J. Stillman, B.R. Hollebone, and J.S. Stillman, *Biochem. Biophys. Res. Comm.,* 1976, **72,** 554.

region is interpreted in terms of overlapping charge-transfer from porphyrin to Fe.[397]

M.c.d. studies have shown that reduction by thermolysed electrons at 77 K of metHb, inositol hexaphosphate–metHb complex, and F-metHb results in mixed high spin–low spin forms, whilst reduction of azido-, CN^--, and imidazoylmetHb leads only to low spin states. At 77 K the imidazoylmetHb α-band is split (555 nm and 559 nm), probably due to a decrease in symmetry of the active-site, which changes at 178 K, being indicative of re-orientation or the appearance of a second low-spin form. Rise in temperature also affects the m.c.d. spectrum of azidometHb, but the other derivatives show only a slight spectral shift.[398]

The markedly different m.c.d. spectra obtained for $oxyP_{450cam}$ and oxyMb have been used to argue against the presence of an axial neutral histidine ligand *trans* to the dioxygen in P_{450}.[399] The m.c.d. spectra have been obtained, in the near i.r. region, for P_{450} from *Pseudomonas putida* in the oxidized, reduced, and carbonmonoxy forms at various temperatures and in the presence of camphor. Oxidized P_{450} with camphor exhibits positive and negative bands at 825 nm and 970 nm respectively, both of which are assigned to Faraday *B* terms. P_{450} in the reduced form in the presence of camphor exhibits at least five bands in the region 650—1175 nm. The heme Fe in both oxidized and reduced forms has a ligand field symmetry lower than C_{4v}. The CO complex has no detectable m.c.d. in the near i.r. Oxidized P_{450} undergoes a transition from high to low spin on decreasing the temperature to liquid nitrogen temperatures.[400]

M.c.d. of several metalloporphyrin complexes of rabbit and human serum hemopexins in the region 300—650 nm have been reported. The heme complexes of both proteins are identical, suggesting similar co-ordination. The Soret region of ferriheme–hemopexin is typical of completely low-spin ferric hemeproteins, and its temperature dependence indicates predominantly Faraday *C* terms. The visible m.c.d. of this complex closely resembles cytochrome-b_5 and other bis imidazole heme derivatives. Ferroheme–hemopexin also exhibits spectra similar to ferrocytochrome b_5. Deuteroheme derivatives of rabbit hemopexin show similar spectra to those of the heme complexes. Co and Ni deuteroporphyrin IX bound to hemopexin do not show the effects seen with Fe porphyrins.[401]

5 Infrared and Raman Spectroscopy

Contributed by R.M. Stephens

Model Compounds.—An unusual peptide structure, the intermolecular hydrogen bonded parallel-chain pleated-sheet β-conformation, has been postulated

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from the spectra of H-(Ile)$_7$-OMe.HCl and H-(Val)$_7$-OMe.HCl. The i.r. and c.d. data were compared with that obtained from H-(Ala)$_7$-OMe.HCl and Me_3CO_2C-(Ala)$_7$-OMe which adopted the more conventional antiparallel β-pleated sheet conformation.[402] The structures of γ-branched homo-oligopeptides have been analysed using results obtained from i.r. and c.d. spectroscopy. The conformations and stability of Me_3CO_2C-(L-X)$_n$-OMe [X = Leu, Phe, or Cha and $n = 2$—7], were shown to be dependent on steric requirements, electronic properties, and hydrophobicity of the side-chains.[403] Polyamides with regular distribution of CO_2H groups in the polymer chain have been prepared from optically active and racemic intermediate diamines based on lysine and ornithine, and their conformations discussed on the basis of spectral results.[404] Analysis of the oligopeptide -$O_2NC_6H_4S$-(Ala-Leu-Z)$_5$-OEt (Z = Gly or Ala) showed that its conformation in the solid state changed from a β-structure to an α-helical form when shear stress was applied to the film.[405] Collagen-related polytripeptides (Gln-Pro-Pro)$_n$, (Gly-Pro-Hyp)$_n$, (Gly-Hyp-Hyp)$_n$, (Gly-Pro-Ala)$_n$, (Gly-Pro-Gly)$_n$, (Gly-Ala-Pro)$_n$, (Gly-Ala-Hyp)$_n$, (Ala-Pro-Pro)$_n$, and (Ala-Hyp-Hyp)$_n$ have been investigated using H–^{2}H kinetics. The strength and order of interpeptide H-bonds of the polytripeptides in the triple-helical conformation depended on the amino-acid composition and residue sequence in the triplets. Correlation of *X*-ray and spectroscopic data for (Gly-Pro-Hyp)$_n$ showed that the increase of the α-helical parameter in the process of dehydration was accompanied with weakening of interpeptide H-bonds.[406]

The structure of Z-Gly-Ser(CMe_3)-Ser-Gly-O$(CH_2)_{17}$Me in dilute solutions of cyclohexane was shown to be either type I or II reverse-turn structure which is a conformation involving four consecutive residues in which the peptide chain folds back on itself with H-bonding between the CO group of the first residue and the NH group of the fourth residue. The i.r. spectra of films cast from dilute solutions also indicated that the conformation was a reverse turn. It may now be possible to calculate this conformation in proteins in solutions if the reverse-turn spectrum is used in combination with those of other conformations.[407] The N–H stretching frequencies in the i.r. spectra of valinomycin-like depsipeptides in CCl_4 have been correlated with those obtained from valinomycin. The N–H frequency for a 4 ⟶ 1 H-bond depends on whether the resulting β-fold structure is isolated or included in an arrangement of fused ten-membered rings.[408] Conformational studies on monamycins (cyclohexadepsipeptide antibiotics) have shown that β-loops are incorporated in their folding, between the NH group of the valine residue and the carbonyl of

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the hydroxypiperazic acid residue.[409] Conformational studies on the group A peptide antibiotics of a mikamycin have also been reported.[410]

Vibration c.d., Raman and i.r. spectra of alanine, H-Ala-Ala-OH, H-Ala-Ala-Ala-OH, H-Gly-Ala-OH, and H-Ala-Gly-OH have been obtained in the 2800—3100 cm^{-1} region. Vibrational assignment of the C–H stretching modes of alanine were presented and an interpretation of the observed vibrational c.d. spectrum was proposed.[411] The i.r. spectra of crystalline amino-acid conjugates of Ph_2CHCO_2H and of *p*-chlorophenoxyisobutyric acid have been analysed so that conclusions about intermolecular interactions could be made. The NH groups as well as the CO amide-groups of the derivatives of the diphenylacetic acid usually took part in the association, but in the other case they generally did not. Special differences were observed in the interactive behaviour when the spectra of some optically active substances were compared with their racemic analogues.[412] An i.r. study on the complexes of the Group IIB metals with *N*-acetyl-L, α-*N*-benzoyl-DL-, and benzoyl-β-alanine showed that there was no interaction between metal ion and the CO ketonic-group and the NH groups. The amino-acids co-ordinated to the metal ions through the carboxylate group.[413] The Raman and i.r. spectra of glycylglycine and its four ^{13}C, two ^{15}N, and two $^{2}H(C)$ substituted species in the H_2O and $^{2}H_2O$ solutions have been studied. The vibrational spectra of the Cu^{II} complexes of glycylglycine were also analysed in relation to pH dependence.[414]

A collection of the i.r. and Raman spectra of amino-acid monomers, dimers, and polymers is available now on microfilm.[415] A comparative study of the conventional three-fold torsional potential functions, and those developed from the study of dipeptide conformations in protein crystal structures have been obtained by comparing calculated and experimentally observed NH–C_αH coupling constants obtained from n.m.r. studies and the relative occupancy of hydrogen-bonded conformations from i.r. studies of dipeptide model-systems. However, no fully satisfactory agreement was obtained from both sets of data for the three functions.[416] The i.r. and Raman spectra of solid β-alanine, taurine, and ciliatine (β-form) have been analysed and normal-mode calculations undertaken. The valence force-fields of the three crystals were very similar.[417] Normal co-ordinate calculations, Raman, and i.r. spectra have been obtained for analogues of rhodopsin and bacteriorhodopsin chromophores.[418] Previously reported interaction constants have been used to calculate the vibrational spectra of two crystalline modifications of polyglycine and poly-L-alanine.[419]

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Polypeptides.—The effect of dimethylformamide (a helix-inducing solvent), $CHCl_2CO_2H$ and CF_3CO_2H (helix-breaking solvents), and urea (a protein denaturant) on the conformation of poly(β-benzyl-L-aspartate) in a monolayer film at the air–water interface have been studied using polarized i.r. spectroscopy of the collapsed film. Breakdown of the hydrophobic hydrated structure was caused by urea, but no direct effect was observed for the other solvents. As expected this polypeptide formed a right-handed α-helical conformation as a monolayer, irrespective of the substrate solution.[420] Investigation of the liquid-crystalline state of poly(γ-benzyl-L-glutamate) in various solvents showed limiting degrees of bonding to acid molecules in dilute and in liquid-crystal solutions. The possibilities for studying the existence of the liquid-crystal order in polymer solutions and for quantitative determination of the degree of orientation of polymer chains and solvent molecules were discussed.[421]

The degree of association between side-chains in carbobenzoxylysine has been determined by dielectric dispersion measurements and i.r. spectroscopy. A strong interaction was shown to exist between side-chains, and the fraction of hydrogen bonds of the side-chains involved in the interaction was estimated.[422] Proton transfer and polarization of hydrogen bonds in polyglutamic acid with various nitrogen bases was investigated by examining absorption bands associated with the carboxy-group. The amide bands indicated that polyglutamic acid had an α-helical conformation except when the NH_2 group was protonated. If the system was hydrated the polymer formed an α-helical conformation. When dried however an antiparallel β-structure was adopted. This conformational transition, which was dependent on the degree of hydration, was reversible.[423] Sequential polypeptides of L-alanine and β-alanine have been polymerized and their conformations determined. The β-amino-acid residue was not incorporated in the α-helix of polyalanine but into a polyproline II helix. Consequently, the β-amino-acid residues should have a considerable conformational freedom around the N-C_β and C_α–C_β bonds.[424]

I.r. spectroscopy showed that poly(L-alanyl-L-alanyl-L-lactic acid) in poly(oxyethylene)films existed as a distorted α-helix. The non hydrogen-bonded ester groups are transverse to the helical axis and are responsible for the distortion.[425] The conformation of polymers and co-polymers of *O,O*-dicarbobenzoxy-L-β-3, 4-dihydroxyphenyl-L-alanine with γ-benzyl-L- and D-glutamates were studied in CH_2Cl_2 and dioxane. The results indicated that the sense of the helix formed was different for the two solvents.[426] The variation in conformation as a function of chain-length was investigated for oligopeptides and polypeptides having a L-Leu-L-Leu-Gly sequence. Far i.r. spectral and *X*-ray data showed that in the solid state dodeca and lower peptides had a β-structure and pentadeca and higher peptides were in the

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α-helical form.[427] Conformational studies of syndiotactic polyleucine using *X*-ray diffraction, n.m.r., i.r., and optical rotation techniques indicated that this polymer had a π-helical conformation.[428] The conformational dependence of the characteristic amide frequencies has been studied for α-helical poly(L-amino-acid) derivatives and poly(D-amino-acid) derivatives. D- and L-amino-acid polymers with the same groups had the same amide frequencies and the results confirm that, in general, polypeptides from L-amino-acids adopt a right handed helical conformation.[429]

Proteins.—The geometrical distortion of the α-helical structure of the globular proteins, sperm whale myoglobin, bacteriophage T4, lysozyme, and hen-egg-white lysozyme has been studied using ^{2}H-exchange in solution. The structural heterogeneity of the polypeptide backbone of the proteins was determined by studying the half-width of the amide *A*-band during successive deuteriation of the protein. The results also revealed a correlation between the degree of geometrical distortion of the α-helical structure and the dynamic accessibility of their peptide groups to a water molecule.[430] Deuterium-exchange techniques were also used to study the conformational changes in aspartate aminotransferease. In the uniliganded pyridoxal form, at pH 6.0, 43 % of the total 411 peptide H-atoms per subunit exchanged within the first ten minutes. An additional 9 % exchanged slowly in the following time period to 360 min. A quite similar exchange curve was obtained from the pyridoxamine form. In contrast, the formation of an equilibrium mixture of the covalent transamination intermediates, which occur in the simultaneous presence of the amino-acid and the keto-acid substrates, resulted in a marked retardation of H-exchange, reflecting a substantial tightening of structure. The exchange reactions of at least two peptide H-atoms per subunit (6 % of the total) were retarded by a factor of six on average.[431] The degree of deuterium incorporation into membrane-bound rhodopsin showed that this protein had a very low solvent accessibility with a considerable number of amide protons being shielded. Such exchange behaviour is expected for compact α-helical segments deeply embedded in the hydrophobic lipid layer.[432] The kinetics of H-exchange for collagens from different animals has been studied by radioisotopic methods (with ^{3}H) and i.r. spectroscopy (with ^{2}H). Although the collagens differed in their amino-acid composition, they were all similar in content of slowly exchanged H-atoms. Each had 1.0 very slowly exchanging and 0.6 slowly exchanging H-atoms per 3.0 amino-acid residues of the triple-stranded collagen molecules.[433]

The conformational mobility of rabbit-muscle tropomyosin, in thin films prepared at 55 % relative humidity from solutions of differing pH values (1—12), was analysed on the basis of H–^{2}H exchange. For all films, the kinetics

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of H–^{2}H exchange indicated the presence of rapidly and slowly exchanging groups. The results indicated that the conformation of tropomyosin remains approximately the same between pH 1—7 but undergoes marked reduction in helix content with an increase in conformational mobility at pH > 7.[434] Detailed studies of the heat denaturation of human serum albumin using H–^{2}H exchange and sedimentation measurements showed that β-structure formed when aggregates appeared in solution. The formation of the β-structure and the aggregation had a related dependence on concentration and temperature.[435]

The reactive group in the active-centre of chymotrypsin is serine which is hydrogen-bonded through histidine to an aspartate residue. I.r. studies showed that both H-bonds in this system are easily polarizable. How the charge-relay mechanism works when a substrate or an inhibitor is attached to the active-centre of the enzyme was discussed.[436] Investigations of the anion-binding sites in transition state analogue complexes of creatine kinase, indicated that when the anions are liganded to various divalent cations, characteristic changes in frequency or in multiplicity are observed. I.r. spectra of the complexes of the anions with enzyme–divalent cation–ADP–creatine species coincided with the vibrational spectra obtained from the simple anion–metal ion complexes in concentrated solutions of the metal–anion salts. These cation-specific responses for the enzyme-bound anions provided cogent evidence for liganding of the anions to the activating divalent cations.[437] The conformation of cleaved histone proteins H3 and H4 was investigated to establish if certain regions were involved in the formation of a tetrameric complex and to locate secondary structure in histone H4. Removal of 36 % and 30 % of the residues from the *N*-termial regions of H4 and H3 did not inhibit complexation but removal of 18 % and 33 % of the proteins from the *C*-terminal ends did. The i.r. spectra of six peptides and the c.d. spectra of eight peptides of H4, indicated two 13-residue helical regions, one assigned to residues 55—67, and the other in the region of residue 84. The *C*-terminal residues of histone H4 adopted a β-structure.[438] The conformation of the non-histone chromatin protein HMG 17 has been studied using c.d., i.r., n.m.r., and small angle neutron scattering. In free solution the protein has little or no secondary or tertiary structure. This is in contrast to other high-mobility-group proteins HMG 1 and 2.[439]

Dry films of thromboplastin apoprotein indicated the presence of α-helical and β-conformations, and addition of phospholipids resulted in a conformational change in the β-structure of the apoprotein. Heat treatment of this protein from *Acetabularia.* The spectral data in the amide *A,* I, II, III, and IV absorption band at 1630 cm^{-1}. Deuteriation studies indicated that some of the apoprotein protons were buried in the hydrophobic lipid phase.[440] It has been

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established that the α-helical structure is predominant in a plant cell-wall protein from *Acetabularia*. The spectral data in the amide *A*, I, II, III, and IV regions showed that the protein conformation was ordered.[441] The conformations of proteins precipitated from *Acetabularia* have also been investigated.[442, 443] The effects of systematically varying the steric and polar groups R and R′ in *N*-alkylamides of the type RCONHR′ on the conformational properties of the amide bond have been related to the structure of globular proteins. As a consequence of very rapid interactions in the steric and polar environments of the peptide hydrogen, conformational flexibility may be induced in the protein backbone. The probability distribution of labile backbone-peptide hydrogens at any given instance is likely to be a determining factor for the overall backbone conformation of a native globular-protein molecule at that instant.[444] The determination of structural changes in antibodies arising from their specific interaction with antigens is an important problem in understanding immunological responses. The adsorption of anti-rabbit serum albumin antibodies on to monomolecular films of rabbit serum albumin, bovine serum albumin and ovalbumin, and of anti-ovalbumin antibodies on to films of rabbit serum albumin and ovalbumin at a Ge-aqueous interface have been studied using polarized attenuated total reflectance i.r. spectroscopy. The intensity of the amide I absorption band indicates that the strengths of binding of these three albumin proteins with anti-rabbit serum albumin is in the order rabbit > bovine $\gg$ ovalbumin; with anti-ovalbumin, it is ovalbumin $\gg$ rabbit. The conformations of the proteins and protein complexes were mainly α-helical and random-coil structures. The average orientation of the transition moments of the amide I and *A*-bands was $\sim$75° with respect to the surface normal. This indicates that the polypeptide chains are on average approximately parallel to the surface of the systems studied. Consequently, the effect of the specific antibody–antigen interaction on the conformation and orientation of the antibody was thought to be negligible in the films studied.[445] Calculation of conformational related-basis spectra from the amide I band of reference proteins of known conformational composition has aided the computation of conformations of globular proteins. The comparison of the percentages of the β-conformation derived from *X*-ray analysis or calculated from the i.r. spectra showed the suitability of the spectra as a basis for the rough estimate of the β-conformation in proteins.[446] In conclusion two reviews on the interpretation of the i.r. and Raman spectra of peptides and proteins have recently been reported.[447, 448]

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6 Nuclear Magnetic Resonance

Contributed by H.W.E. Rattle

Biological n.m.r. is coming of age: as the volume of publications continues to rise steeply, it is fair to say that at last the technique has reached a stage where the majority of them describe work on topics dictated by the biochemical problems rather than by the capability of the machinery. Several new and subtle n.m.r. methods have been introduced, but the main advances remain in the fields of computing and of magnet technology. The field has not been over-reviewed, although readers may be interested by a book on biological magnetic resonance[449] which contains articles on n.m.r. of histones and on ^{23}Na, ^{39}K, and ^{19}F resonance probes, together with others on e.s.r. spectroscopy. Carbon-13 n.m.r. has been reviewed[450, 451] while the study of molecular-switching mechanisms[452] and the application of spectroscopic methods to iron-containing proteins[453] and lanthanide probes to calcium-ion binding-sites[454] are also covered.

Techniques.—Among the methods being newly applied to proteins, that of photochemically induced dynamic nuclear polarization (CIDNP) promises some interesting results. The technique itself was discussed at a NATO Advanced Study Institute[455] and involves introducing a dye such as fluorescein or a xanthene dye at low concentrations into the protein solution: irradiation with laser light can then alter the nuclear magnetization of exposed tyrosyl and tryptophanyl residues, a change that can be detected by n.m.r. difference spectroscopy. The method has been applied to luliberin,[456] pancreatic phospholipase A_2,[457] and to the basic pancreatic trypsin inhibitor[458] where it was able to distinguish between exposed and buried residues. Another method of inducing changes in nuclear magnetization, the nuclear Overhauser effect, also has distinct possibilities in the resolution of single resonances in a crowded spectrum; two groups have developed methods for obtaining difference spectra between Overhauser-enhanced and normal resonance spectra, and have demonstrated their application to lysozyme[459] and to the basic pancreatic trypsin inhibitor.[460] The problem of the resolution of lines and splittings in broadened protein spectra remains acute, despite the availability of high-field spectrometers, and has been attacked by several methods, notably by using a spin–echo technique (T_2 spectra) during data collection and by treatment of collected data by convolution, either with trapezoidal or sinebell functions.

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When the three methods were compared critically, the Hahn spin–echo method emerged slightly ahead.[461] A similar multipulse method ($90°$–τ–$180°$–τ) with time-shared irradiation during data collection[462] enables accurate values for the spin-coupling constant J to be obtained for poorly resolved doublets. The effect of cross-relaxation on T_1 values during interaction between a small ligand and a macromolecule is discussed[463] as is its effect on T_1 values for a number of proteins.[464] Transfer of saturation between solvent protons and the surface residues of superoxide dismutase, followed by rapid cross-relaxation within the protein, are proposed to explain the transfer of saturation from solvent water to histidine-NH residues even in the absence of chemical exchange.[465] Some apparent discrepancies (of the order of two times) between protein rotational correlation times determined by n.m.r. and those determined by light scattering, are explained in terms of small librational movements within the protein with correlation times of the order of 10^{-11}s.[466] This may mean that many published n.m.r. values for rotational correlation times are quite a lot too long. The sources of relaxation are further considered in a study of biomolecules and their components in the solid state,[467] and a spectrometer with a sideways-spinning 20 mm tube probe, used for relaxation measurements and spin-locking experiments on ^{13}C at natural abundance, is described.[468] High field natural-abundance ^{13}C-n.m.r. can be used to study the environments of individual carboxy-groups,[469] although the main problem remains that of assignment.

Turning from instrument-based to sample-based techniques, an allegedly simple method of drying small amounts of solvents such as dimethyl sulphoxide and chloroform for n.m.r. use is described.[470] The permanent attachment of probe molecules may be achieved through the formation of trifluoroacetonyl derivatives of cysteine,[471] or by deuterium labelling of histidines.[472] Other probes described include the hexafluoroarsenate(v) ion,[473] and the ^{113}Cd[474] and $^{35}Cl^-$ ions.[475] In another interesting labelling experiment it is pointed out that ^{18}O causes an isotopic shift in the resonance of ^{31}P (compared to ^{16}O) of 0.0206 p.p.m. and that as a result ^{18}O-labelled phosphate may be used to follow the fate of phosphate in enzyme-catalysed exchange reactions;[476] other papers using this new method will be mentioned later.

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Amino-acids and Peptides.—Extensively modified amino-acids have aroused some interest; in particular the co-enzyme F_{420} from *Methanobacterium* was identified[477] as a highly modified glutamic acid derivative, and the implications for vitamin K-dependent blood coagulation proteins of the metal-binding properties of γ-carboxyglutamic acid discussed.[478] The latter, as incorporated in small peptides, has also been the object of some interesting studies using $^{43}Ca^{2+}$ and $^{25}Mg^{2+}$ ions as resonance probes.[479] Extensive conformational studies of pyridoxal Schiff's bases of amino-acids have been carried out[480, 481] and proton spectra of 17 phenylthiohydantoin derivatives of amino-acids presented.[482] Proline has been shown to exhibit some characteristics of hydrophilic colloids in solution.[483] Natural-abundance ^{15}N spectroscopy has been used to determine the differential rates of proton exchange for the guanidinium nitrogens of arginine.[484] A continuing study of repeated hexa peptides of tropoelastin enabled further estimates of ϕ and χ torsion angles to be made for both the natural peptide[485] and a permutation of its sequence.[486] Conformational data is also reported for fibrinogen-like peptides in dimethylsulphoxide solution[487] and for some X-Pro-Pro tripeptides.[488] The *cis–trans* isomerism of poly(D,L-proline)[489] and the detection of *cis* residues in poly(γ-hydroxy-L-proline) are discussed.[490] Other polypeptides synthesized and investigated by n.m.r. include a series of ^{15}N-labelled polymers containing glycylglycine units[491] and poly(hydroxypropyl-L-glutamine), the mobilities of whose side-chains appeared to be *increased* by large applied pressures.[492] The flexibility of $(Gly\text{-}Lys\text{-}Gly)_n$ was investigated using spin-label e.s.r.[493] Among synthetic cyclic peptides studies we may mention *cyclo*-(Gly-L-Pro-Gly-D-Ala-L-Pro),[494] *cyclo*-$(X\text{-}Pro\text{-}Y)_2$,[495] and the apparent resolution of a discrepancy between reported spectra of *cyclo*-tetraglycyl, in which it is alleged that the workers reporting the alternative results were looking at *cyclo*-octaglyclyl instead![496]

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Peptide Hormones. It is clear that the high signal-to-noise ratio obtainable using the latest spectrometers has aided a solution study of glucagon, which readily aggregates at 'normal' n.m.r concentrations but was studied in monomeric form at very low concentrations;[497] glucagon has also been studied by the technique of optically-detected magnetic resonance.[498] One way round the extreme propensity of glucagon to aggregate at high concentrations is to methylate Met-27. The modified protein forms a carbamino adduct when incubated with $^{13}CO_2$, and the structure may be identified with that of the native protein, but methylglucagon shows far less tendency to aggregate.[499]

An analogue of oxytocin, in which a prolyl residue was introduced at position-3 of the cyclic portion of the molecule, provides some interesting comparisons between results obtained using ^{13}C [500] and ^{1}H [501] resonance; a type II reverse-turn is apparently formed, and T_1 measurements for met-enkephalin and α-endorphin in aqueous solution, again using ^{13}C-resonance, indicate that flexibility of the small peptide varies along its length, and that α-endorphin is flexible with the enkephalin segment little different from the rest of the molecule.[502] Perturbation of the T_1 values in proton spectra of neurophysin by the binding of spin-labelled peptides was interpreted[503] in terms of a second binding-site for peptides immediately adjacent to Tyr-49. A study of the histidine resonances from the α-subunit of porcine lutropin[504] shows that the structural changes in the subunit on binding to the β-subunit to form the intact protein transfer the histidine from a solvent-inaccessible environment to a solvent accessible environment.

The conformation of angiotensin II changes from an all-*trans* to a partly-*cis* form about the His^6-Pro^7 peptide bond in rat uterus *in vitro,* with a corresponding change in potency;[505] 250 MHz ^{1}H studies on *cis–trans* isomerism in Pro-24 of fragments of corticotropin are reported,[506] and also the conformation and aggregation of melanostatin.[507] Investigations of the interaction of 5′-AMP with thyrotropin-releasing hormone indicate that the base stacks with the imidazole moiety of the hormone with a degree of complex formation far higher than its own degree of self-association.[508]

Other Biopeptides. Once almost the only polypeptide antibiotic studied by n.m.r., gramicidin S is still of interest; a complete assignment of its

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^{15}N-spectrum is reported[509] and the conformational changes with temperature studied *via* the shielding effects of the D-phenylalanine ring.[510] Among the many other antibiotics studied, a hydrogen–deuterium exchange study of polymyxin B showed the influence of counter-ions and enabled the identification of two reverse-turns,[511] while the effects of such exchange on the ^{1}H, ^{13}C, and ^{15}N-spectra of viomycin is discussed[512] as an aid to the assignment of resonances. A cyclic-peptide analogue of valinomycin, *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ was found to differ in conformation from the familiar valinomycin bracelet structure and to lack any central cavity,[513] while a new antibiotic from *B. subtilis*, iturin A, was found[514] to form a relatively rigid structure with a small (< 2 Å) cavity into which the backbone carbonyls are directed. A new polypeptide with membrane-exciting properties, trichotoxin A-40, has had most of its ^{13}C-spectrum assigned,[515] as has the nonapeptide bradykinin.[516] One of the most extensively studied peptides reported recently is the basic pancreatic trypsin inhibitor (BPTI). Detailed assignments of the 360 MHz ^{1}H-spectrum[517] and the 90.5 MHz ^{13}C-spectrum[518] are described, as well as the binding of lanthanides to the carboxy-groups of the molecule.[519] A single salt bridge between the terminal residues of the molecule was shown to have marked effects on its stability and internal mobility, contributing about 4.2 kJ mol^{-1};[520] the hydrogen–deuterium exchange rates of eight slowly exchanging NH-protons in this very stable protein were also studied in detail.[521] comparison between the glycoprotein, cow-colostrum trypsin inhibitor, and the molecule and its stability with respect to thermal denaturation is increased as the content of amino-acids with charged side-chains goes up. A detailed comparison between the glycoprotein cow-colostrum trypsin inhibitor and BPTI yields extensive homologies, indicating that the carbohydrate moiety has very limited effects on the spatial structure of the protein part of the molecule.[523]

Enzymes.—The ^{13}C-spectra of labelled human carbonic anhydrase B have been used to obtain dynamic information; carboxymethylation at histidine

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200[524]—[526] provides a probe into co-ordination of this residue with the Zn-atom. The inhibitors methanol and aniline[527] bind in different ways to carbonic anhydrase, the aniline co-ordinated to the metal ion and methanol within a hydrophobic region in the catalytic site; ^{14}N nuclear quadrupole resonance relates the inhibiting effect of benzene sulphonamides on the same enzyme to the sulphamyl group of the inhibitor.[528] The effects of the presence of carbonic anhydrase on the relaxation behaviour of solvent water in the presence of buffer ions,[529] of phosphate in solution,[530] and of dicarboxylates[531] are also discussed.

A series of experiments in which specific ^{13}C-labels were added to dihydrofolate reductase in cultures of *Streptococcus faecium* is described.[532, 533] Internal motions were measured, as were changes with the binding of ligands. Two of the tryptophan residues in dihydrofolate reductase must approach within 3 Å of each other, as shown by ^{19}F-labelling[534] while comparison of the ^{13}C-relaxation parameters of the methionine-methyl resonances of the same protein with theoretical calculations indicates that a better fit is obtained if the motion of the groups is assumed to be restricted.[535] In the glutamate dehydrogenase system, the substrate and co-enzyme appear to enhance each other's binding to the enzyme by greater than an order of magnitude;[536] e.s.r. measurements on spin-labelled glutamate dehydrogenase indicate that the only spin-label site which causes paramagnetic relaxation of bound substrate and co-enzyme is not an SH group: binding of spin label at this site inactivates the enzyme.[537] The co-enzyme of trimethylamine dehydrogenase is apparently covalently bound[538] and appears to be a 6-substituted flavin.[539] The kinetics of co-enzyme-binding to lactate dehydrogenase show that the lifetimes of the NAD–enzyme and NADH–enzyme complexes are $\sim$ 1 ms and $\sim$ 10 ms respectively,[540] and the comparison between the two redox states of the co-enzyme may be sensitively probed using ^{15}N-labelling.[541] N.m.r. has also been used to provide evidence for metal–substrate co-ordination in ethanol binding to cobalt-substituted liver alcohol dehydrogenase.[542]

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Phosphorus resonance is a useful probe of enzymic reactions involving ADP and ATP; an example is found in the differentiation of nucleotide binding-sites on adenylate kinase, in which the metal ion was found to play a role in addition to its catalytic function[543] and another in the bound reactants and products of yeast 3-phosphoglycerate kinase, in which a second ATP-binding site is again discovered.[544] The same technique was used to measure the interconversion rates of bound substrates in a number of kinases.[545] A multi-enzyme complex of glycolytic enzymes was isolated from red-cell ghosts, and the association of the enzymes on combination with 2,3-diphosphoglycerate–monophosphoglycerate mutase studied.[546] Lithium resonance was used as a probe of temperature-dependent changes at the active-site of pyruvate kinase, showing the existence of two states, the more isotropic form being favoured by lower temperatures.[547] Fluorinated substrate analogues containing fluoromethyl groups were used as stereochemical probes of enzyme reactions, showing that pyruvate kinase does not act chirally on the two chemically distinguishable H-atoms of fluoropyruvate, while in contrast methylmalonyl-CoA transcarboxylase removes specifically one of the two prochiral H-atoms in generating the nucleophilic enolate anion at the active-site.[548] Fluorine was also used as a probe in the assignment of a pK value of 8.2 to the active-site Lys-258 of aspartate transaminase.[549] The specific α-deuteriation of amino-acids by *E. coli* aspartate aminotransferase was the subject of kinetic studies[550] and was shown to be first-order with respect to both substrate and enzyme.

The kinetics of refolding of heat-denatured ribonuclease A were studied by ^{1}H-n.m.r., and a new resonance showed the presence of a structural intermediate in the folding;[551] assignments of the ^{13}C-resonances of RNase A are offered in.[552] A starting point for more n.m.r. studies of the enzyme is provided[553] in the introduction of a strong binding-site for lanthanides at its N-terminus, and the binding of EDTA to RNAse has also been studied[554] – it is likely that three of the EDTA carboxy-groups can complex to His-12 and to Lys-7 and Lys-41. Complexing of Pr^{3+} and Dy^{3+} to the phosphate groups of cyclic UMP and cyclic CMP, which are intermediate states in the cleaving of RNA by RNase, has been used to deduce that the C(2)O atom does not, as previously thought, play a decisive role in the mechanism of hydrolysis.[555] A model for the structure of the Michaelis complex formed by RNase with substrate has been proposed following experiments using 2′-deoxy-2′-fluororibose substrate analogues.[556] The structure of the active-site of guanyloribonuclease

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(RNase T_1) was found to differ from that of RNase A[557] in that His-40 and a glutamic acid residue (probably Glu-58), rather than two histidine residues, are found to be responsible for the catalytic activity of the enzyme. The histidine assignments in this study were made with a tritium labelling method; another method proposed is that of photo-oxidation of the histidines in the presence of Methylene Blue.[558] RNase was also among the proteins studied in a series of investigations of the conformational mobilities of globular proteins in which it was possible to distinguish the relaxation of rigid and flexible regions of the protein and of flexible sidechains.[559, 560] The binding of substrate oligosaccharides[561] and the perturbations induced by cobalt ions[562] in the spectrum of lysozyme have also been reported.

The activation of zymogens has been studied *via* the histidine residues of trypsinogen[563] and chymotrypsinogen[564] which had previously had labile NH-protons fully exchanged for deuterium by reversibly unfolding the proteins in ^2H_2O. The complex formation of trypsin and of chymotrypsin with pancreatic trypsin inhibitor appear to be similar. By using a solvent subtraction technique[565] it was possible to observe some low-field hydrogen-bonded NH resonances in ordinary water solutions of serine proteinases, showing a conformational equilibrium between two states which was abolished on binding an inhibitor. The method will undoubtedly have further application for protein solutions. Some ^{15}N-studies on the state of histidine in the catalytic triad of α-lytic protease show that its pK is 7.0, contrary to previous reports that the histidine would be an abnormally weak base. Also, at catalytically active pH-values, an unusual tautomeric form of the imidazole ring is found, probably due to a hydrogen bond to the neighbouring aspartate residue. The implications for the charge-relay system are discussed.[566] A model for the aromatic-binding site of chymotrypsin has been proposed following a series of measurements using ^{19}F-resonance of fluorinated analogues of inhibitors.[567] A conformation for phenacyl-α-chymotrypsin is also reported,[568] and ^{19}F-resonance has been used to follow the interaction between elastase and sodium trifluoroacetate[569] and trifluoroacetylated chloromethyl ketones.[570]

The active-site of an ATPase has been probed by ^7Li resonance[571] and cobalt-labelled ATP.[572] The metal dependence of the phosphate oxygen $\rightleftharpoons$

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water exchange reaction of *E. coli* alkaline phosphatase[573] and human prostatic acid phosphatase[574] has been followed by the ^{31}P–^{18}O technique described earlier, while the zinc stoicheiometry in the enzyme, followed by ^{31}P resonance, indicates two different ways in which two Zn^{2+} ions can bind to the apoenzyme, one of them co-operative.[575] The binding of inorganic phosphate to yeast inorganic pyrophosphatase is also described[576] as is that of purine ribosyl 5′ nucleotides to glycogen phosphorylase *b*.[577]

N.m.r. experiments to determine the form of 2-phosphoglycolic acid bound to triosephosphate isomerase reveal the unexpected result that the enzyme appears to bind the trianionic form of the inhibitor.[578] The mechanism of enzymic racemization of allantoin[579] differs from that of non-enzymic racemization. The reconstitution of copper-free superoxide dismutase with copper[580] and the binding of ^{199}Hg to the same molecule[581] were studied by n.m.r. Synthetase enzymes which have been studied recently include thymidylate synthetase,[582,583] carbamyl phosphate synthetase,[584] and glutamine synthetase.[585] In the last of these, the effect of Mn^{2+} ions at the two metal-binding sites was used to determine distances of ~ 7 Å and ~ 10 Å between the metal ions and the phosphorus atom of covalently bound AMP.

Haem Proteins.--*Haemoglobin.* The mechanism of haemoglobin oxygenation remains of considerable interest, and a number of methods have been used to investigate this large and rather difficult molecule. Interactions between the quaternary state of the molecule and the spin state of the haem have been followed in ferric mixed spin derivatives by measurements of paramagnetic susceptibility[586] and several studies have used mutant haemoglobins. Hb Providence (β-82 Lys-Asx) was used[587] to show that β-82 EF6 Lys is probably one of the binding-sites for Cl^- ions, while two mutations (Hb Osler and McKees Rocks) which are altered at β-14 C2 Tyr indicate[588] that a resonance appearing at –6.4 p.p.m. in the spectrum of normal haemoglobin in H_2O is that of a proton engaged in a hydrogen bond between Tyr β-145 and Val-β98 FG5 which may prove to be an important structural probe. Another probe, this

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[588] G. Viggiano, K.J. Wiechelman, P.A. Chervenick, and C. Ho, *Biochemistry*, 1978, **17**, 795.

time for modifications of the distal environment of the haem pocket, is provided by the ^{13}C-labelled isocyanoethane ($C_2H_5N^{13}C$) which was used in a number of mutant-Hb molecules to show the difference between the environments of ligands bound to the β-chain in its isolated form and as part of the Hb tetramer.[589] The possibility of labelling haemoglobins for n.m.r. by means of alkylation at cysteine β-93[590] and by biosynthetic incorporation of carbon-labelled amino-acids[591] is discussed. With a molecule as large as haemoglobin, with its 3500 protons, it is clearly easier to observe the resonances of small molecules which bind to it: examples this year are studies of the binding of benzene,[592] ^{35}Cl,[593, 594] ATP and cations,[595] and isocyanides.[596] The results of the last study indicate that the chemical shift of the bound isocyanide depends on the structure of the Hb subunit. Subtle changes in the binding between Fe and its proximal histidine in Hb, myoglobin, and cytochrome *c* were shown to affect the chemical shift of ^{15}N in bound labelled-cyanide ion,[597] while Johnson *et al.* report the unambiguous observation of the proton resonance of a distal histidine in soybean leghaemoglobin; its behaviour was consistent with the formation of a salt link between it and bound nicotinate.[598] Detailed studies on the proton resonance hyperfine shifts in insect monomeric haemoglobins (from *Chironomus thummi thummi*),[599, 600] show chemical-shift changes in a vinyl group on the proto-haem prosthetic group which are diagnostic of the *T–R* transition and arise from changes in the co-planarity of the vinyl group with the haem. Two conformational states are detected, a conclusion which is reinforced by a separate study on the same molecules.[601]

Myoglobin. Investigation of the interaction between the iron and proximal histidine in methaemoglobin and metmyoglobin by using hyperfine shifts indicate that the interaction is strongest in the former and stronger in the β-subunits of MetHb than in the α-subunits.[602] New probes into the structures of myoglobins and haemoglobins are reported in the form of ^{13}C–^{57}Fe spin coupling[603] and of cobalt myoglobins and Fe–Co hybrid Hb[604] in which the linkage between the electronic structure of the prosthetic groups and the subunit co-operativity of the Hb was clearly shown. pH effects on the structures of sperm whale and horse deoxymyoglobins were followed using hyperfine shifts:

[589] D. Mansuy, J. Thillet, B. Cendrier, J.Y. Lallemand, and J.C. Chottard, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 217.
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the results suggested that the haem–apoprotein contacts are looser in the acidic than in the alkaline conformations. The structure-modulating group had a pK of 5.7 in both cases.[605] Chemical modification of the haem groups showed that the pK_a for the acid–base transition decreased as the haem 2,4 substituents were made more electron-withdrawing.[606] In a series of studies of a large number of myoglobins from different species, the C-2 resonances of 11 different histidines were assigned.[607] Some of the pK values were compared with an extension of the Tanford-Kirkwood theory, giving good agreement.[608] Relaxation studies on myoglobins were used to investigate the binding of pyridine and 1-methylimidazole to metmyoglobin[609] and also, with measurements made at liquid helium temperatures, to propose a new model for low-lying iron-2+ states.[610]

Cytochromes. Cytochrome *c* has often been used as an example of molecular evolution because of the variable sequences, and many different variants of the molecule have been examined by n.m.r.[611] In all cases except two low-potential cytochromes, the haem environment was found to be similar, even though the folding of the polypeptide chains differed between smaller bacterial, and mitochondrial and larger bacterial cytochromes. The high-spin direction on the haem-*c* periphery is rotated 90° as compared with horse-heart ferricytochrome *c*.[612] The haem environment has been studied in the presence of various nitrogenous ligands;[613, 614] all the haem proton resonances observed at 360 MHz were assigned for ferrocytochrome *c*, a further six assignments being obtained by saturation-transfer experiments in ferricytochrome *c*. Modifications of cytochrome *c* molecules which may prove very useful in future investigations include the attachment of spin-labels to a single lysine (probably 72 or 73) and a single tyrosine (probably 74) residue,[615] of ^{13}C-Me to methionine-80 and methionine-65,[616] of ^{19}F labels to single lysines including Lys-13,[617] and the replacement of Fe by Co in the haem group.[618] In the last of these studies it was possible to compare electron transfer from cobaltocytochrome *c* to ferricytochrome *c*, studied by stopped-flow kinetics, with electron-tunnelling theory, obtaining good agreement between the two. Stop-flow methods, coupled with on-line processing of e.s.r. data, were also used to determine that there are six sites for spin-labelled decylamine on microsomal cytochrome P_{450}.[619]

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Peroxidases. The use of n.m.r. has helped to exclude the possibility of direct contact between haem groups when cytochrome *c* peroxidase interacts with cytochrome *c*.[620] A series of studies on horseradish peroxidase using 220 MHz proton resonance,[621—624] show the presence of ferric Fe in high- and low-spin haem groups of the reaction intermediates termed compound I and compound II; evidence was found against a π-cation radical on the haem ring as a source of the oxidizing equivalent in compound I. The equilibria between horseradish peroxidase and aromatic donors are further discussed.[625]

Metalloproteins. The determination of the active-centres of xanthine oxidase (mol. wt. $\sim$ 280 000 daltons) has been carried out by use of a ^{19}F-labelled extrusion reagent. After tests on *C. pasteurianum* and spinach ferredoxins had proved successful, application to xanthine oxidase provided clear evidence that the molecule contains four Fe_2S_2 sites in its active complex of two identical subunits.[626] The Fe_2S_2 clusters of bacterial ferredoxins cause shifts in the ^{13}C-resonances of cysteinyl residues, and comparison of ferredoxins from several sources indicated that the amino-acid environments of the clusters were similar in each.[627] The addition of thioacetamide to haemocyanin appears to exert an allosteric effect which is capable of reducing the affinitiy of the protein for both oxygen and fluoride ion, as shown by narrowing of the ^{19}F resonance in a solution containing 0.125M-KF.[628]

The structure of plastocyanin has been reviewed[629] and is the subject of several papers.[630—632] Some strongly broadened lines in the proton spectra are agreed to correspond to two histidine residues and one methionine which are proposed as ligands to the Cu^{2+} [632] along with possibly a cysteine or some other residues.[631] The metallothioneins are reported[633, 634] to have a random conformation in their apoprotein form, but well-defined structures in the metal forms, with small differences of the order to be expected from the different sizes and co-ordination preferences of the metals (Zn^{2+}, Cd^{2+}, and Cu^{2+}) which bind to them. The binding of calcium and manganese ions to concanavalin A induces a 'locked' conformation which is primarily responsible for the saccharide-binding activity of the protein;[635] in another study, however, using

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[634] A. Galdes, M. Vasak, H.A.P. Hill, and J.H.R. Kaegi, *F.E.B.S. Letters*, 1978, **92**, 17.
[635] S.H. Koenig, C.F. Brewer, and R.D. Brown, *Biochemistry*, 1978, **17**, 4251.

^{113}Cd resonance, it was concluded that metal binding to the S2-site of the protein was weakened on saccharide binding.[636]

Other Proteins. The exceptional ability of human plasma albumin to bind inorganic anions was the subject of ^{35}Cl quadrupole relaxation measurements on bound chloride ion[637] and of ^{19}F-resonance measurements on a bound fluorinated sulphonate compound.[638] Further ^{35}Cl relaxation experiments[639] reveal that the Cl-binding sites are very free to move about relative to the protein as a whole. Other proteins found in the n.m.r. literature for 1978 are a fragment of troponin C which binds calcium[640] and water relaxation[641] and cross-polarization[642] studies of collagen. The cross-polarization method enables high-resolution natural-abundance ^{13}C spectra to be obtained from solid materials like collagen, and could be a valuable technique for the future. Three studies of mobilities in tobacco mosaic virus (TMV) coat protein are also reported. Two of them utilized spin-labels[643, 644] to obtain overall motion of protein molecules, and the third[645] used ^{1}H-n.m.r. to suggest that the region from Asp-88 to Arg-112 of the protein molecules in a 45 TMV protein particle is remarkably flexible.

The specific interactions between the mouse-myeloma immunoglobulin MOPC 315 with nitrophenyl haptens have been measured by using ^{19}F-labelled haptens;[646] it appears that the presence or absence of the distal constant-regions of the Ig molecule does not affect the binding-site interactions. More detail comes from 270 MHz ^{1}H studies of the binding of trinitrophenyl derivatives to the same Ig;[647] the interaction was the same for a number of hapten derivatives, the ring being stacked with tryptophan-93L (on the light-chain) and confined to a 'box' formed by Trp-93L, Tyr-34L, and Phe 34H (on the heavy-chain), with hydrogen-bonds being formed to Tyr-34L and Asp-36L. Further detailed conclusions have been drawn from comparative ^{31}P studies of the phosphorylcholine-binding mouse-myeloma antibodies M603, W3207, and M167,[648] and comparative ^{19}F-studies of binding of trinitrophenyl haptens to M315, M460, and X25.[649]

Comparison between the short neurotoxins of the venom of the cobras *Naja nigricollis* and *N. naja oxiana* show that in each case the invariant Trp-28 has limited accessibility to solvent and is in proximity to the imidazole ring of His-31 and a carboxy-group, and the invariant Tyr-24 has restricted mobility.

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A common spatial structure for the two toxins is proposed.[650] Honey bee-venom anamin, as well as short neurotoxin from *N. naja oxiana,* is described in.[651] The sea snake erabutoxins a and b, which have been subjects of *X*-ray crystallographic analysis, have been studied at 270 MHz, revealing that in this case Trp-29 is exposed and mobile while Tyr-25 is restricted in motion, with a pK_a as high as 12. Histidine-26 is exposed to solvent, while His-7 is tightly buried in the interior and is not protonated until the toxin is denatured at pH 2.85.[652] The conformation in solution of AM-toxin T, a cyclotetradepsipeptide phytotoxin from *Alternaria mali,* is described.[653]

One of the many components of snake venoms is phospholipase A_2. ^{31}P-Resonance was used to monitor the hydrolysis of phospholipids in human blood serum high-density lipoprotein-3 by α-phospholipase A_2 from *Crotalus adamanteus,* and the kinetics of the hydrolysis of phosphatidylcholine to lysophosphatidylcholine followed.[654] The specificity of phosopholipase A_2, this time from *N. naja naja,* and phospholipase C, from *B. cereus,* towards different phospholipids in mixed micelles is reported.[655] Phospholipase A_2 prefers phosphatidylcholine to phosphatidylethanolamine as a substrate, and requires Ca^{2+} for activity; the difference in specificity may be correlated with differences in n.m.r. chemical-shift patterns caused by the ethanolamine moeity. An n.m.r. study of a tyrosine and two histidine residues in the structure of porcine pancreatic colipase is reported.[656]

Membranes.—Direct studies of membrane structure by n.m.r. are difficult, but some progress is being made; fluorine labelling at tyrosines in M13 coat protein has been used in relaxation measurements[657] on phospholipid vesicles, and specific labelling with ^3H and ^{13}C has been used to study the binding of lyso-lecithin to human-serum high-density apoprotein A-I.[658] Another paper[659] reports the observation of natural-abundance ^{13}C resonances from high-density lipoprotein particles, from which it was possible to conclude that some of the cholesterol in the system is bound very tightly. The major phospholipids of low-density lipoprotein were found to be accessible to small amounts of Pr^{3+}, suggesting that the head groups of all mobile phospholipids are on the surface of the particle in contact with the aqueous medium.[660] The protein-mediated trans-bilayer movement of lysophosphatidylcholine has been studied in vesicles containing glycophorin; such vesicles had a higher content of the lyso-compound on the inner monolayer than vesicles without glycophorin.[661]

[650] A.S. Arsen'ev, A.M. Surin, Yu.N. Utkin, V.I. Tretlin, V.F. Tystrov, V.T. Ivanov, and Yu.A. Ovchinnikov, *Bioorg. Khim.*, 1978, **4**, 197.
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One way of studying membrane systems is to attach a spin-label for e.s.r. measurement: the method is reviewed [662] and it has been used to study the transfer rate of phosphatidylcholine exchange protein,[663] the effect of γ-irradiation of erythrocytes [664] and the association of structural lipoprotein with matrix protein in the *E. coli* outer membrane *in situ*.[665]

Myelin basic protein has been studied by ^{13}C [666,667] and ^{1}H [668,669] resonance. Detailed conclusions were not possible, but some evidence for secondary and tertiary structure was adduced.

Proteins Associated with DNA.—270 MHz studies of the globular regions of histone H1[670] and H5[671] have been reported. Assignments of aromatic resonances and ring-current shifted methyl resonances were facilitated by using interproton Overhauser effects arising from through-space dipole interactions and measured using the NOE difference method.[459] Spectra of histone complexes including H3–H4 tetramer and H2A–H2B dimer confirmed earlier findings of the high mobility of lysine-rich regions of the molecules.[672] Basic regions of histones H2A and H2B are also found to be mobile in chromatin 'core particles' (a core particle consists of a histone octamer $(H2A–H2B–H3–H4)_2$ plus 140 base pairs of DNA) while the apolar regions of H2A and H2B and the whole of the H3 and H4 molecules are immobilized in the protein core of the nucleosome.[673] An interesting ^{31}P-resonance experiment on core particles revealed a single symmetrical resonance from DNA phosphates, indicating that the DNA is not kinked or possibly that the whole system is in rapid motion.[674] Sodium-23 has been used as a probe into the role of monovalent cations in chromatin structure.[675] A chromosome-associated non-histone protein, HMG 17, has been found to have little or no secondary or tertiary structure in solution,[676] in contrast to the other high-mobility-group proteins HMG 1 and 2. Moving to prokaryote systems, some experiments using selectively deuteriated and fluorinated gene V protein have shown that the interaction with DNA involves Tyr-26–base–Phe-13 intercalation.[677] Studies of the structure of ribosomal protein L7/L12[678] and spin-label measurements of

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labelled ribosomes from *E. coli*[679] reveal many details of this large and complex system, including that L7/L12 has a highly mobile domain in the 70*S* ribosome.

Muscle Proteins.—The ^{18}O–^{31}P isotope-shift method mentioned earlier was used to follow the kinetics of oxygen exchange between inorganic phosphate and water, as catalysed by myosin subfragment 1. Essentially all molecules of inorganic phosphate that bind to the enzyme undergo complete exchange and are released as PO_4^{3-}.[680] The interaction of skeletal myosin light-chains with calcium ions is reported.[681] A number of interconverting conformational forms of rabbit tropomyosins were distinguished during titration of the protein by separation of the resonances of two histidines into a number of components;[682] the existence of a specific kinase or phosphatase that can distinguish between α-and β-tropomyosins has been inferred from radioactive labelling and ^{31}P-resonance studies of the two components.[683] A structural basis for the observation that the Ca^{2+} threshold for the activation of tension in some myofibrils is increased in the presence of high Mg^{2+} concentrations is proposed[684] following ^1H-n.m.r. study of the calcium and magnesium induced structural changes in troponin C. Muscle parvalbumins have been studied by ^{113}Cd,[685] ^{43}Cu,[686] and ^{19}F[687]-resonance; changes in structure with temperature and pH, and characterization of different binding sites, are reported.

Glycoproteins and Water.—A few studies of glycoproteins have been reported: a structural basis for the antifreeze activity of blood glycoproteins from antarctic fish is proposed,[688] while 360 ^1H-n.m.r. has been used to determine the type of branching in complex asparagine-linked glycan-chains of glycoproteins,[689] the primary structure of a complex asparagine-bound glycopeptide from horse-pancreas RNase,[690] and the structure of the single glycan of rabbit serotransferrin.[691]

Study of the magnetic resonance of water in protein systems still produces some useful results. A note of warning concerning the effects of cross-relaxation on the relaxation of water in complex systems, such as tissues, is sounded:[692] this effect should be taken into account when calculating results. The amount of water bound to proteins in dilute solutions[693] and to

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glycogen,[694] the state of water in soybean protein preparations,[695] the water phase-transitions in collagen–water systems,[696] and the diffusion of water adsorbed on proteins using a new pulsed-gradient magnetic-field programme[697] are all reported. Others worthy of mention are the formation of microsomal cytochrome P450 complexes[698] and two studies on the hydration of haemoglobin.[699, 700] In the last of these it was shown that spin transfer between protein and water molecules is effective enough to cause the entire solute–solvent system to relax as one unit.

7 Mössbauer Spectroscopy

Contributed by D.P.E. Dickson

During 1978 ^{57}Fe-Mössbauer spectroscopic studies have been reported on haem proteins, iron–sulphur proteins, and iron-storage proteins, and also on model compounds which emulate the more complex proteins but whose properties may be more readily understood. The aim of most of this work is to obtain data concerning the electronic structure and spatial arrangement of the iron atom or atoms in the active-centre of the protein. This information should be helpful in understanding the properties of the active-centre in relation to the biological functions of these proteins. Theoretical work is necessary in order to obtain calculated values of the Mössbauer parameters, derived on the basis of particular models for the active-centre, which can be compared with the experimental data.

Two investigations reported in 1978 involve measurements on whole organisms or organs which are interpreted in terms of previous measurements on isolated proteins. Mössbauer spectra obtained from whole cells of the purple, photosynthetic bacterium *Chromatium minutissimum*[701] at 77 K contain a number of overlapping quadrupole-split doublets with parameters consistent with those of oxidized and reduced cyotchromes and ferredoxins. However, because of the poor quality of the spectra, which results from the low content of iron in the samples, a proper quantitative analysis is impossible. Papaefthymiou *et al.*[702] obtained spectra from healthy heart-tissue and heart tissue from patients with thalassemia, a genetic blood disease which leads to premature breakdown of the haemoglobin and consequent iron overload. The spectra of the healthy tissue show very little absorption while the spectra of the diseased tissue show considerable absorption, indicating the presence of appreciable amounts of iron. The spectra are essentially identical to those of

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[702] G.C. Papaefthymiou, K.S. Kaufman, R.B. Frankel, and A. Rosenthal, *J. Appl. Phys.,* 1978, **49,** 1815.

the iron-storage protein ferritin, and indicate that the iron is in this or a similar form. From the superparamagnetic behaviour which is observed in the spectra as a function of temperature it is estimated that the iron is contained in particles with diameters between about 60 and 90 Å. Work of the above type is likely to increase in the future and it is in this area that Mössbauer spectroscopy may be able to make significant contributions to biology and medicine.

An investigation which does not fall under any of the headings discussed above is a study of the enzyme protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*[703] which catalyzes the cleavage of protocatechuate to *β*-carboxy-*cis,cis*-muconate with the incorporation of two atoms of molecular oxygen. The enzyme contains iron but the chemical nature and ligand geometry of the iron atoms are as yet unknown. Mössbauer spectra have been obtained from the reduced form of the enzyme in the temperature range 1.5—200 K and in applied magnetic fields of up to 5.5 T. These spectra indicate that all the iron atoms are equivalent, with Mössbauer parameters consistent with a high-spin ferrous assignment. The applied-field spectra were fitted to a spin-Hamiltonian which yielded a consistent set of parameters. The results give a zero-field splitting parameter D of $-(6 \pm 1)$ cm^{-1} with a large rhombicity, and also show that the electric field gradient (e.f.g.) tensor is rotated relative to the zero-field splitting, indicating a point symmetry of monoclinic or lower. The electronic-spin relaxation rate of the ferrous ion was found to be unusually slow ($< 10^7$ s^{-1}) at 4.2 K.

The Mössbauer effect depends on how tightly the Mössbauer atom is bound into the molecule and in turn how tightly this molecule is bound in the sample. Information on this can be derived from the value of the recoil-free fraction or *f*-factor which relates to the absorption intensity of the Mössbauer spectrum. The degree of hydration of proteins may have a significant effect. A number of investigations of this type using haem-proteins, iron–sulphur proteins, and ^{57}Fe-labelled chymotrypsin and blood serum albumin have been reported.[704—706]

Haem Proteins.—Measurements reported on the oxyhaemoglobin of various species,[707, 708] including worms, insect larvae, fish, and mammals show only insignificant differences in the Mössbauer chemical-shifts but considerable differences in the quadrupole splittings. This is discussed in terms of the effect of changes in the amino-acid sequence on the symmetry of the haem group and the electronic structure of the low-spin ferrous ion.

[703] R. Zimmermann, B.H. Huynh, E. Münck, and J.D. Lipscomb, *J. Chem. Phys.*, 1978, **69**, 5463.
[704] O.V. Belonogova, E.N. Frolov, S.A. Krasnopol'skaya, G.P. Atanasov, V.K. Gnis, E.N. Mukhin, A.A. Levina, A.P. Andreeva, G.I. Likhtenstein, and V.I. Gol'danskii, *Doklady Akad. Nauk S.S.S.R.*, 1978, **241**, 219.
[705] V.E. Prusakov, O.V. Belonogova, E.N. Frolov, R.A. Stukan, V.I. Gol'danskii, G.I. Likhtenstein, and L. May, *Proc. Int. Conf. Mössbauer Spectroscopy*, 1977, **1**, 309, Documentation Office, Central Institute of Physics, Bucharest, Roumania.
[706] I.G. Gusakovskaya, T.I. Larkina, S.I. Pirumova, N.S. Ovanesyan, and V.A. Trukhtanov, *Biofizika*, 1978, **28**, 228.
[707] D. Guleva, T. Bonchev, I. Vasilev, and S. Teofilov, *Bulg. J. Phys.*, 1977, **4**, 636.
[708] D. Guleva, T. Bonchev, I. Vasilev, and S. Teofilov, *Proc. Int. Conf. Mössbauer Spectroscopy*, 1977, **1**, 315, Documentation Office, Central Institute of Physics, Bucharest, Roumania.

The nature of the interaction of molecular oxygen with the haem group in haem proteins is of considerable interest. The problem concerns the geometry of the dioxygen ligand and whether Fe^{2+}–O_2 or Fe^{3+}–O_2^- provides a better description of the electronic configuration. The relevance of Mössbauer spectroscopy to this problem is that the temperature variation of the Mössbauer quadrupole splitting is dependent on the electronic structure of the iron atom in the haem group. Huynh *et al.*[709] have made calculations for oxyhaemoglobin based on a neutral-oxygen model (*i.e.* Fe^{2+}–O_2) which are consistent with Mössbauer and vibrational spectra which had previously been interpreted in terms of the Fe^{3+}–O_2^- configuration. Two calculations were made, one of the SCF–Cl–Pariser-Parr-Pople type, the other using the SCF–Xα multiple scattering method. The calculated values of the e.f.g. tensor components and hence the Mössbauer quadrupole splitting and asymmetry parameter are compared with those from other theoretical models and with the measured values and appear to show a much better agreement with the experimental data than the values calculated using the other models. However, the authors consider that the dichotomy between Fe^{2+}–O_2 and Fe^{3+}–O_2^- in previous discussions may be too simplistic. The overall charge distribution appears to be of less importance than the nature of the bonding and the attendant orbital populations, although these calculations show no net charge transfer to oxygen.

Bade and Parak[710] have obtained Mössbauer spectra from sperm whale oxygenated myoglobin over a range of temperatures. The spectra show a well resolved quadrupole-split doublet with a temperature-dependent splitting. The temperature dependence of the corresponding e.f.g. tensor, calculated using a low-spin ferric-term scheme, gives good agreement with the experimental data. The Mössbauer data can thus be understood in terms of an Fe^{3+}–O_2^- configuration and this can also explain the diamagnetic character by an exchange coupling between Fe^{3+} and O_2^- to give a ground-state singlet. In order to keep the complex diamagnetic at room temperature an exchange coupling with $J \gg 300\ cm^{-1}$ is necessary.

Sharma and Kolman[711] have calculated e.f.g. components for the high-spin ferric ion in methaemoglobin using a molecular orbital treatment. The four nitrogen atoms in the haem group and the eight nearest carbon atoms are found to give the dominant contribution. The contribution from the next nearest atoms is an order of magnitude smaller and all other contributions are negligible. The resulting e.f.g. leads to a value of 1.60 $mm\ s^{-1}$ for the Mössbauer quadrupole splitting in good agreement with experimental values.

The study of the effects of various types of radiation on haem proteins may be useful in understanding their stability under different conditions. The photodissociation of ^{57}Fe-enriched carboxymyoglobin at temperatures between 20 and 90 K has been studied by Mössbauer spectroscopy.[712] Below 50 K the irradiation yields a stable product which is ascribed to a high-spin ferrous compound. Following photodissociation, recombination occurs. This has been

[709] B.H. Huynh, D.A. Case, and M. Karplus, *J. Amer. Chem. Soc.*, 1977, **99**, 6103.
[710] D. Bade and F. Parak, *Z. Naturforsch. Teil. C.*, 1978, **33**, 488.
[711] R.R. Sharma and R. Kolman, *J. Chem. Phys.*, 1978, **68**, 2516.
[712] Y. Maeda, T. Harami, H. Sakai, and Y. Morita, *Proc. Int. Conf. Mössbauer Spectroscopy*, 1977, **1**, 299, Documentation Office, Central Institute of Physics, Bucharest, Roumania.

studied by Mössbauer spectroscopy[713] using a constant-velocity drive set to a velocity corresponding to one of the spectral lines of the photodissociation product. By measuring the absorption intensity at this velocity as a function of time it is possible to follow the recombination process (*i.e.* the disappearance of the photodissociation product). The resulting curves obtained at low temperatures were analysed in terms of two exponentials corresponding to two activation energies. The spectra also show a decrease in the linewidth with time during the recombination process.

Huynh *et al.*[714] have investigated the oxidized (ferric) form of the haem protein cytochrome c_2 from *Rhodospirillum rubrum*, using Mössbauer spectroscopy and electron paramagnetic resonance (e.p.r.). The Mössbauer spectra were obtained in applied magnetic fields of up to 5 T. The data were analysed within the framework of the ligand-field model commonly used to evaluate low-spin ferric haem compounds. The analysis of the applied-field Mössbauer spectra shows that the determinant of the electronic g-tensor, *i.e.* the product $g_x g_y g_z$, is positive for cytochrome c_2. A similar analysis has shown this determinant to be also positive in the case of cytochromes b_5, c, and P450, and chloroperoxidase. Knowing the sign of $g_x g_y g_z$ is helpful in removing ambiguity from the interpretation of e.p.r. data in terms of ligand field theory. The sign reflects the strength of the symmetry distortions.

Mössbauer spectroscopy data is discussed in a review by Mallick *et al.*[715] on the electronic structure of iron in haemin fluoride, chloride, bromide, iodide, and hydroxide (haematin) and myoglobin fluoride and metmyoglobin. The aim is to understand the relationship between the electronic structure of these haem compounds and the biochemical effects associated with the oxygen-binding properties of myoglobin and haemoglobin (co-operativity, conformational changes, *etc.*). Mössbauer chemical-shifts, quadrupole splittings, and hyperfine fields are compared with other sources of information on hyperfine interactions and associated properties such as electron nuclear double resonance (ENDOR), *X*-ray photoelectron spectroscopy (XPS or ESCA), and magnetic resonance. Trautwein[716] considers data from Mössbauer spectroscopy and other techniques in a discussion of the electronic and magnetic structure of various iron-containing complexes, including haem compounds.

The important role of haem iron in the reversible binding of oxygen by haemoglobin and myoglobin has led to extensive studies of the electronic structures of the iron ion in oxyhaemoglobin and oxymyoglobin. The deoxy forms are now also receiving appreciable attention, both experimental and theoretical. Bacci[717] discusses the fact that previous workers have all used relatively complicated models with a large rhombic ligand-field component, the origin of which has been attributed either to the imidazole ring of the histidine amino-acid residue, which is oriented normal to the haem plane, or to some

[713] H.E. Marcolin, R. Reschke, and A. Trautwein, *Proc. Int. Conf. Mössbauer* 1977, **1**, 301, Documentation Office, Central Institute of Physics, Bucharest, Roumania.
[714] B.H. Huynh, M.H. Emptage, and E. Münck, *Biochim. Biophys. Acta* 1978, **534**, 295.
[715] M.K. Mallick, S.K. Mun, S. Mishra, J.C. Chang, and T.P. Das, *Hyperfine Interactions*, 1978, **4**, 914.
[716] A. Trautwein, *J. Mol. Structure*, 1978, **45**, 199.
[717] M. Bacci, *J. Chem. Phys.*, 1978, **68**, 4907.

inequivalence of the four nitrogen atoms in the haem plane. Instead of this the author uses a model with only $^5B_2$ and ^5E levels together with a β_1 vibrational mode for a square pyramidal Fe^{II} complex. This model gives reasonable fits to the experimental temperature dependence of the Mössbauer quadrupole splitting for both deoxyhaemoglobin and deoxymyoglobin. Curves comparing the temperature dependence of the quadrupole splitting with and without the vibronic coupling suggest that the vibrational models may play a significant role in deoxyhaemoglobin and deoxymyoglobin and possibly also in other analogous systems.

In order to investigate the intermediate-spin ferrous state ($S = 1$), which is thought to exist in a number of haem proteins, Lang *et al.*[718] have investigated the square-planar compound $\alpha, \beta, \gamma, \delta$-tetraphenylporphinatoiron^{II} (a possible analogue for proteins and enzymes which may have iron in this spin and oxidation state). Mössbauer spectra were obtained from the compound over a range of temperatures and in applied magnetic-fields. The chemical shift and essentially temperature independent quadrupole splitting are consistent with the $S = 1$, intermediate spin ferrous, assignment. The low temperature applied magnetic-field spectra are interpreted in terms of a crystal-field model involving a low-lying $^3A_{2g}$ state which is split by spin-orbit coupling into a ground-state singlet and a doublet lying 80 cm^{-1} above it.

Summerville *et al.*[719] have investigated the tricyanomethanide salt of 5,10,15,20-tetraphenylporphinato-iron(III), a six-co-ordinate iron complex with iron in the intermediate-spin ferric state, which may be a suitable model compound for certain haem proteins in which iron is in this state. The Mössbauer spectrum obtained from this complex at 298 K is a quadrupole-split doublet with a chemical shift of 0.30 mm s^{-1} and a quadrupole splitting of 3.18 mm s^{-1}, parameters consistent with the $S = \frac{3}{2}$ assignment.

In a ^{57}Co emission Mössbauer study[720] spectra were obtained from Co^{II} octaethylporphyrin ($Co^{II}OEP$) and its pyridine and oxygen adduct, $CO^{II}OEP.Py.O_2$, in frozen solution at 20 K. The spectra observed in such an emission experiment are from the ^{57}Fe to which the ^{57}Co decays. Two chemical species of $Fe^{II}OEP$ were observed, while the adduct appears to exist as both $Fe^{II}OEP.Py.O_2$ and $Fe^{II}OEP.Py_2$. The complex with oxygen is changed to the bis-pyridine adduct by evacuation at about −30 °C.

Dolphin *et al.*[721] have investigated a series of high-spin ferric porphyrin complexes Fe(P)Cl, FE(P)OMe, and $[Fe(P)]_2$ where P = octaethylporphyrin or tetraphenylporphyrin. Mössbauer spectra obtained at 4.2 K and in applied magnetic fields of up to 5 T were analysed using a spin-Hamiltonian to give the crystal-field splittings and the magnetic hyperfine interaction and quadrupole interaction parameters. In each of the monomeric complexes the hyperfine field is large, approximately 50 T, the quadropole splitting is positive, and the e.f.g. has effectively axial symmetry, but the zero-field splittings of the methoxides are substantially smaller than those of the chlorides. In the dimers

[718] G. Lang, K. Spartalian, C.A. Reed, and J.P. Collman, *J. Chem. Phys.*, 1978, **69**, 5424.
[719] D.A. Summerville, I.A. Cohen, K. Hatano, and W.R. Scheidt, *Inorg. Chem.*, 1978, **17**, 2906.
[720] H. Sakai, Y. Maeda, H. Ogoshi, H. Sugimoto, and Z. Yoshida, *Chem. Letters*, 1978, **4**, 353.
[721] D.H. Dolphin, J.R. Sams, T.B. Tsin, and K.L. Wong, *J. Amer. Chem. Soc.*, 1978, **100**, 1711.

the two high-spin Fe^{3+} ions are antiferromagnetically coupled to give a non-magnetic spin singlet ground-state. The temperature dependence of the spectra indicate slight relaxation broadening in the case of the dimeric complexes.

Mössbauer spectroscopy has been used in a study of complexes of ferri-porphyrins with histidine-containing dipeptides.[722]

In a series of very low temperature Mössbauer measurements by Groves *et al.*[723—725] a transition to a magnetically ordered state was observed in haemin at about 0.13 K. The high-spin ferric ions in crystalline haemin are sufficiently close together for exchange interactions to produce magnetic ordering at sufficiently low temperatures. At 4.2 K the Mössbauer spectrum is a quadrupole-split doublet which begins to broaden below 0.2 K, with the full six-line magnetic hyperfine pattern being observed at 0.08 K. They are marked line-broadening effects, even at temperatures well below the ordering temperature, which are attributed to relaxation behaviour.

The haemochromes are a group which provide simple models of known structure for several ferrous haem proteins. James *et al.*[726] have obtained Mössbauer spectra from a series of bis(piperidine)-haemochromes and carbonyl-piperidine-haemochromes. The data suggest that replacement of the axial amine by CO causes pronounced changes in the Fe–porphyrin bonding, *i.e.* a substantial *cis*-effect. From this work it appears that the porphyrins act as 'electron sinks' whereby they are able to modify their σ- and π-bonding characteristics towards iron in order to accommodate the requirements of the axial ligands. Such an ability may well be partly responsible for the diverse functions in which haem proteins participate in biological functions. In this investigation of a series of closely related iron(II) porphyrins the reactivity of the various members is correlated with the differences in their Mössbauer parameters and the corresponding differences in their electronic structure.

Cytochrome oxidase has an important function as the terminal oxidation–reduction enzyme in mitochondrial respiration but it is as yet little understood. Latest evidence suggests that it contains a haem group coupled to copper *via* an imidazole bridge. Petty and Wilson[727] have prepared a possible model compound for this enzyme which contains a binuclear iron–copper site with a bipyrimidine bridge. Its usefulness as an analogue for the electronic structure of the cytochrome oxidase active-site is discussed in terms of Mössbauer spectroscopy, e.p.r., magnetic, and electrochemical measurements. The lack of any temperature dependence of the Mössbauer spectrum indicates that the coupling between the Fe^{2+} and Cu^{2+} ions does not lead to any spin-equilibrium effects.

[722] V.N. Luzgina, E.I. Filippovich, A.F. Vanin, R.P. Evstigneeva, and L.I. Fainshtein, *Zhur. obshchei Khim.*, 1977, **47**, 2115; *J. Gen. Chem.* 1977, **47**, 1938.

[723] J.L. Groves, A.J. Becker, L.M. Chirovsky, W.P. Lee, and C.S. Wu, *Hyperfine Interactions*, 1978, **4**, 930.

[724] G.W. Wang, J.L. Groves, A.J. Becker, L.M. Chirovsky, W.P. Lee, T.E. Tsai, and C.S. Wu, *Hyperfine Interactions*, 1978, **4**, 910.

[725] G.W. Wang, L.M. Chirovsky, W.P. Lee, A.J. Becker, and J.L. Groves, *Nuclear Instrum. Methods.*, 1978, 155, 273.

[726] B.R. James, J.R. Sams, T.B. Tsin, and K.J. Reimer, *J.C.S. Chem. Comm.*, 1978, **17**, 746.

[727] R.H. Petty and L.J. Wilson, *J.C.S., Chem. Comm.*, 1978, **11**, 483.

Iron–Sulphur Proteins.—Werber *et al.*[728] have carried out Mössbauer spectroscopic measurements on a two-iron ferredoxin isolated from the extreme halophile, *Halobacterium* of the Dead Sea. The spectrum of the protein in the oxidized state consists of two overlapping quadrupole-split doublets of equal intensity corresponding to two inequivalent Fe^{3+}-ions. In the reduced form the spectrum consists of two well resolved doublets corresponding to an Fe^{3+}–Fe^{2+} pair of ions. The spectra and parameters are generally similar to what has been observed in other two-iron ferredoxins although the degree of inequivalence of the Fe^{3+} ions in the oxidized protein is somewhat greater than in other two-iron ferredoxins. Mössbauer measurements on the whole bacterium gave considerably different spectra from those of the isolated ferredoxin, indicating that there are appreciable quantities of other iron-containing components present.

The degree of inequivalence between the iron atoms of the iron–sulphur proteins having four-iron active-centres has been of interest for some time. Middleton *et al.*[729] have obtained Mössbauer spectra from both oxidized and reduced forms of the four-iron ferredoxin from *Bacillus stearothermophilus*, and have analysed them using computer fits to theoretical spectra obtained from a spin-Hamiltonian. The fact that a consistent set of parameters was obtained from fits to spectra taken over a wide range of temperature and applied magnetic-field can be regarded as a good indication of the reliability of the fitting procedure. The results lead to a model for the oxidized centre in which all four iron atoms have the same valence, intermediate between ferric and ferrous, with one pair spin-up and the other pair spin-down. On reduction the extra electron goes predominantly to one pair of iron atoms which become ferrous with the other pair remaining substantially unchanged. While this model has been previously proposed on the basis of a more qualitative interpretation of the spectra of a number of these proteins, the present quantitative evidence leaves little doubt that the essential features of this model must be incorporated into any description of the ground-state electronic structure of the four-iron centre. Using the above model the authors obtained relationships between the spin-Hamiltonian parameters for individual iron atoms and those for the coupled centre, which may give insight into the relationship between the e.p.r. and Mössbauer spectra.

Mössbauer measurements carried out on tetrakisthiophenolate iron(II) between 4.3 and 19 K in applied magnetic fields of up to 6 T confirm the validity of this complex as a reduced rubredoxin analogue.[730]

Calculations have been made of the ground and excited states of both redox states of $Fe(SH)_4$,[731] an idealized and simplified model of the active-site of rubredoxin, which is basically similar to some of the models that have been synthesized. The calculations establish that the ground state is high-spin in

[728] M.M. Werber, E.R. Bauminger, S.G. Cohen, and S. Ofer, *Biophys. Struct. Mechanism*, 1978, **4**, 169.

[729] P. Middleton, D.P.E. Dickson, C.E. Johnson, and J.D. Rush, *European J. Biochem.*, 1978, **88**, 135.

[730] A. Simopoulos, N. Sfarnas, A. Kostikas, and D. Coucouvanis, *Proc. Int. Conf. Mössbauer Spectroscopy*, 1977, **1**, 311, Documentation Office, Central Institute of Physics, Bucharest, Roumania.

[731] R.A. Bair and W.A. Goddard, *J. Amer. Chem. Soc.*, 1978, **100**, 5669.

both redox forms, in agreement with what is observed in both the protein and the synthetic models. The calculated charge distribution leads to a value of –0.235 for the ratio of the Mössbauer quadrupole-splittings in the oxidized and reduced states, in good agreement with the experimental values of approximately –0.23 for the protein and approximately –0.17 for the model compounds.

Wong *et al.*[732] have made Mössbauer spectroscopy and magnetic-susceptibility measurements on the compounds $(CpFeS)_4$, $(CpFeS)_4PF_6$, and $(CpFeS)_4(PF_6)_2$ which contains the cubane-cluster anions tetrathioxotetra (η^5-cyclopentadienyl) tetra-iron$^{0,1+,2+}$. The compounds represent possible models for the various redox states of the four-iron centre of certain iron–sulphur proteins.

The Mössbauer parameters indicate high electron density at iron due to the bond with the electron-rich cyclopentadienyl ligand. Mössbauer measurements in applied magnetic fields indicate that the unpaired spin in $(CpFeS)_4PF_6$ does not interact with the Fe-sites and occupies a ligand-based molecular orbital. The redox electrons fill metal-ligand orbitals which are predominantly of ligand nature.

The enzyme nitrogenase is known to contain iron, molybdenum, and inorganic sulphur. One possibility is that some of the active-centres in nitrogenase are modified forms of a four-iron iron–sulphur protein centre, with a molybdenum atom replacing one of the iron atoms. Wolff *et al.*[733] have investigated the compound $(Et_4N)_3[Mo_2Fe_6S_9(SEt)_8]$ which contains two $MoFe_3S_4$ cubane-type clusters which may be a possible analogue for the active-centres found in nitrogenase. The low-temperature Mössbauer spectra show magnetic hyperfine interactions indicative of a slowly relaxing paramagnetic ground-state.

Iron-storage Proteins.—Ferritin is an iron-storage protein which contains a concentrated inorganic core of iron in a protein envelope. The core consists predominantly of FeOOH with varying amounts of phosphate. The size of the cores can be determined from the temperature dependence of the Mössbauer spectra as a result of superparamagnetic behaviour. In a Mössbauer study[734, 735] of natural ferritin, and ferritin reconstituted in the presence and absence of phosphate, the spectra were analysed to give the distribution of core sizes. The results show that natural ferritin has a larger contribution from smaller sized cores, while the ferritin reconstituted in the presence of phosphate has a narrower distribution of core sizes centred on a smaller mean diameter.

[732] H. Wong, D. Sedney, W.M. Reiff, R.B. Frankel, T.J. Meyer, and D. Salmon, *Inorg. Chem.*, 1978, **17**, 194.

[733] T.E. Wolff, J.M. Berg, C. Warrick, K.O. Hodgson, R.H. Holm, and R.B. Frankel, *J. Amer. Chem. Soc.*, 1978, **100**, 4630.

[734] J.M. Williams, D.P. Danson, and C. Janot, *Phys. Med. Biol.*, 1978, **23**, 835.

[735] D.P. Danson, J.M. Williams, and C. Janot, *Proc. Int. Conf. Mössbauer Spectroscopy*, 1977, **1**, 307, Documentation Office, Central Institute of Physics, Bucharest, Roumania.

8 Dissociation and Association of Proteins

Contributed by E.J. Wood

Analytical Ultracentrifugation.—*Techniques.* A very useful compilation has appeared containing articles on the investigation of enzyme structure by ultracentrifugion and related methods.[736] The first half of the book deals with the determination of molecular weight by sedimentation equilibrium, osmometry, and detergent-gel electrophoresis, as well as the determination of partial specific volumes and dry weights. The second half of the book is concerned with methods for the investigation of interacting systems and contains articles on the practical aspects of obtaining and evaluating data from transport experiments and from the kinetics and equilibrium binding of small molecules by proteins. In addition to this volume, a number of articles have appeared offering procedures and detailed assistance to the non-expert user of the ultracentrifuge. Amongst these may be mentioned a simple procedure that can be used to test for convection in the ultracentrifuge cell and the steps that can subsequently be taken to avoid it.[737] Another group reports a method to help in the alignment of the Rayleigh optical system.[738] Such alignment is essential for high precision work and the method involves the construction of a simple, externally adjustable, motor-driven slit assembly for the Beckman Model E ultracentrifuge. Wei and Deal[739] have described a simple, fast, and reliable width-method for evaluating diffusion coefficients from the spreading of dc/dx *vs.* c curves with time. Its major advantage is claimed to be that it allows use of virtually all of the available data instead of only one height and one width from each curve. However, it is unlikely to find a great deal of use in the investigation of interacting protein systems where the technique of quasi-elastic light-scattering offers so much more precision and speed (see Bloomfield and Lim).[736]

Several groups have developed further the methods for the automatic acquisition and analysis of ultracentrifuge data, and much of the emphasis has been on (*a*) low cost, and (*b*) using commercially available data-handling systems. Thus Kim *et al.*[740] describe a system for collecting data from the commercially-available Beckman Model E scanner system in which the output is digitized and then stored on digital cassette tape, to be processed later on any suitable computer. They tested both a commercial and an inexpensive, home-built digitizer. Another group[741] used a Hewlett–Packard 9810A desk-top computer in conjunction with a digital voltmeter linked to the output of the Model E scanner and a magnetic tape cassette unit for data storage. With this system it was possible to process both sedimentation velocity and sedimentation equilibrium data and algorithms were developed for handling data characterized by a relatively high level of noise.

[736] Methods in Enzymology, ed. C.H.W. Hirs and S.N. Timasheff, 1978, Vol. 48, 'Enzyme Structure, Part F,' Academic Press, New York and London.
[737] D.B. Millar and J.P. Christopher, *Analyt. Biochem.*, 1978, **90**, 33.
[738] W.F. Stafford, *Analyt. Biochem.*, 1978, **88**, 104.
[739] G.J. Wei and W.C. Deal, *Analyt. Biochem.*, 1978, **87**, 433.
[740] C.S. Kim, G. Moross, W. Moyer, G. Kaufman, R. MacColl, and M.R. Edwards, *Analyt. Biochem.*, 1978, **86**, 371.
[741] D. Inners, S.H. Tindall, and K.C. Aune, *Analyt. Biochem.*, 1978, **87**, 477.

The availability of an air-driven, table-top high-speed preparative ultracentrifuge, the Beckman Airfuge®, stimulated Schachman and his colleagues to develop a method for molecular weight determination by an equilibrium, depletion technique.[742] The rotor holds six tubes of ~100 μl capacity and produces centrifugal fields of up to 160 000 × g. It was shown experimentally that the logarithm of the fraction of protein or macromolecule remaining in the upper 40 μl of the sample solution at the end of a run varied almost linearly with its molecular weight. Calibration with a series of macromolecular species of known molecular weight allowed the determination of the molecular weights of unknowns in the range 1—15 × 10^4 daltons. Only small amounts of material were required and accuracies of about 10 % were achieved.

A model E ultracentrifuge fitted with scanner optics has been used by Pestka and Luk[743] to obtain isokinetic patterns of moving zones by monitoring a fixed point in the analytical cell as a function of time. Sedimentation coefficients could be calculated by simple ratio if the value for any component in a mixture were known. O'Brien's group has re-investigated the methods for sedimentation coefficient determination by sucrose density centrifugation with special reference to detergent-extracted membrane-bound proteins.[744] They found that the method of Martin and Ames[745] gave the most accurate results for $s_{20,w}$ when both standards and unknowns had the same $\bar{v}$, and when the gradient was made in water or dilute buffer solutions. For all other cases they give an alternative procedure. Prusinier[746] has described a general approach for the design of an initial purification procedure for any biological particle by means of a refinement of the technique[747] whereby a series of centrifugations in fixed-angle rotors is used to obtain data on the sedimentation behaviour of the particle.

Theory. Rowe[748] has re-investigated the problem of the concentration dependence of transport processes such as sedimentation, and gives a simple general treatment applicable to limiting concentrations in the absence of charge effects or other forms of specific interactions between solute particles. The treatment initially assumes that at very low concentrations, the velocity of sedimenting particles relative to solvent (*i.e.* bulk solvent adequately removed from the immediate vicinity of the particles) is unchanged by varying the particle concentration. The problem of predicting the velocity (which of necessity is measured in a cell-fixed frame of reference) at particle concentration c then resolves itself into one of finding a procedure for the transposition of velocities between the two frames of reference, the cell-fixed and the volume-fixed (*i.e.* relative to solvent). This is done by considering the volume flux associated with particle migration, without reference to whether this corresponds to movement of particles, solvent, or both, and specifying in a solvent-fixed frame of

[742] M.A. Bothwell, G.J. Howlett, and H.K. Schachman, *J. Biol. Chem.*, 1978, **253**, 2073.
[743] S. Pestka and D. Luk, *Analyt. Biochem.*, 1978, **89**, 178.
[744] R.D. O'Brien, C.A. Timpone, and R.E. Gibson, *Analyt. Biochem.*, 1978, **86**, 602.
[745] R.G. Martin and B.N. Ames, *J. Biol. Chem.*, 1961, **236**, 1372.
[746] S.B. Prusinier, *J. Biol. Chem.*, 1978, **253**, 916.
[747] N.G. Anderson, *Analyt. Biochem.*, 1967, **23**, 72.
[748] A.J. Rowe, *Biopolymers*, 1977, **16**, 2595.

reference using (*a*) the frictional properties, and (*b*) the sedimentation properties, of the system. Progress has been made with the practical application of the method to interacting systems such as the self-association of myosin and of α-chymotrypsinogen.[749] In another theoretical study Van Holde and Weischet[750] present a method which allows the calculation of a distribution of sedimentation coefficients from the boundaries of sedimentration-velocity experiments with mono- or pauci-disperse solutes. For two solutes, differences in sedimentation coefficient as small as 20 % could be resolved, and in the absence of heterogeneity and concentration-dependence effects it was also possible to obtain reasonably accurate values for diffusion coefficients.

Applications. In a study of detergent-extracted acetylcholine receptor protein from *Torpedo* electric tissue it was found that two molecular weight species could be separated by sucrose density-gradient centrifugation.[751] The molecular weights of these were determined, without prior determination of the extent of detergent bound to them, by adjusting the solvent density with ^2H_2O in order to blank out the contribution of bound detergent to the sedimentation potential. The two appeared to be related as monomer and dimer with mol. wt. 250 000 and 500 000, sedimentation coefficients 8.6S and 12.8S, and Stokes radii 7.3 nm and 9.5 nm. Another monomer–dimer system to be investigated was that of bacterial α-amylase.[752] The system was shown by equilibrium and sedimentation velocity to be in equilibrium, with an association constant of 2.4×10^3 M^{-1}. It was also shown that Zn^{2+} bound exclusively to the dimer. In a further examination of the acid dimerization of α-chymotrypsin, chemically modified derivatives were investigated.[753] For example blocking the carboxy-group of Tyr-146 or methylation of His-57 completely eliminated the ability to dimerize. It was concluded that dimerization in solution was best explained by electrostatic interaction between these two side-chains, but that the interactions in the crystal form might well be different.

The interactions between purified ribosomal proteins continue to attract much interest. Neither protein S6 nor S18 from the *E. coli* 30S ribosomal subunit tended to self-associate over the concentration range studied, but it was shown by sedimentation equilibrium that when mixed together they interacted with an association equilibrium constant of 6.6×10^4 M^{-1} at 3 °C.[754] The sedimentation equilibrium data were collected and processed by the method described in ref. 741. Sedimentation equilibrium continues to be a valuable technique for the study of the self-association of apo-lipoprotein.[755] Both native and reduced apo-lipoproteins A-II from human high density lipoprotein underwent reversible self-association and this process was independent of temperature between 5 and 30 °C. As previously reported the native protein appeared to exist in a monomer–dimer–trimer equilibrium, but after reduction

[749] C.H. Emes and A.J. Rowe, *Biochim. Biophys. Acta*, 1978, **537**, 110 and 125.
[750] K.E. van Holde and O.W. Weischet, *Biopolymers*, 1978, **17**, 1387.
[751] J.A. Reynolds and A. Karlin, *Biochemistry*, 1978, **17**, 2035.
[752] R. Tellam, D.J. Winzor, and L.W. Nichol, *Biochem. J.*, 1978, **173**, 185.
[753] M.J. Gorbunoff, G. Fosmire, and S.N. Timasheff, *Biochemistry*, 1978, **17**, 4055.
[754] V. Prakash and K.C. Aune, *Arch. Biochem. Biophys.*, 1978, **187**, 399.
[755] T.-L. Teng, D.L. Barbeau, and A.M. Scanu, *Biochemistry*, 1978, **17**, 17; S. Formisano, H.B. Brewer, and J.C. Osborne, *J. Biol. Chem.*, 1978, **253**, 354.

by dithiothreitol the patterns of self-association were better described as a monomer–dimer–tetramer equilibrium. The apparent weight average molecular weight for apolipoprotein A-I varied with protein concentration, but also M_w^{app} increased with ionic strength and decreased with rotor speed, the latter indicating that the system was pressure-sensitive. Although the volume changes found with associating protein systems are usually small, these changes can result in quite large effects at the pressures generated in the ultracentrifuge (*e.g.* > 150 atm. at the bottom of the cell). From a study of the effects of pressure it was concluded that there was a change in $\bar{v}$ upon association of 5.5×10^2 ml g^{-1} and that this being the case the sedimentation equilibrium data could be interpreted in terms of a monomer–dimer–tetramer–octomer system.

Gel Filtration.—Although a wide variety of pH ranges and salt concentrations have been suggested for the separation of macromolecules by gel filtration on agarose gels, a recent study suggests that Sepharose gel particles undergo major morphological changes at high salt concentrations and at acidic pH values. Efficiency of separation is therefore likely to be adversely affected under these conditions and Lin and Castell[756] provide evidence that the best separations are to be achieved near neutral pH and at low salt concentrations. In addition to separation, gel filtration can also be used to study ligand binding. The strategy of using gel filtration to detect and purify binding proteins has been investigated.[757] The method involves gel filtration of, say, a cell-free extract containing one or more proteins that will complex non-covalently a given ligand, with labelled ligand. If label emerges in a sharp peak together with protein, then the ligand is assumed to be protein-bound. However, a negative result does not necessarily show that binding protein is absent: a positive result will only be obtained if the complex has a small dissociation constant or dissociates slowly. A simple model of zonal gel filtration was analysed numerically to establish how the capacity of a protein–ligand complex to survive intact on the column depends on experimental variables and on the kinetic characteristics of the complex.

The association–dissociation equilibrium of the bifunctional enzyme from *E. coli,* aspartokinase I–homoserine dehydrogenase I was investigated by a gel filtration technique.[758] A computer-controlled single photon counting spectrophotometer was used to monitor at 215 or 220 nm the equilibrium gel permeation of the enzyme on Sephadex G-200. The data suggested that there was a dimer–tetramer equilibrium with $K_{ass} = 2.6 \times 10^7$ M^{-1}, and both dimer and tetramer, molecular radii 53.8 Å and 70 Å, possessed dehydrogenase activity. Treatment of the complex with chaotropic salts generated a monomeric form devoid of activity and having a mol. wt. ~80 000 (see also ref. 749). The same spectrophotometer was also used to study the self-association of haemoglobin β-chains into tetramers.[759] The tetramers bound oxygen with a higher affinity than did the dissociated chains, implying the existence of at least

[756] A.W.S.M. Lin and D.O. Castell, *Analyt. Biochem.*, 1978, **86**, 345.
[757] I.A. Nimmo and A. Bauermeister, *Biochem. J.*, 1978, **169**, 437.
[758] L.P. Vickers, G.K. Ackers, and J.W. Ogilvie, *J. Biol. Chem.*, 1978, **253**, 2155.
[759] R. Valdes and G. K. Ackers, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 311.

two conformational states for the β-chains. In contrast the self-association of α-chains into dimers was not linked to oxygenation.

The technique of using matrix-bound subunits of proteins to investigate association–dissociation properties was used by Bickerstaff and Price to study rabbit-skeletal muscle creatine kinase,[760] and by Takahashi and Gross to study the self-association of the light-harvesting chlorophyll *a/b* protein from chloroplast membranes.[761]

Light Scattering.—Few studies have appeared in which conventional light-scattering techniques have been used, but in contrast, transport methods coupled with laser light-scattering experiments have found considerable usage. Künzler and Lustig[762] have pointed out the advantages of such a combination of comparatively rapid methods for the characterization of large, fragile particles such as the metastable structures involved in virus assembly. In addition to using conventional light-scattering with a modified form of Zimm plot to obtain radii of gyration and external radii of various pre-heads, they determined molecular weights and partial specific volumes simultaneously by measuring sedimentation and diffusion coefficients in buffers containing different amounts of ^2H_2O. Diffusion coefficients and Stokes radii were determined by quasi-elastic light-scattering. In a somewhat similar way Kunitake *et al.*[763] studied the molecular weight distribution in the very low density lipoprotein fraction from serum by first performing a density-gradient flotation experiment and then scanning the centrifuge tube with a laser. The molecular weight distribution was calculated from the intensity of scattered light at points down the tube, and in addition, the diffusion coefficient distribution was derived from the intensity fluctuations of the scattered laser light. From the data it was possible to construct the distribution of molecular weights, buoyant densities, and frictional coefficients for the lipoproteins in the polydisperse sample.

By combining transport by electrophoresis with laser light-scattering, Haas and Ware[764] studied the dissociation of human carboxyhaemoglobin tetramers at pH values above 10. The data were interpreted in terms of an increase in net electrical charge on the dimer of at least 2.8—4.4 upon dissociation. It was shown by photon correlation spectroscopy that the translational diffusion coefficients of oxyhaemoglobins A and S were identical over the concentration range 0.1—37 g dl^{-1}, and there was no evidence for the aggregation of HbS at high concentrations.[765] Kam *et al.*[766] have made a systematic study using quasi-elastic light-scattering of the factors controlling crystal growth in proteins. Successful crystallization depends on the competition between the growth rates for compact three-dimensional structures and long-chain forms which lead to an amorphous precipitate. The size and shape distribution of small aggregates

[760] G.F. Bickerstaff and N.C. Price, *Biochem. J.*, 1978, **173**, 85.
[761] M. Takahashi and E.L. Gross, *Biochemistry*, 1978, **17**, 806.
[762] P. Künzler and T. Hohn, *J. Mol. Biol.*, 1978, **122**, 191; P. Künzler and A. Lustig, *ibid.*, 1978, **122**, 121.
[763] S.T. Kunitake, E. Loh, V.N. Schumaker, S.K. Ma, C.M. Knobler, J.P. Kane, and R.L. Hamilton, *Biochemistry*, 1978, **17**, 1936.
[764] D.D. Haas and B.R. Ware, *Biochemistry*, 1978, **17**, 4946.
[765] C.R. Jones, C.S. Johnson, and J.T. Penniston, *Biopolymers*, 1978, **17**, 1581.
[766] Z. Kam, H.B. Shore, and G. Feher, *J. Mol. Biol.*, 1978, **123**, 539.

of lysozyme were monitored during the pre-nucleation stage. The advantage of this procedure is its speed compared with the present, empirical approach to crystal growing where it is often necessary to wait weeks or months before the effect of changing one of the controlling parameters can be observed by visual inspection.

Laser-Raman spectroscopy was used to study the structure of the capsid of turnip yellow mosaic virus.[767] The frequencies and intensities of the bands in the Raman spectrum are influenced by the secondary structure of macromolecular, hydrogen-bonding interactions, and the environments of the various molecular subgroups. It was shown that the secondary structure of coat-protein molecules was comprised of 9 ± 5 % α-helix, 43 ± 6 % β-sheet, and 48 ± 6 % irregular conformation and was not altered by the removal of the RNA from the capsid.

Turbidity measurements seem to be the most favoured method of investigating assembly to macrostructures such as microtubules and sickle cell haemoglobin fibres, and many such studies have appeared. These include the demonstration of a delay time in deoxy-HbS polymerization in concentrated phosphate buffer,[768] promotion of tubulin polymerization by guanosine 5′-methylene diphosphonate, a GDP analogue which is resistant to hydrolysis,[769] the effect of non-tubulin proteins and polycations (*e.g.* polylysine) on tubulin polymerization,[770] nucleation and elongation of tubulin polymerization in the presence of GDP and GTP,[771] and the effect on polymerization of blocking sulphydryl groups in tubulin.[772]

Transport Studies.—Previous studies with the lectin, concanavalin A, have been extended using the analytical band—sedimentation technique.[773] This technique can be used down to initial concentrations of 0.2 ng ml^{-1} and is thus more sensitive to molecular dispersity than boundary sedimentation. Solutions of protein, or buffer, were layered on to buffer in each of the sectors of a double-sector cell. Centrifugation was performed at several different temperatures in a Beckman model E instrument fitted with a split-beam scanner interfaced to a computer. The concanavalin dimer–tetramer equilibrium was analysed by comparing the band-sedimentation results with predictions for a dimer–tetramer model system. Over the concentration range investigated the polymerization reaction was temperature dependent, and for 4 °C $\leq t \leq$ 23 °C, K_{eq} varied from 1.9×10^4 M^{-1} to 6.9×10^5 M^{-1}. It was concluded from the results obtained that the two dimers associate through a large, hydrophobic binding-region and this conclusion was supported by crystallographic evidence.

Another interesting example of subunit interaction between dimers is that of the abnormal human haemoglobin, Hb Hirose. This has a serine in place of

[767] K.A. Hartman, P.E. McDonald-Ordzie, J.M. Kaper, B. Prescott, and G.J. Thomas, *Biochemistry*, 1978, **17**, 2118.
[768] K. Adachi and T. Asakura, *J. Biol. Chem.*, 1978, **253**, 6641.
[769] I.V. Sandoval, J.L. Jameson, J. Niedel, E. MacDonald, and P. Cuatrecasas, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3178.
[770] J.C. Lee, N. Tweedy, and S.N. Timasheff, *Biochemistry*, 1978, **17**, 2783.
[771] M.F. Carlier and D. Pantaloni, *Biochemistry*, 1978, **17**, 1908.
[772] Y. Ikeda and M. Steiner, *Biochemistry*, 1978, **17**, 3454.
[773] M. Huet and J.M. Claverie, *Biochemistry*, 1978, **17**, 236.

tryptophan at position C-3 (37)β, and was studied by sedimentation velocity and gel chromatography.[774] Under conditions where liganded haemoglobin A was largely tetrameric, Hb Hirose was essentially all dissociated into $\alpha\beta$ dimers, with a tetramer–dimer dissociation constant of about 1.2 μM. This latter value is 10^6-fold greater than that for deoxy-HbA, and is similar to that for oxy-HbA. Active enzyme sedimentation and sedimentation equilibrium were used to study malate dehydrogenase from both cytoplasm and mitochondria.[775] No changes in the kinetic parameters of the enzymes were observed over a wide range of enzyme concentrations, and no evidence of dissociation of the dimer was found over the same range under the conditions of measurement of kinetic activity.

Sedimentation velocity experiments in conjunction with electron microscopy are important in elucidating the mechanisms of polymerization of such macrosystems as virus coats and microtubules. Such studies are relevant in deciding for example, the extent to which the nucleic acid controls the size and shape of the protein coat. Thus in most strains of alfalfa mosaic virus the cylindrical virus particles may be classified into a small number of length classes each comprising one type of RNA. These studies have been extended by Driedonks *et al.*[776] in terms of a model with distinct nucleation and elongation steps. Irrespective of the type of nucleic acid present, coat-protein polymerization was a co-operative process.

An extensive investigation of tubulin polymerization using sedimentation velocity and other methods has appeared.[777] The oligomeric species were characterized and the conditions for their existence defined: thus the 30S to 6S mass ratio varied with total protein concentration, suggesting that they existed in equilibrium. The 6S species was favoured at low pH, the 30S oligomer at pH 6.5—7.4, and at pH 7.4 increasing amounts of the 18S oligomer were formed. High salt concentrations had a (reversible) depolymerizing effect. The next step was to investigate the structure of the ring-shaped oligomers and to define the conditions, including the presence of non-tubulin proteins, for ring formation. In fact, non-tubulin factors (high molecular weight proteins and the *tau* proteins) were found to be active in promoting ring-formation. The high molecular weight proteins promoted 30S oligomer formation, while the *tau* protein promoted the formation of a 20S oligomer and the data indicated that the 20S species was a single ring and that the 30S species was a two-layered ring bearing projections of high molecular weight protein. It also seemed that the organization in the ring was conserved in the microtubule. Fixation of the 30S oligomers with glutaraldehyde allowed them to be visualized by electron microscopy. This allowed refinement of the model for the structure as a helix of 29 tubulin dimers and 5 high molecular weight proteins. In sedimentation

[774] J. Sasaki, T. Imamura, T. Yanase, D.H. Atha, A. Riggs, J. Bonaventura and C. Bonaventura, *J. Biol. Chem.*, 1978, **253**, 87.

[775] C. Frieden, J. Honegger and H.R. Gilbert, *J. Biol. Chem.*, 1978, **235**, 816.

[776] R.A. Driedonks, P.C.J. Krijgsman, and J.E. Mellema, *J. Mol. Biol.*, 1978, **124**, 713; *European J. Biochem.*, 1978, **82**, 405.

[777] J.M. Marcum and G.G. Borisy, *J. Biol. Chem.*, 1978, **253**, 2825; R.B. Vallee and G.G.Borisy, *ibid.* p. 2834; R.B. Scheele and G.G. Borisy, *ibid.*, p. 2846; J.M. Marcum and G.G. Borisy, *ibid.*, p. 2852.

experiments in which the hydrostatic pressure was increased by increasing rotor velocity and overlayering with oil, it was observed that depolymerization of the 30S oligomer took place. This indicated that the polymerization of the 6S protein to form oligomer was accompanied by a positive volume change. This effect was used to demonstrate the existence of a rapidly reversible equilibrium between the components. At a simpler level, the reversible dissociation of the $\alpha\beta$ dimer of tubulin was studied by sedimentation equilibrium and sedimentation velocity measurements.[778]

Electron Microscopy.—Much work is being done on the assembly of viruses and electron microscopy is obviously one of the most valuable techniques in this field. The assembly process is highly ordered and organized and involves the participation of nucleic acids as well as protein–protein interactions. Assembly is regulated by the assembly pathway rather than the order of transcription or translation, and, in addition it seems that some steps require the intervention of proteins that act as tools or catalysts but which are not incorporated into the completed virus. Bacteriophage T4 assembly, for example, proceeds *via* a prehead consisting of a shell and a core. The latter is required for head assembly but is not part of the final head. Protein P20, a minor but essential prehead protein, directs the assembly of core proteins to give a structure like the core inside a prehead.[779] If P20 is not present, then core proteins assemble to irregular aggregates. The kinetics of T4 head assembly have been studied,[780] and *X*-ray diffraction results have been used to assess the extent of distortion artefacts associated with specimen preparation for electron microscopy.[781] The assembly of tobacco mosaic virus protein has been reviewed.[782]

Electron microscopic techniques have been applied to the elucidation of the arrangement of the α and β-subunits of tubulin in microtubules. Image processing of negatively stained zinc-induced sheets showed an 82 Å repeat, and it was possible to visualize the arrangement and shapes of the two-heterodimers contained in each unit-cell.[783] However, these zinc-induced sheets differ from microtubule precursor sheets in their lattice parameters. Improved negative staining methods were used by Crepeau *et al.*[784] to show that the two types of subunit alternate along individual protofilaments in microtubules themselves.

High-resolution electron microscopy was used to show that in the iron-containing protein, ferritin, there was a strong attachment of the mineralized iron to the polymerized protein subunits.[785] Following procedures which damaged the molecule (*e.g.* freezing and thawing, boiling and cooling), small portions of the disrupted shells remained closely apposed to their core crystallites.

[778] H.W. Detrich and R.C. Williams, *Biochemistry*, 1978, **17**, 3900.
[779] R. van Driel and E. Couture, *J. Mol. Biol.*, 1978, **123**, 713.
[780] M.K. Showe and L. Onorato, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4165.
[781] W.C. Earnshaw, J. King, and F.A. Eiserling, *J. Mol. Biol*, 1978, **122**, 247.
[782] P.J.G. Butler and A.C.H. Durham, *Adv. Protein Chem.*, 1977, **31**, 188.
[783] T.S. Baker and L.A. Amos, *J. Mol. Biol.*, 1978, **123**, 89.
[784] R.H. Crepeau, B. McEwan, and S.J. Edelstein, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 5006.
[785] W.H. Massover, *J. Mol. Biol.*, 1978, **123**, 721.

Several enzyme complexes were studied by electron microscopy, including acetyl-CoA carboxylase which showed filamentous structures,[786] branched chain α-keto acid dehydrogenase complex in which the dihydrolipoyl transacylase part had the appearance of a cube with dehydrogenase molecules distributed over its surface,[787] and yeast fatty acid synthetase which existed as two types of ovate structure, one consisting of a 'plate' with four arch-like subunits and one of a 'plate' with six 'arches'.[788]

Immuno-electron microscopy was used along with computerized multidimensional scaling to construct a three-dimensional model of the arrangement of the proteins in the 30S ribosomal subunit.[789] Dimers between ribosomal subunits were constructed by using antibodies bivalent for a single ribosomal protein. The location of the antigenic site was then located by negative staining techniques.

Kinetic Studies.—*Protein–Protein Interactions.* The mechanism by which *E. coli* arginine decarboxylase dissociates from decamer to dimer was investigated by a stopped-flow method.[790] The dimer contains a sulphydryl group which is not available in the decamer and which was detected by reaction with 5,5′-dithiobis (2-nitrobenzoic acid) and following the increase in absorbance at 412 nm. It was found that dissociation involved (*a*) the binding of one Na^+ ion, (*b*) dissociation of one proton, and (*c*) irreversible dissociation of subunits, in that order. The dissociation process occurred in stages. In a second, theoretical paper[791] possible combinations of these events leading to the experimental observations were set out.

Wodak and Janin[792] have examined protein–protein interactions by a computer procedure using the system pancreatic trypsin-inhibitor–trypsin as a model. A simplified model was presented in which each residue is replaced by one interaction centre and all possible modes of interaction between inhibitor and enzyme active-site generated systematically. The elimination of side-chain detail made possible the scanning of a much larger number of configurations than would otherwise be feasible, although complementarity and specificity were not properly represented in the simplified model.

The kinetics of the binding of structural protein *soc* (small outer capsid protein) to the head shell of bacteriophage T4 were studied.[793] It was shown that the relationship between the binding velocity, V, *soc* concentration, and the ratio f of unoccupied to the total number of binding-sites was approximately $V \propto [soc]^{1.4} \times f^{2.2}$ at 37 °C and that the rate of binding decreased sharply as the reaction proceeded. Other interactions that have been studied are the kinetics of assembly of the large subunit of *E. coli* ribosomes from 23S and 5S

[786] F. Ahmad, P.M. Ahmad, L. Pieretti, and G.T. Watters, *J. Biol. Chem.*, 1978, **253**, 1733.
[787] F.H. Pettit, S.J. Yeaman, and L.J. Reed, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4881.
[788] J.K. Stoops, E.S. Awad, M.J. Arslanian, S. Gunsberg, S.J. Wakil, and R.M. Oliver, *J. Biol. Chem.*, 1978, **253**, 4464.
[789] P.T. Gaffney and G. Craven, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 3128.
[790] E.A. Boeker, *Biochemistry*, 1978, **17**, 258.
[791] E.A. Boeker, *Biochemistry*, 1978, **17**, 263.
[792] S.J. Wodak and J. Janin, *J. Mol. Biol.*, 1978, **124**, 323.
[793] T. Ishii, Y. Yamaguchi, and M. Yanagida, *J. Mol. Biol.*, 1978, **120**, 533.

RNA and all the proteins,[794] and the dissociation of plasma blood-clotting factor XIIIa.[795]

Protein–Small Molecule Interactions. Changes in absorbance were used to study the kinetics of the binding of bilirubin to human serum albumin.[796] This reaction is important in animals as one of the functions of albumin is to protect the individual from the toxic effects of bilirubin, the final catabolic product of haem metabolism. When observed at 380 nm, at least three kinetic events could be distinguished, a bimolecular association step ($k_1 = 3.8 \times 10^7$ M^{-1} s^{-1}) and two relaxation steps ($k_2 = 40.2$ s^{-1} and $k_3 = 3.8$ s^{-1}). When observations were made at 420 nm, only the two relaxations were seen indicating that the association step was isosbestic at this wavelength. The dissociation rate was measured by mixing albumin–bilirubin complex with free albumin and observing at 485 nm. The rate constant was 0.01 s^{-1}, leading to a minimum value for the equilibrium constant $\sim 4 \times 10^9$ M^{-1}. In another study the kinetics and equilibrium of the binding of steroids to human corticosteroid-binding globulin (transcortin) were investigated by fluorometric methods.[797]

The binding of the antimitoic agent colchicine prevents assembly and causes disassembly of microtubules. The association of tubulin with this substance involved an initial fast step, constituting most of the reaction, followed by a slower step (5—10 % of the bound colchicine). The data could be explained by a mechanism requiring a colchicine-induced conformational change in the protein:

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PC)'$$

and the results indicated that the second step was essential in order to obtain the nearly irreversible binding of colchicine believed to be responsible for causing assembled microtubules to dissociate.[798] The complicated kinetics of the enzyme aspartokinase–homoserine dehydrogenase I from *E. coli* and its inhibition by threonine have been studied extensively by stopped-flow methods.[799] This enzyme possesses two distinct and separate sites for threonine binding, and the co-operative inhibition of the homoserine dehydrogenase activity by threonine could best be described by rapid equilibrium binding of threonine to the high-affinity site followed by site–site interactions.

Many papers on the binding of ligands by haemoglobins and myoglobins have appeared in the last year. Szabo[800] showed that the way in which co-operativity in haemoglobin is manifested kinetically depended on the nature of the ligand, by comparing the kinetics of binding of NO, O_2, and CO with those for bulky isocyanides. Alberding *et al.*[801] have produced a stochastic theory of

[794] G. Sieber and K.H. Nierhaus, *Biochemistry*, 1978, **17**, 3505.
[795] J.M. Freyssinet, B.A. Lewis, J.J. Holbrook and J.D. Shore, *Biochem. J.*, 1978, **169**, 403.
[796] R.D. Gray and S.D. Stroupe, *J. Biol. Chem.*, 1978, **253**, 4370.
[797] S.D. Stroupe, G.B. Harding, M.W. Forsthoefel, and U. Westphal, *Biochemistry*, 1978, **17**, 177.
[798] D.L. Garland, *Biochemistry*, 1978, **17**, 4266.
[799] C.F. Bearer and K.E. Neet, *Biochemistry*, 1978, **17**, 3517.
[800] A. Szabo, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2108.
[801] N. Alberding, H. Frauenfelder, and P. Hänggi, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 26.

ligand binding and have applied it to the binding of CO by myoglobin at partial pressures from 1—10^3 bars. Chevion *et al.*[802] determined the low temperature e.p.r. spectra and room-temperature, ligand dissociation rate-constants for human NO-haemoglobins A and F (foetal) as a function of pH and inositol hexaphosphate concentration. The results were consistent with an increased stability of a ligated low-affinity structure in the foetal protein. Sharma and Ranney[803] also studied the dissociation kinetics of $Hb(NO)_4$ and Hb(NO) by reaction with an excess of deoxymyoglobin. Their results indicated that the ligand dissociation from $Hb(NO)_4$ was co-operative and also that the $R \longrightarrow T$ transition, brought about with or without inositol hexaphosphate, changes the ligand affinity of one type of the chains much more than of the other. Many groups have studied the kinetics of CO binding, including binding to isolated α and β-chains,[804] binding to the valency hybrids $\alpha_2^{+}\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{+}$,[805] the pico-second photodissociation and subsequent recombination in CO-Hb,[806] and binding to singly reduced methaemoglobins (Hb $Fe_3^{3+}Fe^{2+}$).[807] The binding of CO to fish haemoglobin (*Brevoortia tyrannus*) which shows a Root effect was investigated.[808]

Protein–Small Molecule Equilibria.—*Theory and Techniques.* Kunisawa and Otsuka[809] have constructed a general partition function to describe the co-operative effects in proteins composed of more than two subunits. This function was applied in the analysis of the mechanism of the haem–haem interactions in haemoglobins by curve fitting. Their study is based on the assumption that the strain energy due to ligand binding is stored in weak contacts between subunits. In the minimum co-operative unit (*i.e.* a pair of subunits) two competing mechanisms may operate: expansion or contraction of the weak contacts which results in negative co-operativity, and alternation of the contacts which can produce positive co-operativity. In considering the crucial role in many biological processes of the kinetic and thermodymanic properties of ligands interacting with membrane-receptors, Thakur and Delisi[810] present a method for characterizing the free energy and affinity distributions of a heterogeneous population of molecules interacting with a homogeneous population of ligands. They derive expressions for the moments as functions of experimental binding curve characteristics and then construct the distribution as an expansion over a Gaussian basis set.

A number of workers have given consideration to the interpretation of Scatchard plots of binding data. Thus Baghurst *et al.*[811] present an equation in Scatchard format which describes the binding of a ligand to a two-state

[802] M. Chevion, A. Stern, J. Peisach, W.E. Blumberg, and S. Simon, *Biochemistry,* 1978, **17,** 1745.
[803] V.S. Sharma and H.M. Ranney, *J. Biol. Chem.,* 1978, **253,** 6467.
[804] N. Alberding, S.S. Chan, L. Eisenstein, H. Frauenfelder, D. Good, I.C. Gunsalus, T.M. Nordlung, M.F. Perutz, A.H. Reynolds, and L.B. Sorensen, *Biochemistry,* 1978, **17,** 43.
[805] H.S. Rollema, A. Raap, and S.H. de Bruin, *European J. Biochem.,* 1978, **83,** 313.
[806] L.J. Noe, W.G. Eisert, and P.M. Rentzepis, *Proc. Nat. Acad. Sci. U.S.A.,* 1978, **75,** 573.
[807] K. Ho, M.H. Klapper, and L.M. Dorfman, *J. Biol. Chem.,* 1978, **235,** 238.
[808] W.H. Saffran and Q.H. Gibson, *J. Biol. Chem.,* 1979, **253,** 3171; S.J. Torkelson and Q.H. Gibson, *J. Biol. Chem.,* 1978, **253,** 7331.
[809] T. Kunisawa and J. Otsuka, *J. Theoret. Biol.,* 1978, **74,** 559.
[810] A.K. Thakur and C. Delisi, *Biopolymers,* 1978, **17,** 1075.
[811] P.A. Baghurst, L.W. Nichol, and D.J. Winzor, *J. Theoret. Biol.,* 1978, **74,** 523.

acceptor system (*e.g.* either an isomerizing or a polymerizing one), and use it to formulate a general set of conditions for the occurrence of critical points in Scatchard plots. Evidence is presented to show that there is an exact correspondence between the appearance of critical points in the plots and points of inflection in the associated binding curves. Whitehead[812] gives a method in which the 'Hill exponent' and free energies of interaction may be determined from double-reciproal Eadie–Hofstee–Scatchard and Hanes plots and from the simple plot of ligand binding against free ligand concentration.

A flow dialysis procedure has been devised in which the steady state is reached in less than 30 sec, and allows the determination of dissociation constants (K_d) of 10^{-7} M and lower.[813] In this type of procedure ('rate-of-dialysis') a complete set of data can be collected with a single protein sample in a much shorter time than with equilibrium dialysis, and is therefore applicable to labile materials. An ultra-filtration device is also described in the same article which does not disturb binding equilibria because it only uses about 1 % of the total solution, and which allows simultaneous measurements on six samples in a few minutes. The methods were tested with glycogen phosphorylase and the allosteric ligand 5′-AMP. Russo and Engel[814] have presented a general method for determining binding constants for thin-film dialysis data.

Examples. Haemoglobin. As usual many papers dealing with the equilibrium binding of oxygen and allosteric ligands to haemoglobin have appeared in the past year. Groth *et al.*[815] analysed oxygen-binding data for whole blood and for concentrated haemoglobin solutions at different pH and pCO_2 values, and different 2,3-bis-phosphoglycerate concentrations. Their results were consistent with the view that 2,3-bis-phosphoglycerate acts primarily as a quaternary effector and that quaternary transition most likely takes place after oxygenation of the second subunit in the tetramer, and that the probability of existence of intermediate states of oxygenation were low. Bucci *et al.* [816] studied the interaction of the effector benzenehexacarboxylate with ionizable groups in human haemoglobin A as well as in two mutant human haemoglobins. In HbA it bound preferentially to the deoxy form, lowering its oxygen affinity, but in Hb Providence-Asp (β82 Lys ⟶ Asp) the interaction was greatly reduced above pH 7.5. Perutz and his co-workers[817] studied the inositol hexaphosphate (IHP)-induced change from quaternary oxy to the deoxy structure ($R \longrightarrow T$) in derivatives of human, trout IV and carp methaemoglobins. It was shown that the spectral change in the u.v. region induced by IHP was a reliable indicator of the $R \longrightarrow T$ transition. Trout HbIV was also investigated by Wyman *et al.*[818] They concluded that the essential feature of the functional behaviour of this haemoglobin and which is characteristic of the haemoglobins of teleost fish,

[812] E.P. Whitehead, *Biochem. J.*, 1978, **171**, 501.
[813] K. Feldmann, *Analyt. Biochem.*, 1978, **88**, 225.
[814] S.F. Russo and R.G. Engel, *Biopolymers*, 1978, **17**, 837.
[815] T. Groth, L. Garby, and C.H. de Verdier, *J. Mol. Biol.*, 1978, **121**, 507.
[816] E. Bucci, A. Salahuddin, J. Bonaventura, and C. Bonaventura, *J. Biol. Chem.*, 1978, **253**, 821.
[817] M.F. Perutz, J.K.M. Sanders, D.H. Chenery, R.W. Noble, R.R. Pennelly, L.W.M. Fung, C. Ho, I. Giannini, D. Pörschke, and H. Winkler, *Biochemistry*, 1978, **17**, 3640.
[818] J. Wyman, S.J. Gill, H.T. Gand, A. Colosimo, B. Giardina, H.A. Kuiper, and M. Brunori, *J. Mol. Biol.*, 1978, **124**, 161.

was the proton-induced shift in the allosteric equilibrium constant. In a further study of this haemoglobin[819] the oxygen binding was investigated as a function of pH at pressures of up to 10 atm. of pure oxygen. The results showed the extreme proton–oxygen linkage which gives rise to a Root effect. Trout HbIV (the major component in trout blood) was shown to have very special properties that enable it to operate as a pump for injecting oxygen into the swim-bladder in the face of a high opposing pressure, namely by an extremely low oxygen affinity at acid pH values. In order to obtain binding data at high partial pressures of oxygen, a spectrophotometer cell was constructed capable of operating at up to 25 atmospheres.

Kilmartin's group has studied the relation of the Bohr group salt bridges to co-operativity in haemoglobins. These salt bridges can be broken by raising the pH or by removing the amino-acid residue concerned. It could be shown that K_T (the O_2 association constant for the *T* state) was increased upon breaking the salt bridges in the *T* state and this is in contradiction of crystallographic data on Hb Kansas (Asn-102$\beta \longrightarrow$ Thr) which show no breakage of Bohr group salt bridges on ligation of the *T* structure.[820] In terms of the Perutz mechanism, ligation of the *T* state cause tertiary-structure changes culminating in the breakage of salt bridges: the bonds changed or broken in this pathway were supposed to be partially responsible for the free energy of co-operativity. Kilmartin *et al.*[821] propose that the contradictory findings in HbA and Hb Kansas are resolved if it is accepted that the unusual properties of the liganded *T* state of Hb Kansas are part of a general occurrence and would be expressed by any haemoglobin with low ligand-affinity, however caused. This would imply that there is no unique stereochemical mechanism for haemoglobin.

A new technique has been devised utilizing the observation that in sickle-cell anaemea, the oxygen affinity is inversely related to the intracorpuscular haemoglobin concentration in the cells.[822] This led to the proposal that the decreased oxygen affinity was a direct consequence of intracellular HbS polymerization and that therefore the oxygen affinity could be used as an index of HbS polymerization. On this basis a sensitive method was developed for measuring the minimum gelling concentration of HbS.

Enzymes. It was concluded from a study of the glucose-induced quenching of tryptophan fluorescence of isozymes A and B of hexokinase that the intrinsic dissociation constants, K_M and K_D for binding of glucose to the monomeric and dimeric forms of the B isozyme, were within a factor of two of each other ($K_D/K_M \leq 2$).[823] It was noted that values of K_M were influenced by the ionic strength, so much so that it is meaningless to compare K_M and K_D values measured at different ionic strengths as has frequently been done in the literature. In another study, using fluorometric methods,[824] the co-operativity

[819] M. Brunori, M. Coletta, B. Giardina, and J. Wyman, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4310.
[820] J.V. Kilmartin, K. Imai, R.T. Jones, A.R. Faruqui, J. Fogg, and J.M. Baldwin, *Biochim. Biophys. Acta*, 1978, **534**, 15.
[821] J.V. Kilmartin, N.L. Anderson, and S. Ogawa, *J. Mol. Biol.*, 1978, **123**, 71.
[822] R.E. Benesch, R. Edalji, S. Kwong, and R. Benesch, *Analyt. Biochem.*, 1978, **89**, 162.
[823] I. Feldman and D.C. Kramp, *Biochemistry*, 1978, **17**, 1541.
[824] B. Birdsall, A.S.V. Burgen, J. Rodgrigues de Miranda, and G.R.K. Roberts, *Biochemistry*, 1978, **17**, 2102.

of the binding of ligands (2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate) to dihydrofolate reductase was investigated. These ligands can be regarded as 'fragments' of the inhibitor methotrexate. It was shown that the presence of one fragment not only altered the affinity but also altered the specificity of the binding-site. Thus the binding of *p*-aminobenzoyl-L-glutamate to enzyme–2,4-diaminopyrimidine complex was 54-fold more tight than to enzyme alone, and alkyl substitution on the amino nitrogen of *p*-aminobenzoyl-L-glutamate led to up to a 130-fold increase in affinity for the enzyme-2,4-diaminopyrimidine complex. The binding of adenylyl imidodiphosphate (an ATP analogue) to phosphofructokinase was also studied by fluorescence methods.[825]

Subunit Structure of Proteins.—There appears to be no diminution in the use of SDS-electrophoresis ('detergent-gel') methods for characterizing the subunit structures of proteins rapidly and inexpensively. Lambin[826] has investigated the reliability of molecular weight determinations using polyacrylamide gradient gels. The results confirmed the reliability, with 40 proteins, of the relationship: $\log(\text{mol.wt.}) = a \log(T) + b$, where T is the polyacrylamide concentration in the gradient reached by a protein, a is the slope and b is the y intercept of the regression line of the plot of log(mol.wt.) *vs.* T%. Molecular weights of unreduced and reduced proteins, and of glycoproteins, it is claimed, can be determined with the same accuracy as other proteins. A two-dimensional electrophoresis method was introduced which takes advantage of the migration anomalies shown by some polypeptides on gels of different porosities, and was used to investigate the subunit structure of yeast and bovine cytochrome oxidases.[827] Kapp and Vinogradov[828] have described a method for removal of SDS from proteins using an ion-retardation resin.

Many multisubunit protein systems have been investigated by detergent-gel methods including RNA polymerase I from *Acanthamoeba castellani* which contains at least ten subunits,[829] fatty acid synthetase from *Mycobacterium smegmatis* which appears to be an oligomer of identical multifunctional subunits of mol. wt. 290 000,[830] acetyl-CoA carboxylase from avian liver which is composed of two non-identical subunits both of mol. wt. 230 000,[831] and ferritin which has only two types of subunit of mol. wt. 21 000 and 19 000, the relative proportions of which vary in different tissues as multiple isoferritins and which are hybrids of these two subunits.[832] Another hybrid to be investigated was the artificial hybrid, A_2B_2, formed by incubating rabbit-muscle phosphofructokinase (A_4) with rabbit-liver phosphofructokinase (B_4).[833] Ligandin, the rat-liver protein that binds bilirubin, azodye carcinogens,

[825] N.M. Wolfman, W.R. Thompson, and G.G. Hammes, *Biochemistry*, 1978, **17**, 1813.
[826] P. Lambin, *Analyt. Biochem.*, 1978, **85**, 114.
[827] R.O. Poyton, E. McKemmie, and C. George-Nascimento, *J. Biol. Chem.*, 1978, **253**, 6303.
[828] O.H. Kapp and S.N. Vinogradov, *Analyt. Biochem.*, 1978, **91**, 230.
[829] S.R. Spindler, G.L. Duester, J.M. D'Alessio, and M.R. Paule, *J. Biol. Chem.*, 1978, **253**, 4669.
[830] W.I. Wood, D.O. Peterson, and K. Bloch, *J. Biol. Chem.*, 1978, **253**, 2650.
[831] J.C. Mackall, M.D. Lane, K.R. Leonard, M. Pendergast, and A.K. Kleinschmidt, *J. Mol. Biol.*, 1978, **123**, 595.
[832] P. Arosio, T.G. Adelman, and J.W. Drysdale, *J. Biol. Chem.*, 1978, **253**, 4451.
[833] F. Gonzalez and P.G. Kemp, *J. Biol. Chem.*, 1978, **253**, 1493.

steroids, and many other compounds, was shown by detergent-gel electrophoresis, isoelectric focusing in the presence of urea, and cross-linking experiments, to be composed of two polypeptide chains of mol. wt. 25 000 and 22 000.[834] Cross-linking experiments were also used to investigate the subunit arrangements in ribulose 1,5-bis-phosphate carboxylase,[835] the state of self-association of proteins apo-A-I and apo-A-II from human high density lipoprotein,[836] and the relationship between aggregation state and catalytic activity in bovine-liver glutamate dehydrogenase.[837]

Two interesting respiratory protein systems were studied by a variety of methods and their subunits isolated. Thus the haemoglobin of the killifish contained four distinguishable components, α^a, α^b, β^a, and β^b, which form the tetrameric isohaemoglobins found in native haemoglobin. Hb I had the structure $\alpha_2^a \beta_2^b$ and HbIV the structure $\alpha_2^a \beta_2^a$, while other isohaemoglobins were heterotetramers consisting of all four chains.[838] In another study it was found to be possible to isolate two types of polypeptide chain from the haemocyanin of *Murex fulvescens*.[839] Both had a mol. wt. of 250 000—300 000, and both were required to be present for re-formation of the native molecule of mol. wt. 9×10^6.

The re-assembly of immunoglobulin M (IgM) following mild reductive cleavage was studied by Wilde and Koshland.[840] The IgM molecule consists of five molecules similar in size to IgG plus a small sulphydryl-containing polypeptide called the J-chain. Repolymerization was found to require the presence of J-chain and a disulphide exchange system such as monofunctional thiol (*e.g.* mercaptoethylamine) and/or a disulphide exchange enzyme.

The interactions of the various proteins present in ribosomes continue to be studied intensively. Lambert *et al.*[841] used cross-linking agents containing disulphide bonds to investigate the topography of proteins in the 30S subunit, and Spierer and his colleagues[842] have examined the function of 5S RNA in the structure of ribosomes. This group has studied the stoicheiometry, co-operativity, and stability of interactions between the ribosomal proteins L5, L18, and L25 from the 50S subunit and 5S RNA.

[834] M.M. Bhargava, I. Listowsky, and I.M. Arias, *J. Biol. Chem.*, 1978, **253**, 4116.
[835] H. Roy, A. Valeri, D.H. Pope, L. Rueckert, and K.A. Costa, *Biochemistry*, 1978, **17**, 665.
[836] J.B. Swaney and K. O'Brien, *J. Biol. Chem.*, 1978, **253**, 7069.
[837] L. Zeiri and E. Reisler, *J. Mol. Biol.*, 1978, **124**, 291.
[838] P.A. Mied and D.A. Powers, *J. Biol. Chem.*, 1978, **253**, 3521.
[839] M. Brouwer, M. Ryan, J. Bonaventura, and C. Bonaventura, *Biochemistry*, 1978, **17**, 2810.
[840] C.E. Wilde and M.E. Koshland, *Biochemistry*, 1978, **17**, 3209.
[841] J.M. Lambert, R. Jue, and R.R. Trant, *Biochemistry*, 1978, **12**, 5406.
[842] P. Spierer and R.A. Zimmermann, *Biochemistry*, 1978, **17**, 2474; P. Spierer, A.A. Bogdanov, and R.A. Zimmermann, *Biochemistry*, 1978, **17**, 5394.

3
Peptide Synthesis

BY I. J. GALPIN
Appendices compiled by M. F. J. GALPIN

1 Introduction

In the last year relatively few 'milestones' in peptide synthesis have been passed. Greater attention appears to have been focused on the actual synthesis of often quite large peptides, and the efforts directed towards the development of new protecting-groups has diminished. Many workers in this area now clearly consider the synthesis of peptides of 20—30 residues a realistic target for their efforts whether it be by conventional solution methods or by solid-phase techniques. Base labile protecting groups, particularly in solid-phase synthesis, have become a major point for consideration as their use avoids the fairly destructive treatment with acidolytic reagents.

A great deal of activity has been shown in the area of the opiate peptides and it is a certainty that many hundreds of enkephalin analogues will have been synthesized during the last year. Unfortunately much of the interesting information will not see the light of day as these compounds are of such great interest pharmaceutically. Although the opiate peptides perhaps dominate the scene at present there has been considerable progress in many other areas.

With so many analogues and homologues of physiologically active peptides being synthesized it is clear that a wide range of preparative and analytical methods should be used, and in this vein it is interesting to note the greatly increased use of h.p.l.c. and partition chromatography.

The proceedings of the 14th Japanese peptide symposium[1] have been reviewed where appropriate although much of the material has already appeared in the literature.

A general review[2] and the 4th volume of the Chemistry and Biochemistry of amino-acids, peptides, and proteins[3] have appeared. In addition reviews or books describing the following peptides and related areas have been published, somatropin,[4] lipotropin,[5] thyroid hormones,[6] vasopressin,[7] and ACTH.[8]

[1] T. Nakajima, 'Proceedings of the 14th Symposium on Peptide Chemistry', Hiroshima 1976, Protein Research Foundation, Osaka, 1977.
[2] I.J. Galpin, *Chem. in Britain*, 1978, **14**, 181.
[3] 'Chemistry and Biochemistry of Amino-acids, Peptides and Proteins', Volume 4, ed. B. Weinstein, Marcel Dekker, New York and Basle, 1977.
[4] C.H. Li, 'Hormonal Proteins and Peptides, Growth Hormone and Related peptides', Volume 4, Academic Press, New York, 1978.
[5] C.H. Li, 'Hormonal Proteins and Peptides, Lipotropin and Related Peptides', Volume 5, Academic Press, New York, 1978.
[6] C.H. Li, 'Hormonal Proteins and Peptides, Thyroid Hormones and Analogues', Volume 6, Academic Press, 1978.
[7] Annual research reviews, 'Anti-diuretic hormone', ed. M.L. Farsling, Eden Press, Montreal, 1977.
[8] 'ACTH and Related Peptides', Annals of New York Academy of Sciences, Volume 297, ed. D.T. Krieger and W.F. Ganony, N.Y. Academy of Sciences, 1978.

The tone of this years research was greatly saddened by the death of Professor George Wallace Kenner. All those who knew him will remember his diverse contributions which are particularly well expressed in his recent Bakerian Lecture[9] the last sentence of which clearly showed his optimism regarding the subject. 'What I have tried to persuade you is that the total synthesis of proteins may yet follow the stages of alpinism referred to by Mummery[10] with reference to the Grépon "an inaccessible peak – the most difficult climb in the Alps – an easy day for a lady".'

The general format of this year's coverage follows that established previously, with lists of synthetic peptides and useful derivatives being presented as appendices.

2 Methods

Protective Groups.—*Established Methods of Amino-group Protection.* The recently introduced reagent,[11] di-t-butyl pyrocarbonate (1) has now been more

$$Bu^tO{\cdot}CO{\cdot}O{\cdot}CO{\cdot}OBu^t$$

(1)

widely applied and its use in the acylation of trifunctional amino-acids has been described.[12] The analogous di-t-amylpyrocarbonate[13] has also been synthesized and used to prepare the corresponding amino-acid derivatives. Removal of Boc protection continues to give rise to difficulty, particularly in the presence of methionine and/or tryptophan. However, further detailed studies of deprotection using sulphonic acids appears to give a solution to these problems.[14,15] Anhydrous *p*-toluene sulphonic acid 2M in dioxane in the presence of 2 % anisole cleaves Boc from Boc-Trp-OH without side-reactions. However, a mixture of 2 % anisole and 2 % ethane dithiol is more efficient as a scavenger. Thioanisole also appears to be a useful scavenger both in the removal of Boc and Z(OMe) protection by sulphonic acids. Some degradation of methionine cannot be avoided and this residue is thus best introduced as its sulphoxide if methane sulphonic acid is to be used for deprotection.[16] This deprotection regime is particularly useful in solid-phase synthesis as side-chain benzyloxycarbonyl-groups and the backbone benzylic ester linkage are totally stable to this reagent in contrast to the more usual 50 % TFA–CH_2Cl_2 which is generally used.

Alternative methods for the removal of benzyloxycarbonyl groups have been reported. Catalytic transfer hydrogenation using cyclohexa-1,4-diene[17] or

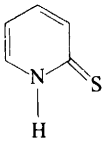
[9] G.W. Kenner, *Proc. Roy. Soc.*, 1977, **A353**, 441.
[10] A.F. Mummery, 'My climbs in the Alps and Caucasus', Chapter 6, Oxford: Basil Blackwell, 1936.
[11] L. Moroder, A. Hallett, E. Wünsch, O. Keller, and G. Wersin, *Z. physiol. Chem.*, 1976, **357**, 1651.
[12] V.F. Pozdnev, *Bioorg. Khim.*, 1977, **3**, 1605.
[13] V.F. Pozdnev, *Bioorg. Khim.*, 1978, **4**, 1273.
[14] K. Suzuki, N. Endo, K. Nitta, and Y. Sasaki, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 2198.
[15] K. Suzuki, N. Endo, K. Nitta, and Y. Sasaki, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 2204.
[16] N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 650.
[17] A.M. Felix, E.P. Heimer, T.J. Lambros, C. Tzougraki, and J. Meienhofer, *J. Org. Chem.*, 1978, **43**, 4194.

hydrazine[18] in place of cyclohexene as hydrogen donor allows removal using palladium black as catalyst. Side-chain benzyl derived functions and the nitrofunction of N^G-nitroarginine are also removed. Using the first hydrogen donor a marked solvent dependance is shown, following the order HOAc >

H_2^+—Gly—NH_2 ^-O_2C—Gly—NH_2

(2)

EtOH > DMA > MeOH > DMF; HMPA,TFE,PhOH,TFA,THF,DMSO, and IPA cannot be used. In ethanol Z is readily removed from Z-Met-OH; however Z-Cys(Bzl)-OH is totally stable even if liquid ammonia is used as solvent. This is in contrast to the straightforward removal by direct hydrogenlysis in liquid ammonia reported last year.[19] The isolation[20] of a carbamate salt (2) from the hydrogenolysis of Z-Gly-NH_2 under neutral conditions has provided substantiation of the assumed mode of removal of the Z group. As these salts can be directly acylated their presence often passes unnoticed.



(3)

The use of 2-thiopyridone (3) for the selective removal of *o*-nitrophenylsulphenyl groups has been described.[21] A 3—5 fold excess of the reagent in glacial HOAc,MeOH,DMF, or CH_2Cl_2 gave a quantitative cleavage in 5 minutes, 20—30 minutes being required if a 5—10 fold excess was used when the protected peptide was attached to a polymeric support. The soluble mixed disulphide was produced with no modification of tryptophan or other side-reactions, this being in contrast to related thiol reagents which caused considerable side-reactions.[22] The utility of this method was demonstrated by a solid-phase synthesis of leucine enkephalin.

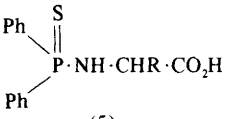
Vinyl phenylthiocarbonate has been introduced as a reagent for the introduction of the vinyloxycarbonyl (VOC) function,[23] this intermediate being preferable to the unstable chloroformate. The new reagent was prepared in 65 % yield by reaction of the lithium enolate (4) with phenylthiochloroformate.

CH_2=CH—OLi

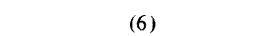
(4)

[18] M.K. Anwer, S.A. Khan, and K.M. Sivanandaiah, *Synthesis*, 1978, 751.
[19] A.M. Felix, M.H. Jimenez, T. Mowles, and J. Meienhofer, *Internat. J. Peptide Protein Res.*, 1978, **11**, 329.
[20] I. Schön and L. Kisfaludy, *Z. Naturforsch.*, 1978, **33**, 1196.
[21] A. Tun-Kyi, *Helv. Chim. Acta*, 1978, **61**, 1086.
[22] W. Kessler and B. Iselin, *Helv. Chim. Acta*, 1966, **49**, 1330.
[23] A.J. Duggan and F.E. Roberts, *Tetrahedron Letters*, 1979, 595.

The reagent was stable to light and does not polymerize, being stable to base, and giving the VOC derivative when treated with amino-acids at pH 9.5 (Gly 80 %, Phe 60 %, Pro 77 %.)



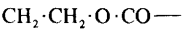
(5)



(6)

The range of diphenylthiophosphinyl amino-acids (5) has been extended,[24] these derivatives were prepared directly from the thiophosphinyl chloride and the corresponding amino-acid. A range of other dialkylthiophosphinyl amino-acids were also described, including the dimethyl and diethyl derivatives, these were both cleaved more rapidly than the diphenyl derivatives on treatment with 80 % HOAc.

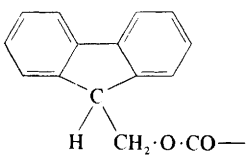
Two papers have appeared describing the use of *N,O*-bis-trimethylsilyl-amino-acids[25, 26] (6), these derivatives have increased nucleophilicity and hence are readily acylated on the protected amino-function. Work up generally leads to the hydrolysis of the trimethylsilyl ester and the generation of the free carboxylic acid.



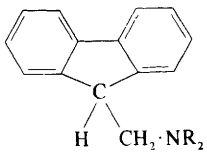
(7)

The synthesis of dipeptides using base labile 2-phosphonioethoxycarbonyl (7) groups in water has been described.[27] Methyldiphenylphosphonioethoxy-carbonyl-leucine was coupled with phenylalanine t-butyl ester using a water soluble carbodi-imide in the presence of 1-hydroxybenzotriazole. At low pH the more sensitive triphenylphosphonioethoxycarbonyl group could also be used. The pronounced acid stability of these groups allowed acidolytic cleavage of the ester function to be carried out generating the protected dipeptide acid.

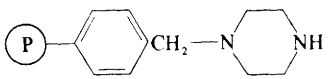
Considerable attention has been paid to the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group (8), particularly with reference to



(8)



(9)

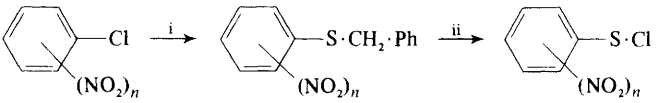


(10)

[24] M. Ueki and S. Ikeda, ref. 1, p l.
[25] E.P. Krisin, V.N. Karelsky, A.A. Autonov, and E.D. Glinka, *Khim. prirod. Soedinenii*, 1978, 482 (*Chem. Abs.*, 1978, **90**, 6684f).
[26] S.V. Rogozhin, Yu.A. Davidovich, and A.I. Yurtanov, *Izvest. Akad. Nauk S.S.S.R. Ser. khim.*, 1978, 657 (*Chem. Abs.* 1978, **89**, 110 347p).
[27] H. Kunz, *Angew. Chem. Internat. Edn.*, 1978, **17**, 67.

its use in solid-phase synthesis (see Vol. 10 of these Reports, p. 332). Normally removal is carried out by treatment with morpholine or piperidine, these bases scavenge the dibenzolfulvene which is generated in the cleavage, yielding adducts of the type shown (9). As such compounds can be difficult to remove in solution experiments, Carpino *et al.*[28] have developed a solid reagent which can be used for the removal of Fmoc. The solid reagent (10) was prepared from chloromethylpolystyrene by reaction with t-butyloxycarbonyl piperazine, when reaction occurs with dibenzofulvene a totally insoluble adduct is formed which can be removed from the reaction medium.

New Methods of Amino-group Protection. An extensive investigation into the use of further substituted *o*-nitrophenylsulphenyl amino-acid derivatives has been carried out[29] with a view to increasing the acid-stability of this type of group. In general the substituted sulphenyl chloride was prepared by the route



Reagents: i, NEt_3 or KOH, $Ph.CH_2.SH$; ii, SO_2Cl_2

Scheme 1

shown in Scheme 1, the sulphenyl chloride was then treated with the amino-acid to produce the corresponding derivative. 2,4,6,-Trinitrophenylsulphenyl (2,4,6-Tnps), 2,4,-dinitrophenylsulphenyl (2,4-Dnps), and 4-methyl-2-nitrophenylsulphenyl (Nmps) amino-acid derivatives were prepared. 2,4-Dnps was cleaved at the same rate as Nps when 0.5 M-HCl in dioxane was used to effect the cleavage however the 2,4-Dnps derivatives were relatively more stable when 80 % HOAc was used as indicated in Table 1.

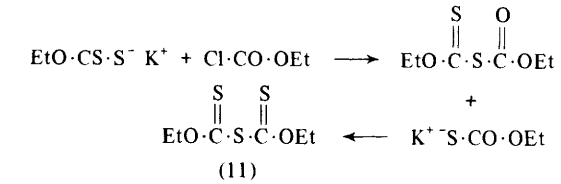
Table 1 *Cleavage of substituted Nps groups by 80 % acetic acid at 23 °C*[29]

Amino-acid	$t_{0.5}$/h *Nps*	$t_{0.5}$/h *2,4-Dnps*	$t_{0.5}$/h *Nmps*
Ala	4.5	29.8	1.7
Arg (NO_2)	5.3	77.0	
Ser (Bu^t)	4.0	50.3	
Cys (Bzl)	2.0	58.0	

The utility of the 2,4-Dnps group was demonstrated by the synthesis of *S*-benzylcysteinyl peptides. A polymeric nitrophenylsulphenyl chloride was also synthesized[30] and has been used for amino-protection. The insoluble protecting group could be removed under the usual conditions which are employed for Nps removal, and could be regenerated and used repeatedly for amino-protection.

The preparation of ethoxythiocarbonyl (Etc) amino-acids and their esters

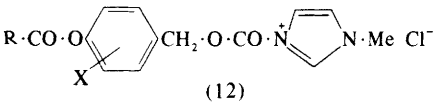
[28] L.A. Carpino, J.R. Williams, and A. Łopusinski, *J.C.S. Chem. Comm.*, 1978, 450.
[29] H. Ito, K. Ogawa, T. Iida, and I. Ichikizaki, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 296.
[30] H. Ito, K. Ogawa, and I. Ichikizaki, *Chem. Letters*, 1978, 7.



Scheme 2

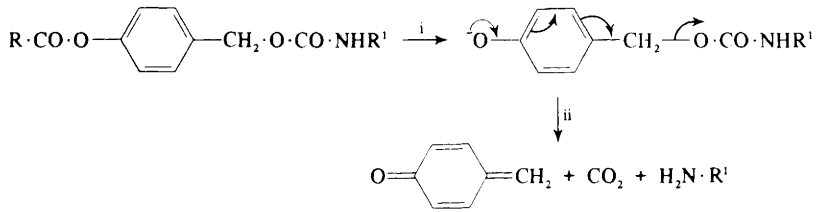
has been described.[31] The Etc group is introduced using the compound (11) which is prepared by the route outlined in Scheme 2, the reaction with amino-acids is carried out at pH 8—10 and gives on almost quantitative yield of the Etc derivative. The Etc group which shows a λ_{max} 254 nm, may be converted to the 1,2,4-dithiazolidine-3,5-dione (Dts) group[32] using chlorocarbonylsulphenyl chloride as outlined in last year's report.

A study of esters, carbonates, thiocarbonates, and carbonates of *p*-hydroxybenzyloxycarbonylglycine has been carried out.[33] The derivatives were



(12)

prepared using the compounds of type (12), the free amino-function was liberated by weak alkali by 1,6 elimination as indicated in Scheme 3. The hydrolysis which may be followed at 290 nm, gives rise to the corresponding

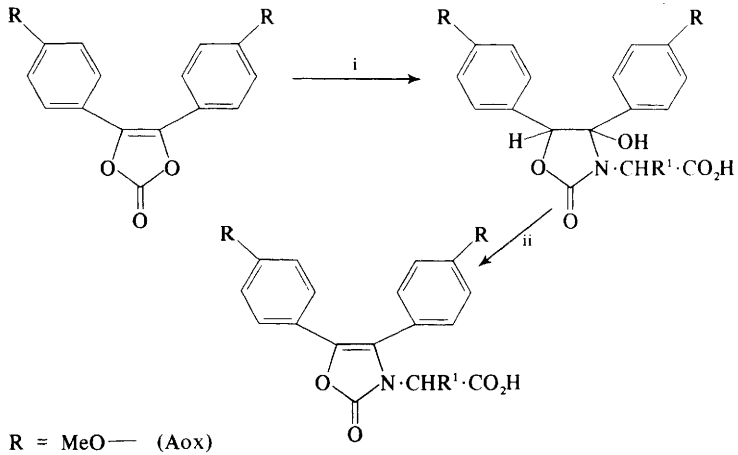


Reagents: i, Na_2CO_3 or H_2O_2–NH_3; ii, H^+

Scheme 3

quinonemethide which must be trapped out of the reaction mixture in order to prevent the possibility of the addition by the liberated amino-function. As these derivatives are generally more stable to TFA than the corresponding Z compounds it is possible that they may be used orthogonally with the more usual acid-labile protecting groups. The 4,5-diaryl-4-oxazolin-2-one amino-acid derivatives (13) have been prepared[34] by condensation of the oxazolinone

[31] G. Barany, B.W. Fulpius, and T.P. King, *J. Org. Chem.*, 1978, **43**, 2930.
[32] G. Barany and R.B. Merrifield, *J. Amer. Chem. Soc.*, 1977, **99**, 7363.
[33] G. LeCorre, E. Guibé-Jampel, and M. Wakselman, *Tetrahedron*, 1978, **34**, 3105.
[34] M. Kanaoka and Y. Kurata, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 660.



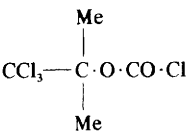
R = MeO— (Aox)
R = Cl— (Cox) (13)

Reagents: i, $NH_2.CHR^1.CO_2H$, $Me_4N^+OH^-$, HCl; ii, TFA

Scheme 4

with the amino-acid in the presence of tetramethylammonium hydroxide as shown in Scheme 4. The derivatives, which are stable to NaOH in EtOH (aq.), anhydrous HF and HBr in acetic acid, and are readily cleaved by hydrogenolysis over a palladium catalyst, were used to prepare AOX-pentagastrin.

The use of the supernucleophilic Co^I phthalocyanine anion for the removal of the trichloro-t-butyloxycarbonyl group, TCBOC (14), has been



(14)

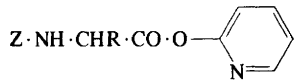
demonstrated[35] by the synthesis of a tripeptide. The TCBOC group is readily introduced by acylation of amino-acids under Schotten Baumann conditions using the stable and distillable chloroformate. The group is inert under the conditions used to remove methyl and t-butyl esters and thus provides an orthogonal protection scheme.

Protection of Terminal Carboxy-groups. Schmidt and Steindl[36] have described the synthesis of a number of *N*-protected amino-acid t-butyl thioesters. These esters are prepared by reaction of lithium t-butylmercaptide with the *N*-protected amino-acid *N*-hydroxysuccinimide ester or the corresponding mixed anhydride formed from either ethyl or isobutyl chloroformate. The preparation of crystalline *N*-Z, Boc, and Tfa derivatives in good yield was

[35] H. Eckert, M. Listl, and I. Ugi, *Angew. Chem. Internat. Edn.*, 1978, **17**, 361.
[36] U. Schmidt and F. Steindl, *Synthesis*, 1978, 544.

described. The t-butylthioesters serve a dual role in that they may be used in the usual way as a semi-permanent carboxy-protecting group or they may be activated by the addition of mercuric trifluoroacetate. When used in this way they may be used as acylating agents in coupling reactions.

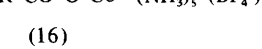
A number of *p*-toluenesulphonates and hydrobromides of β-trichlorethyl amino-acid esters have been prepared.[37] The hydrobromides were obtained by activating the benzyloxycarbonyl amino-acid with dicyclohexylcarbodi-imide



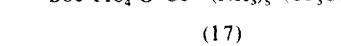
(15)

and 2-pyridone; the intermediate activated ester (15) was then treated with β-trichloroethanol for six days and finally the benzyloxycarbonyl function removed by treatment with HBr–HOAc. The *p*-toluene sulphonates were obtained by a two day azeotropic distillation of the amino-acid; and β-trichloroethanol in carbon tetrachloride using anhydrous *p*-toluene sulphonic acid. The best yield was given by 8—10 molar equivalents of the alcohol and 2—3 molar equivalents of the sulphonic acid, the use of toluene in place of carbon tetrachloride was unsatisfactory as the elevated temperature gave rise to increased side-reactions. Although the β-trichloroethyl esters are usually smoothly removed by treatment with zinc in acetic acid occasionally problems have been encountered during deprotection. Cleavage may be efficiently achieved for smaller peptides, but as the size of the peptide chain increases more side-reactions are observed.

Protection of the carboxy-function of *N*-protected amino-acids by the formation of a cobalt(III) pentamine inorganic ester (16) has been studied.[38] These as inorganic esters are kinetically inert and are resistant to hydrolysis by strong acid. This allows *N*-Boc deprotection to be carried out by treatment with 50 % TFA–CH_2Cl_2 or HBF_4, the tetrafluoroborate salts are particularly useful in synthesis owing to their high solubility in DMF. These esters may be acylated under standard conditions using Boc-amino-acids in the presence of dicyclohexylcarbodi-imide; in the presence of hydroxybenzotriazole, a dipeptide and the protected tetrapeptide (17) were synthesized using this method.



(16)



(17)

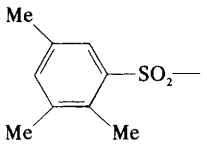
Final deprotection to the corresponding free-acids was achieved by treatment with sodium bicarbonate, cleavage being complete in 1—2 minutes at room temperature giving yields in excess of 90 %. The esterification of benzyloxycarbonyl amino-acids with ethanol or methanol using BF_3–Et_2O as catalyst has

[37] J.F. Carson, *Synthesis*, 1979, 24.
[38] S.S. Isied and C.G. Kuehn, *J. Amer. Chem. Soc.*, 1978, **100**, 6752.

been studied.[39] The *N*-protected amino-acids always gave a high yield of the alkylester but with free amino-acids the yields were very variable ranging from 14—82 %.

Frequently peptide hydrazides are required as the immediate precursor for azide couplings, generally these are obtained by direct hydrazinolysis of alkyl esters or by deprotection of suitable protected hydrazides. An alternative route to peptide hydrazides has been developed[40] in which the peptide free-acid is condensed with 1.1 equivalents of hydrazine in the presence of dicyclohexylcarbodi-imide–hydroxybenzotriazole. This avoids the possibility of side-reactions ensuing from the use of excess hydrazine and gives no observable racemization (less than 0.2 %), generally a yield of 65—75 % was obtained. This method is a distinct improvement over earlier related methods[41] which utilize the reaction of hydrazine with mixed anhydrides or active esters as it can be used at any amino-acid residue whereas the former method was restricted to glycine in order to eliminate racemization.

Side-chain Protection and Reactions of Amino-acids. The use of crystalline picrates or nitrates of *N*-benzyloxycarbonylarginine *p*-nitrophenylester[42] as protection for the guanidine function have been described. These salts represent useful activated unsubstituted derivatives of arginine which are in short supply. The salts couple efficiently in the presence of hydroxybenzotriazole but in the absence of this additive they decompose without effecting

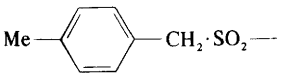


(18)

acylation. The mesitylene-2-sulphonyl(Mts) group (18) has also been proposed for protection of the guanidine function of arginine.[43] The group, which is cleaved by methane sulphonic acid, trifluoromethane sulphonic acid, and anhydrous HF is stable to TFA, 1 M-NaOH, hydrogenolysis, and sodium in liquid ammonia. Introduction is achieved by reaction of a suitable *N*-protected arginine derivative [Z,Z(OMe) or Boc] with the sulphonyl chloride. The group appears to be more labile than the *p*-toluenesulphonyl and has been successfully used for arginine protection in a synthesis of substance P. When cleavage of Mts is carried out using methane sulphonic acid in the presence of anisole tyrosine hydroxy-functions become partially mesitylene sulphonated. This side-reaction can be suppressed by the additional use of *o*-cresol or phenol plus thioanisole.

[39] T. Yamada, N. Isono, A. Inui, T. Miyazawa, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Jap.*, 1978, **51**, 1897.
[40] S.S. Wang, I.D. Kulesha, D.P. Winter, R. Makofske, R. Kutny, and J. Meienhofer, *Internat. J. Peptide Protein Res.*, 1978, **11**, 297.
[41] H. Zahn, J. Meienhofer, and E. Schnabel, *Acta Chim. Soc. Hung.*, 1965, **44**, 109.
[42] J.D. Glass and M. Pelzig, *Internat. J. Peptide Protein Res.*, 1978, **12**, 75.
[43] H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J.C.S. Chem. Comm.*, 1978, 482.

The use of Ti^{III} as a selective reducing agent for the removal of N^G-nitroarginine has been illustrated[44] by the synthesis of arginine vasotocin and the molluscan cardiac stimulant (H-Phe-Met-Arg-Phe-NH_2). Removal of the nitro function is achieved by treatment for 1 h at room temperature with an aqueous methanolic solution buffered with ammonium acetate to pH 4—6 with a 20 % excess of $TiCl_3$. Changes in pH caused an increase in the number of side-products with some ornithine being produced, however none of the routinely employed protecting groups were affected by this reagent.



(19)

The *p*-tolylmethylsulphonyl (Pms) group (19) has been used as a protecting group for the side-chain amino-function of lysine.[45] The Pms function which is introduced by reaction of the Cu^{II} complex of lysine with the corresponding sulphonyl chloride is removed in 60 min by treatment with anhydrous HF at 20 °C in the presence of anisole. It is stable to TFA, dilute HCl, and to hydrogenolysis using palladium black. Syntheses of neurotensin and FTS (facteur thymique serique)[46] (20) were carried out using Pms for protection of lysine.

Glp-Ala-Lys-Ser-Glu-Gly-Gly-Ser-Asn-OH

(20)

An investigation into the kinetics of the thiolysis of N^{im}Dnp histidine-containing peptides by mercaptoethanol[47] has shown that the observed pH optimum of 8.5—9.0 occurs because at higher pH (> 9) oxidation of the thiol is more rapid than the cleavage of Dnp.

A short communication[48] and more detailed account[49] of the removal of *S*-substituted cysteine derivatives by mercuric trifluoroacetate have been presented. *S*-t-butyl,-*p*-methoxybenzyl, and -1-adamantyl cysteine were all smoothly cleaved by mercuric trifluoroacetate in acetic acid, (mercuric acetate in trifluoroacetic acid also cleaves these groups but less rapidly) the free cysteine being liberated from the mercaptide with mercaptoethanol.

Interestingly the *S*-benzyl group is claimed to be stable under these conditions and would thus provide a means of differential *S*-protection when used with either of the preceding groups. A solid-phase synthesis of oxytocin and solution synthesis of somatostatin were used to evaluate the method using *p*-methoxybenzyl for cysteine protection. The *p*-nitrobenzyloxycarbonyl

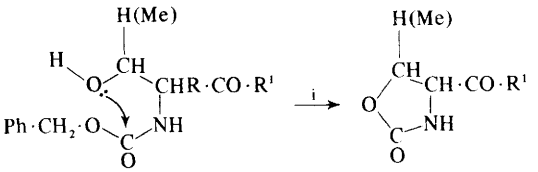
[44] R.M. Freidinger, R. Hirschmann, and D. Veber, *J. Org. Chem.*, 1978, **43**, 4800.
[45] T. Fukuda, C. Kitada, and M. Fujino, *J.C.S. Chem. Comm.*, 1978, 220.
[46] J. Bach, M. Dardenne, J. Pleace, and J. Doca, *Nature*, 1977, **266**, 55.
[47] H.J. Goren and M. Fridkin, *Internat. J. Peptide Protein Res.*, 1978, **11**, 1.
[48] O. Nishimura, C. Kitada, and M. Fujino, ref. 1, p. 49.
[49] O. Nishimura, C. Kitada, and M. Fujino, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 1576.

function was used for N^{α}-protection in the somatostatin synthesis as this could be removed by catalytic hydrogenation in the presence *S*-methoxybenzylcysteine using palladium black in 80 % acetic acid at 50 °C.

$Bu^{t}\cdot O\cdot CO\cdot S-$ (21) $\qquad$ $Ph\cdot CH_2\cdot O\cdot CO\cdot S-$ (22)

The t-butyloxycarbonylsulphenyl (SCB) (21) and benzyloxycarbonylsulphenyl (SZ) (22) groups have been evaluated as protecting–activating groups for the thiol function of cysteine.[50] The Boc-sulphenyl chloride was relatively unstable, but the Z-sulphenyl chloride could be treated with *S*-tritylcysteine derivatives to yield the corresponding Cys(SZ) compounds. The SZ function can thus be used in a similar way to the methoxycarbonylsulphenyl group which was developed by the Ciba–Geigy group[51] for the formation of unsymmetrical cystine peptides in their elegant insulin synthesis. The SZ group was stable to TFA and to mild base although it was cleaved by 40 % HBr–acetic acid and was not stable enough to be subjected to conditions of hydrazinolysis or hydrolysis.

Many varied side-reactions have been observed during peptide synthesis, *O*-acylation of unprotected serine and threonine has been found on several occasions. The rate of acylation of model compounds by Boc-glycine *p*-nitrophenyl ester has been shown to be dependant on the removal of the side-chain hydroxylic proton.[52] The cyclization of the hydroxylic side-chain with the carbonyl function of a benzyloxycarbonyl N^{α}-protecting group under alkaline conditions has been utilized (see Scheme 5) in the synthesis of a



(23)

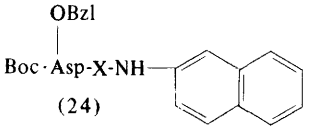
Reagents: i, basic conditions (OH^-)

Scheme 5

5-methyl-2-oxo-oxazolidine-4-carboxylic acid (23) analogue of tuftsin.[53] Thus clearly this side-chain functionality must be protected when *N*-terminal benzyloxycarbonyl serine or threonine are exposed to alkaline conditions.

The rearrangement of peptides with the general structure (24) continues to receive attention; in the case of (24*a*) the $t_{0.5}$ for the formation of the cyclic

[50] K. Nokihara and H. Berndt, *J. Org. Chem.*, 1978, **43**, 4893.
[51] B. Kamber, *Helv. Chim. Acta*, 1973, **56**, 1370.
[52] S.K. Girin and Y.P. Shvachkin, *Zhur. obshchei Khim.*, 1978, **48**, 902.
[53] Y. Stabinsky, M. Fridkin, V. Sakuth, and Z. Spirer, *Internat. J. Peptide Protein Res.*, 1978, **12**, 130.



(24) (24*a*) X = Gly

succinimide derivative may be as low as 2 min.[54] Interestingly the addition of acidic phenols (pentachloropehnol or 2,4-dinitrophenol) in the presence of hydroxybenzotriazole increases the $t_{0.5}$ to greater than 500 min. The use of the *p*-nitrobenzyl ester group fro β-carboxyl protection has also been found to minimize rearrangement when protected peptides are deprotected with methane sulphonic acid[55, 56] in the presence of anisole. A five fold excess of zinc in 90 % acetic acid may also be used to cleave the nitrobenzyl ester function, in this case no α to β rearrangement was observed in contrast to the use of zinc in hydrochloric acid. The same deprotection method may also be used for the removal of N^{α}-*p*-nitrobenzyloxycarbonyl or *S*-*p*-nitrobenzyl-cysteine protection.

Bodanszky and his co-workers have studied this rearrangement in great detail[54, 57, 58, 59] using β-naphthylamides to provide easy detection by u.v. The selective catalysis of active ester couplings by hydroxybenzotriazole or *p*-dimethylaminopyridine inhibits ring closure and the required β-benzylaspartyl peptides are produced in high yield.[57] The base catalysed ring closure also shows considerable dependance on the adjacent residue (see Table 2).

Table 2 *Values of $t_{0.5}$ for the base catalysed rearrangement of Boc-Asp (OBzl)-X-α-naphthylamides*[57,59]

X	$t_{0.5}$
Gly	3
Ala	10
Val	400
Leu	20
Ile	730
Met	100
Phe	20
Ser	0.5
Thr	1

Clearly the very slow rearrangement of the valyl and isoleucyl peptides is due to increased steric interactions. In the case of X = Pro no rearrangement at all was observed.[58] The use of β-phenacyl esters as protection for aspartic acid was advanced,[59] although it appears that the use of this protecting function should be limited to solid-phase synthesis. In solution synthesis under

[54] J. Martinex and M. Bodanszky, *Internat. J. Peptide Protein Res.*, 1978, **12**, 277.
[55] K. Suzuki, N. Endo, K. Nitta, and Y. Sasaki, ref. 1, p. 45.
[56] K. Suzuki and N. Endo, *Chem. and Pharm Bull. (Japan)*, 1978, **26**, 2269.
[57] M. Bodanszky, J.C. Tolle, S.S. Deshmane, and A. Bodanszky, *Internat. J. Peptide Protein Res.*, 1978, **12**, 57.
[58] M. Bodanszky and J.Z. Kwei, *Internat. J. Peptide Protein Res.*, 1978, **12**, 69.
[59] M. Bodanszky and J. Martinex, *J. Org. Chem.*, 1978, **43**, 3071.

conditions where basic conditions prevail, the phenacyl esters cannot be used, the risk being particularly great if the adjacent residue is aspartic acid, glycine, serine, or threonine. It was reassuring to see that when t-butyl esters were used for side-chain carboxy-group protection the rate of the side-reaction was much slower, in most cases giving no appreciable problem.

General Deprotection of Peptides. Several further studies have been made of the deleterious side-reactions which occur during acidolytic deprotection of tryptophanyl, tyrosyl, and methionyl peptides. The use of methane and ethane sulphonic acids in acetic acid or trifluoroethanol gives an efficient removal of *p*-methoxybenzyloxycarbonyl or t-butyloxycarbonyl group[60, 61] giving minimal side-reactions with tryptophan or methionine sulphoxide. Deprotection of Z(OMe)-Trp-OH by TFA gave four by products[62] as identified by n.m.r. and m.s. The major product coming from 2′-electrophilic attack on the indole ring; in contrast to the deprotection of Boc-Trp-OH where the 1′-t-butyl-substituted tryptophan is the major by product no 1′*p*-methoxybenzyl tryptophan was observed. The addition of thioanisole–2 % ethane dithiol–skatole or dimethyl-sulphide–2 % ethane dithiol–skatole as scavengers enabled a 97—99 % recovery of tryptophan to be achieved.

$$CF_3 \cdot CO \cdot O \cdot Bu^t$$

(25)

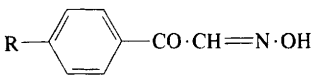
It has been shown[63] that under the conditions used for the removal of Boc or Bu^t esters by TFA the main alkylating agent is t-butyltrifluoroacetate (25). The ester (25), which may be easily formed by reaction of isobutylene with trifluoroacetic acid, is very rapidly generated at the start of the acidolytic cleavage of the t-butyl-based protecting groups. It reacts readily with methionine and tryptophan and any scavengers which may be present. From a detailed kinetic study it was concluded that very little protection was provided by the widely used scavenger, anisole; the highest degree of protection being shown by the use of ethane-1,2-dithiol. Attention was also drawn to the fact that the scavenger should be added to the TFA immediately prior to the cleavage reaction, as frequently it reacts with TFA thus becoming ineffective.

Thioanisole was found to be particularly useful for suppressing side-reactions during the removal of acid-labile groups from the hydroxy function of tyrosine.[64] In the presence of anisole *O*-Benzyltyrosine gave 13 % 3-benzyltyrosine when treated with triflormethanesulphonic acid in TFA (30 min at 0 °C) whereas in the presence of thioanisole only 0.8 % of the 3-substituted product was observed. Similarly, when treated with anhydrous hydrogen fluoride in the presence of thioanisole less than 0.3 % of the substituted tyrosine derivative was obtained.

[60] H. Ogawa, S. Funakoshi, and H. Yajima, ref. 1, p. 53.
[61] H. Yajima, N. Fujii, H. Ogawa, H. Irie, S. Shinagawa, and M. Fujino, ref. 1, p. 57.
[62] H. Ogawa, T. Sasaki, H. Irie, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 3144.
[63] B.F. Lundt, N.L. Johansen, A. Vølund, and J. Markussen, *Internat. J. Peptide Protein Res.*, 1978, **12**, 258.
[64] Y. Kiso, H. Isawa, K. Kitagawa, and T. Akita, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 2562.

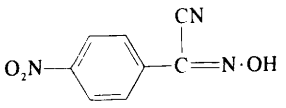
A study[65] of the removal of benzylic based protecting groups from a synthetic sample of protected insulin B-chain compared cleavage by (*a*) sodium in liquid ammonia, (*b*) anhydrous hydrogen fluoride, and (*c*) hydrogen bromide in TFA using ethanethiol as a scavenger. The results showed that deprotection using method (*c*) gave the cleanest product with the minimum number of side-reactions.

Formation of the Peptide Bond.—The most commonly employed coupling reagent in peptide synthesis, *N,N'*-dicyclohexylcarbodi-imide, has again been investigated mechanistically.[66] Using the coupling between tritiated *N*-formylphenylalanine and leucine methyl ester as a model system the formation of various intermediates was monitored. In the absence of a nucleophile after 1 hour 36 % of a compound which was tentatively identified as the *O*-acylurea was present along with 17 % symmetrical anhydride and 4 % azlactone. On prolonged reaction more symmetrical anhydride was formed at the expense of the *O*-acylurea, both of these activated compounds gave the dipeptide when leucine methyl ester was added as would be expected. It was concluded that the inactive *N*-acylurea is not formed by rearrangement of the *O*-acylurea and that under normal coupling conditions only about 1.5 % of the *O*-acylurea is present. Furthermore, amines were found to catalyse the formation of *N*-acylurea from carboxylic acid and dicyclohexylcarbodi-imide. It appears that there is considerable variation in the rate of formation of *N*-acylurea and that the rate is dependant on the size of the amino-acid

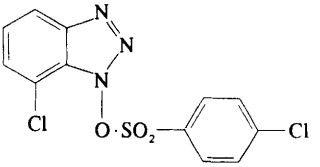


(26) R = F, Cl, Br or OMe

side-chain.[67] Isonitroso compounds of the type shown (26) have been used as additives in dicyclohexylcarbodi-imide couplings to suppress racemization.[68] From the brief details available it seems that electron donating groups in (26) give compounds which suppress racemization whereas electron withdrawing groups, for example in compound (27), give rise to additives which are not effective.



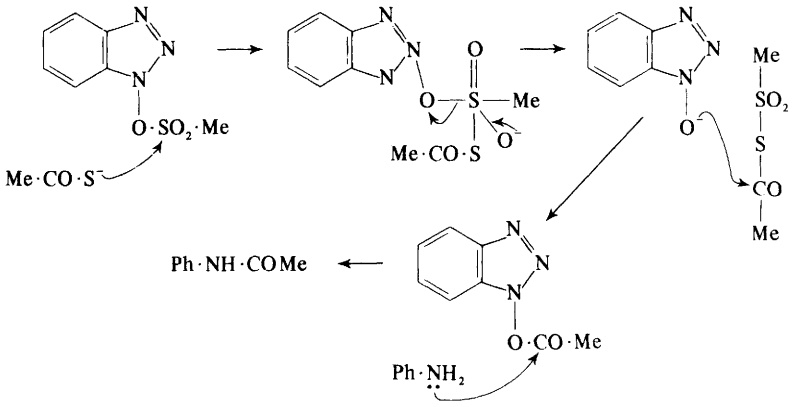
(27)



(28)

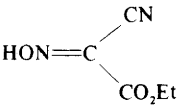
[65] G. Losse and W. Naumann, *J. prakt. Chem.*, 1978, **320**, 651.
[66] A. Arendt and A.M. Kotodziejczyk, *Tetrahedron Letters*, 1978, 3867.
[67] J. Izdebski, T. Kubiak, D. Kunce, and S. Drabarek, *Roczniki Chem.*, 1978, **52**, 539. (*Chem. Abs.*, 1978, **90**, 23 662q).
[68] J. Przybylski and H. Miecznikowska, *Roczniki Chem.*, 1978, **52**, 1179 (*Chem. Abs.*, 1978, **89**, 180 355p).

A range of sulphonates of strongly acidic *N*-hydroxy-compounds related to hydroxybenzotriazole was investigated[69] and the most useful was found to be (28). The reagent could be used either to prepare an active ester (essentially a two-step reaction) which could then be aminolysed, or in a one-pot coupling reaction. The formation of active-ester was rapid in both cases (1 h r.t. with NEt_3 as base) and using the first method the ester could be obtained in high isolated yield. The mechanism of the activation of carboxylate by such sulphonates has been studied using thioacetic acid in the presence of triethylamine. The mechanistic sequence follows the pathway outline in



Scheme 6

Scheme 6 demonstrating that nucleophilic attack does not take place at the 3-nitrogen with displacement of methanesulphonate. In a modified Bodansky test for racemization (28) gave 23.9 % racemization, however the addition of 2-hydroxyimino-2-cyanoacetate (29) reduced the figure to 11.3 %. Under the same conditions dicyclohexylcarbodi-imide gave 43.3 % racemization, reduced



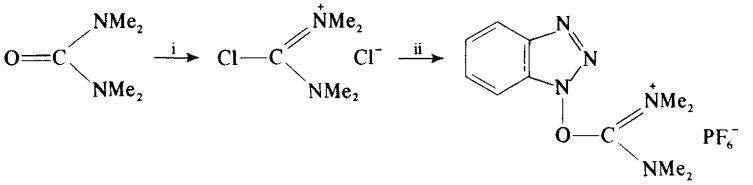
(29)

to 17.7 % on the addition of (29). It is interesting to note that dicyclohexylcarbodi-imide plus 6-chlorohydroxybenzotriazole was not effective in achieving a low level of racemization.

The coupling agent *O*-benzotriazolyl-*N*,*N*-tetramethyluronium hexafluorophosphate (30) (HBTU) was prepared[70] by the route outlined in Scheme 7, and a range of test dipeptides were synthesized in up to 90 % yield using equimolar

[69] M. Itoh, H. Nojima, J. Notani, D. Hagiwara, and K. Takai, *Bull. Chem. Soc. Japan*, 1978, **51**, 3320.

[70] V. Dourtoglou, J.-C. Ziegler, and B. Gross, *Tetrahedron Letters*, 1978, 1269.



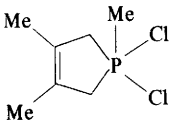
(30)

Reagents: i, $COCl_2$–toluene; ii, HOBt

Scheme 7

quantities of each component. The coupling which was believed to proceed through the hydroxybenzotriazolyl active-ester showed 25—39 % racemization in the Young test when acetonitrile was used as solvent.

The application of 1,3,4-trimethyl-Δ^3-phospholene-1,1-dichloride (31) as a coupling reagent has been described.[71] The cyclo-adduct which was prepared by reaction of 2,3-dimethylbuta-1,3-diene with methyl phosphonous dichloride,[72] was used to prepare four tripeptides by a fragment coupling approach. The dipeptide (32) was prepared from benzyloxycarbonylproline



(31)

Z-Pro-Leu-OMe

(32)

and leucine methyl ester hydrochloride using 1.1 equivalents of (31), 3 equivalents of triethylamine and approximately 0.5 equivalents of *N*-methylimidazole. The coupling yields varied from 30—70 % but unfortunately the Anderson test and the Young test both showed an appreciable level of racemization which would restrict the use of the reagent in actual peptide synthesis.

$$(EtO)_3P + Br_2 \longrightarrow (EtO)_2POBr$$

(33)

Conditions: − 30 — − 25 °C

Scheme 8

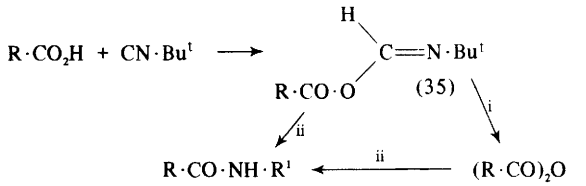
The use of diethylphosphobromidate (33) prepared by the route shown in Scheme 8 has been demonstrated.[73] The crude compound which is stable at room temperature was analytically and spectroscopically pure with further purification not being required. Reaction with protected amino-acids at 15 °C gave a mixed anhydride of the type (34) using 2,6-lutidine as a catalyst. Careful control of the reaction conditions allowed racemization to be minimized and the reagent was found to be particularly useful in the coupling of hindered amino-acids.

[71] E. Vilkas, M. Vilkas, and J. Sainton, *Tetrahedron Letters*, 1978, 2933.
[72] E. Vilkas, M. Vilkas, and J. Sainton, *Nouv. J. Chim.*, 1978, **2**, 307.
[73] A. Gorecka, M. Leplawy, J. Zabrocki, and A. Zwierzak, *Synthesis*, 1978, 474.



(34)

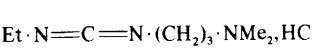
Coupling using t-butylisocyanide has been studied.[74] Initial attack of a protected peptide acid gave the intermediate (35) which could either react with excess carboxylate to give the symmetrical anhydride or with amino-component to yield a dipeptide (see Scheme 9). Very variable yields were observed using this method.



Reagents: i, RCO_2H; ii, R^1NH_2

Scheme 9

Symmetrical anhydrides are used extensively in solid-phase synthesis, being prepared *in situ* by interaction of *N*-alkoxycarbonylamino-acids with dicyclohexylcarbodi-imide. The use of the water-soluble carbodi-imide (36) has permitted symmetrical anhydrides to be isolated in a high state of purity.[75] 2

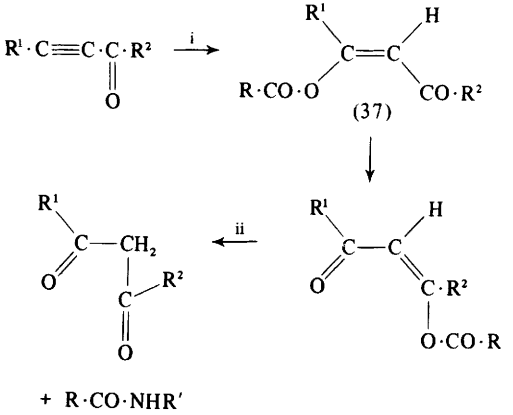


(36)

Moles of the carboxylic acid are treated with 1 mole of the di-imide (36) in dichloromethane at 0 °C. Washing with dilute acid and bicarbonate removed the corresponding urea and any *N*-acylurea which might have been produced, leaving the pure symmetrical anhydride in the organic phase. The isolated anhydrides were all stable at −5 °C but the *N*-t-butyloxycarbonyl anhydrides were more unstable than the corresponding *N*-benzyloxycarbonyl compounds; in fact the t-butyloxycarbonyl anhydrides were totally decomposed within 5 h at room temperature (23 °C). The reagent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) which acts through the formation of the ethoxycarbonyl mixed anhydride was used to prepare optically pure benzoyl and acetyl amino-acid anilides and *p*-nitroanilides.[76]

The use of 'push–pull' olefins in amide-bond formation has been described.[77, 78] These compounds which contain an electron donating group at

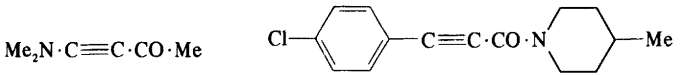
[74] H. Aigner and D. Marquarding, *Tetrahedron Letters*, 1978, 3325.
[75] F.M.F. Chen, K. Kuroda, and N.L. Benoiton, *Synthesis*, 1978, 928.
[76] F.M.F. Chen and N.L. Benoiton, *Canad. J. Biochem.*, 1978, **56**, 38.
[77] M. Neuenschwander, U. Lienhard, H.-P. Fahrai, and B. Hurni, *Helv. Chim. Acta*, 1978, **61**, 2429.
[78] U. Lienhard, H.-P. Fahrai and M. Neuenschwander, *Helv. Chim. Acta*, 1978, **61**, 1609.



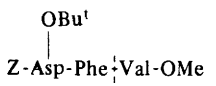
Reagents, i, $R.CO_2H$; ii, $R'.NH_2$

Scheme 10

one end of the acetylenic bond and an electron withdrawing group at the other form enol esters (37) when treated with carboxylic acids (*N*-protected amino-acids) according to Scheme 10. The acetylenes (38)[79] and (39)[80] were both used to prepare dipeptides in very high yields (80—90 %), with either reagent, hydroxylic side-chains did not require protection and the observed level of racemization was low. In the Anderson test (39) gave 1—2 % racemization and



(38) gave no observable racemization; for (39) the Young test showed the racemization to be 4.7 % in dichloromethane and 12.9 % in dimethyl-formamide. Using a g.c. based method racemate levels of 2.1 % (CH_2Cl_2) to 2.7 % (DMF) were detected in the preparation of the tripeptide (40) coupling at

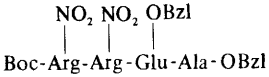


the point indicated. The high yields and low levels of racemization indicate that the 'push–pull' acetylenes could well have considerable potential in peptide synthesis although they still need to be evaluated in the preparation of larger peptides.

Comparisons of various coupling methods have been made by several authors. In a fragment combination approach on a polystyrene polymeric support dicyclohexylcarbodi-imide with pentafluorophenol or 2,2′dipridydi-

[79] H.J. Gais, *Angew. Chem. Internat. Edn.*, 1978, **17**, 597.
[80] M. Neuenschwander, H.-P. Fahrni, and U. Lienhard, *Helv. Chim. Acta*, 1978, **61**, 2437.

sulphide plus triphenyl phosphine gave the highest yields[81] for the synthesis of ovine insulin B-chain. The use of dicyclohexylcarbodi-imide plus hydroxybenzotriazole has also been claimed to give the best yields in the synthesis of the insulin B-chain.[82] The pentafluorophenyl active-ester gave the highest yield

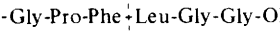


(41)

of the tetrapeptide (41) when eight coupling methods were examined[83] in the formation of the arginylarginine bond. The infrequently used coupling reagent *N,N'*carbonyldi-imidazole has been shown to react with *C*-protected peptides at the amino-terminus giving the corresponding hydantion: the reaction must therefore be anticipated as a side-reaction in couplings involving this reagent.[84]

The use of 2-nitro-4-sulphophenyl esters,[85] 2-fluoro-1,3,5,-trinitrobenzene[86] and an insoluble poly-*p*-succinimido-*o*-nitrophenol[87] in peptide synthesis have also been described.

The revival of interest (see also Vol. 10 of these Reports, p. 326) in peptide bond formation by the reverse action of proteolytic enzymes continues. Most attention has focused on the use of α-chymotrypsin,[88—90] this enzyme catalyses peptide-bond formation between *N*-acylated aromatic amino-acids and hydrophobic amino-acids as the carboxyl component. In order to obtain high yields of product the coupling should be carried out at neutral pH, with a relatively high concentration of the enzyme; a large excess (10-fold) of one of the components also promotes efficient reaction. The main reason for this is to force the equilibrium to the right thus causing precipitation of product; if the product was soluble in the Tris buffer no synthesis was observed as the equilibrium position could not be displaced. The method was used to prepare several dipeptides in high yield,[89] and also to effect the preparation of the



(42)

hexapeptide (42) in 48 % yield.[88] Although in many examples a totally aqueous system was used, in others the addition of organic co-solvents *e.g.* 10 % dimethylformanide[88] was required. A detailed study of the effects of adding organic co-solvents, up to 85 % butanediol or 60 % glycerol, on the equilibrium constant in hydrolytic situations was evaluated for chymotrypsin

[81] K.D. Kaufmann and R. Dolling, *J. prakt. Chem.*, 1978, **320**, 814.
[82] G. Losse, H. Stange, and B. Schwenzer, *Z. Chem.*, 1978, **18**, 97.
[83] K. Neubert and H.D. Jakubke, *J. prakt. Chem.*, 1978, **320**, 217.
[84] F. Esser and O. Roos, *Angew. Chem. Internat. Edn.*, 1978, **17**, 467.
[85] A.A. Gershkovich and S.B. Serebryany, *Bioorg. Khim.*, 1978, **4**, 1129.
[86] K. Inonata, H. Kinoshita, H. Fukuda, K. Tanabe, and H. Kotake, *Bull. Chem. Soc. Japan*, 1978, **51**, 1866.
[87] S.V. Rogzhin, Yu.A. Davidovich, S.M. Andreev, and T.A. Mrachkovskaya, *Bioorg. Khim.*, 1978, **4**, 599.
[88] K. Morihara and T. Oka, ref. 1, p 9.
[89] T. Oka and K. Morihara, *J. Biochem. Japan*, 1978, **84**, 1277.

and trypsin.[90] The equilibrium constant for the hydrolytic reaction drops dramatically on addition of organic solvents, consequently the addition of such solvents tends to promote the back synthetic reaction. It was suggested that h.p.l.c. should be used as a tool for studying the reaction mixtures in order to maximize the rate of the synthetic reaction.

Pepsin has also been used for peptide-bond formation[91] between aromatic amino-acid residues. *N*-benzyloxycarbonyl may be used for amino-protection but *N*-t-butyloxycarbonyl protected amino-acids did not couple at all. A high degree of stereospecificity was observed as *N*-benzyloxycarbonyl-D,L-phenylanine coupled with L-phenylalanine amide to give only the L,L product, the D-phenylalanine derivative being isolated from the reaction mixture. Thus this enzymic method may be used for the preparation of optically pure peptides which may be used as optical standards, also it may be used to resolve *N*-protected racemic mixtures of amino-acids.

$$\text{Z-Leu-Gln-Gly}\overset{\downarrow}{}_{1}\text{Leu-Val-NH}_2$$

(43)

A microbial metalloproteinase (prolisin) has been used[92] in the fragment coupling indicated (43) to prepare the 23—27 portion of secretin. Small amounts of organic solvents (dioxane, n-butanol, or methanol) promoted the reaction which was carried out in the presence of a serine proteinase inhibitor obtained from potato. As has been found with other enzymic methods t-butyl oxycarbonyl peptides could not be used whereas the corresponding benzyloxy-carbonyl peptides gave a maximum yield of 87 % when Tris–HCl plus dioxane was used as the solvent. The product was removed by filtration several times during the coupling reaction.

Racemization.—Some of the data relating to individual coupling methods were discussed in the previous section. A general discussion is also presented in a review by Lebl and Jost.[93]

A particularly interesting paper has been published which attempts to establish a quantitative relationship between the structure of the amino acids being coupled and the degree of racemization.[94] The investigation was carried

$$\text{Boc-A}_i\text{-A}_j\text{-OH} + \text{H}_2\text{N-A}_k\text{-OMe} \longrightarrow \text{Boc-A}_i\text{-A}_j\text{-A}_k\text{-OMe}$$

where: A_i = residue which may participate in oxalone formation
A_j = activated amino-acid
A_k = amino component

Scheme 11

out on various model systems according to Scheme 11, the degree of epimerization was obtained by integration of the methyl ester signal for the diastereoisomeric mixture, Boc-L-A_i-L-A_j-L-A_k-OMe and Boc-L-A_i-D-A_j-L-A_k-OMe in the ^{1}H n.m.r. spectrum at 250 mHz. Usually a separation of

[90] G.A. Homandberg, J.A. Mattis, and M. Laskowski, jun., *Biochemistry*, 1978, **17**, 5220.
[91] A. Pellegrini and P.L. Luisi, *Biopolymers*, 1978, **17**, 2573.
[92] Y. Isowa, T. Ichikawa, and M. Ohmori, *Bull. Chem. Soc. Japan*, 1978, **51**, 271.
[93] M. Lebl and K. Jost, *Chem. Listy*, 1978, **72**, 252 (*Chem. Abs.*, 1978, **88**, 191 360s).
[94] B. Castro, J.R. Dormoy, and D.Le Nguyen, *Tetrahedron Letters*, 1978, 4419.

greater than 3 Hz. was observed, with 7—10 Hz. being more general; it was found that for phenylalanine an impractical chemical shift difference of less than 2 Hz was observed and in contrast to earlier work[95] it was concluded that the presence of phenylalanine was not required in order to observe a chemical shift difference for the methyl ester signals. Using benzotriazolyloxytris (dimethylamino) phosphonium hexafluoro phosphate (BOP reagent) thirteen reference tripeptides were prepared containing five non-functional amino-acids, this allowed prediction of the level of racemization for 125 couplings, the preparation of 52 references would allow calculation of racemization for all the protein amino-acids (excluding glycine and proline) *i.e.* 5832 cases. An excellent agreement was obtained for the predicted value of racemization as compared to that found in practice as shown in Table 3. The 'epimerization

Table 3 *Predicted and observed values of racemization*[94] *for coupling between residues 2 and 3 in tripeptides*

	Predicted	*Observed/%*
Phe-Ala-Val	7.4	7.5
Phe-Ala-Leu	4.5	4.5
Phe-Ala-Ile	5.3	5.3
Val-Ile-Val	34	32.5
Leu-Val-Ile	15	11.4
Val-Val-Leu	18.9	17.2

$$\rho_{ijk} = \frac{LDL}{LLL} = \frac{\rho_{ioo} \times \rho_{ojo} \times \rho_{ook}}{\rho_{ooo}^2} \qquad (44)$$

ratio' ρijk may be obtained from the expression (44) which was derived from an analysis of the kinetics[96] of the rate of coupling *versus* epimerization. It remains to be seen whether or not such a predictive theory can be applied to amino-acids with functionalized side-chains; if generally applicable it would allow the coupling points in fragment couplings to be chosen so as to reduce the racemization to an absolute minimum.

It has also been found that the extent of racemization depends on the configuration of the amino-acids undergoing coupling.[97] Thus when acetyl-L-leucine was coupled with L-leucine methyl ester using dicyclohexylcarbodi-imide 40 % racemization was observed whereas coupling with D-leucine methyl ester gave 13 % racemization. In the case of the L–L coupling the rate of epimerization was high compared to the rate of coupling but in the L–D case the rate of epimerization was relatively low.

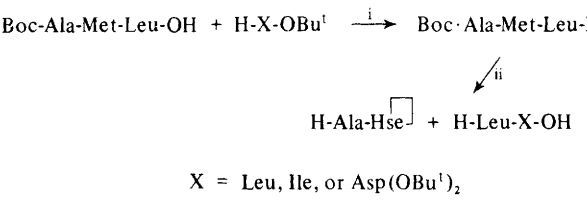
The peptide (45) has been developed as a test peptide for racemization in solid-phase synthesis.[98] The single D-amino-acid diastereoisomers were all separable on a standard amino-acid analyser using a sulphonated polystyrene resin eluted with 0.2M citrate buffer. Colour yields were determined using

[95] J.S. Davies and M.N. Ibrahim, *Tetrahedron Letters*, 1977, 1453.
[96] P.S. Kemp, S.L.H. Choong, J. Pekoak, *J. Org. Chem.*, 1974, **39**, 3841.
[97] A. Arendt, A.M. Kołodziejczyk, and T. Sokotowska, *Tetrahedron Letters*, 1978, 2711.
[98] S.B.H. Kent, A.R. Mitchell, G. Barany, and R.B. Merrifield, *Analyt. Chem.*, 1978, **50**, 155.

H-Leu-Ala-Gly-Val-OH (45)

Boc-Val-O·CH_2·Pam·Resin (46)

standard peptides and a detection limit of 0.01 % could be achieved. Using this method it was shown that when the test peptide (45) was prepared starting from (46)[99] no racemization (less than 0.02 %) was observed. This finding demonstrates the fact that racemization does not cause a problem when stepwise solid-phase synthesis is carried out.



X = Leu, Ile, or Asp(OBu^t)$_2$

Reagents: i, coupling reagent; ii, CF_3CO_2H; CN.Br/AcOH

Scheme 12

A new test method for determining racemization is based on the approach outlined in Scheme 12.[100, 101] The leucyl dipeptides may be analysed using the amino-acid analyser, allowing separation of any diastereoisomers which may have been formed. Various coupling methods are compared and it is claimed that the use of dicyclohexylcarbodi-imide plus additives, especially *N*-hydroxy-5-norbornene-2,3-dicarboxamide (HONB), gave results which are superior to those obtained by the azide method. It is interesting to note that significant racemization was observed in some of the known coupling methods which have been found, by the Young or Anderson test, to be free of racemization. The separation of diastereoisomeric dipeptides by liquid chromatography has also been reported.[102]

A study of racemization during the synthesis of fragments of α-bungarotoxin has been made[103] using a method employing g.c. on capillary columns. It was shown that during the hydrolysis of several proteins racemization of some amino-acids, particularly proline, aspartic acid, and methionine may occur. In addition it was found that acid hydrolysis of protected α-bungarotoxin gave rise to a much larger degree of racemization than when the free protein was hydrolysed. For example approximately 30 % D-valine was found in the protected peptide but only 0.99 % in the corresponding deprotected material. This finding establishes the important principle, that protecting groups should always be removed from synthetic peptides before acid hydrolysis, when the hydrolysate is to be used for the determination of racemization.

[99] A.R. Mitchell, B.W. Erickson, M.N. Ryabtsev, R.S. Hodges, and R.B. Merrifield, *J. Amer. Chem. Soc.*, 1976, **98**, 7357.

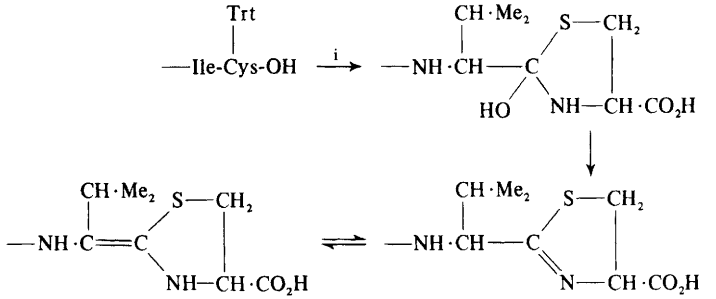
[100] M. Fujino, C. Kitada, and I. Yoshida, ref. 1, p. 28.

[101] C. Kitada and M. Fujino, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 585.

[102] N.A. Samoilova, S.M. Andreev, V.A. Tsiryapkin, Yu.A. Davidovich, S.V. Rogozhin, *Bioorg. Khim.*, 1978, **4**, 725.

[103] S.V. Vitt, M.A. Myagkova, M.B. Saporovskaya, S.B. Nikitina, and V.M. Belikov, *Bioorg. Khim.*, 1978, **4**, 154.

The racemization during acid hydrolysis of residues adjacent to a *C*-terminal cysteine residue has been shown to be particularly facile.[104] Using a g.c.–m.s. method it was found that the hydrolysis proceeds through a thiazoline derivative as indicated in Scheme 13. The initially formed thiazoline makes the



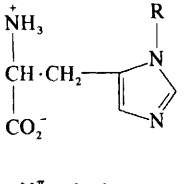
Reagent: i, H^+

Scheme 13

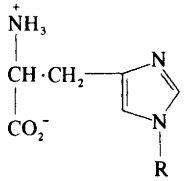
α-hydrogen on the adjacent residue particularly labile and thus give rise to racemization at this position. It is becoming increasingly clear that g.c. utilizing capillary columns with chiral stationary phases is a very useful method of analysing racemic mixtures. *N*-Lauryl valine t-butylamide[105] and polysiloxanes with bonded amino-acids[106] have both been used in the context, although the latter might find more general usage owing to its much greater stability to high temperatures (up to 240 °C).

In a study of the racemization of histidine containing peptides[107] it was found that N^{α},N^{im}-protected histidine with benzyloxycarbonyl, t-butoxycarbonyl, or *p*-toluene sulphonyl for side-chain protection could be coupled with very little racemization using DMF or dichloromethane as solvent; however, N^{im}-benzyl gave a higher level of racemization. The increased level of racemization was concluded to be due to insufficient masking of the basic imidazole function by the benzyl group. The situation is, however, rather more complex, as has been demonstrated by Jones and Ramage.[108] For most histidine derivatives it is unclear which of the two imidazole nitrogen atoms is bonded to the substituent, the two possibilities are fundamentally different as can be seen from (47). Most, if not all direct acetylations, tosylations, and benzylations of the imidazole ring of histidine give rise to the τ substituted product. However by the routes outlined in Scheme 14 it has been possible to prepare both N^{π} and N^{τ} substituted derivatives, the configuration being confirmed using an n.m.r. method.[109] When N^{α}-benzyloxycarbonyl-N^{τ}-phenacyl-L-histidine was

[104] W. Woiwode, H. Frank, G.J. Nicholson, and E. Bayer, *Chem. Ber.*, 1978, **111**, 3711.
[105] H. Frank, G.J. Nicholson, and E. Bayer, *J. Chromatog. Sci.*, 1977, **15**, 174.
[106] H. Frank, G.J. Nicholson, and E. Bayer, *Angew. Chem. Internat. Edn;* 1978, **17**, 363.
[107] S. Terada, A. Kawabata, N. Mitsuyasu, H. Aoyagi, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1978, **51**, 3409.
[108] J.H. Jones and W.I. Ramage, *J.C.S. Chem. Comm.*, 1978, 472.
[109] H.R. Mathews and H. Rapoporte, *J. Amer. Chem. Soc.*, 1973, **95**, 2297.

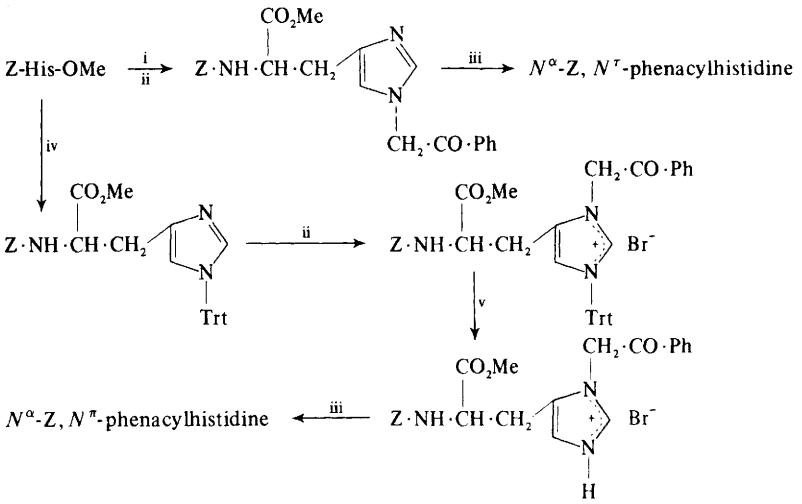


N^{π}-substituent



N^{τ}-substituent

(47)



Reagents: i, $AgNO_3$; ii, $Ph.CO.CH_2.Br$; iii, NaOH; iv, trt.Cl, 3 days; v, $HOAc–H_2O$, 100 °C, 10 min

Scheme 14

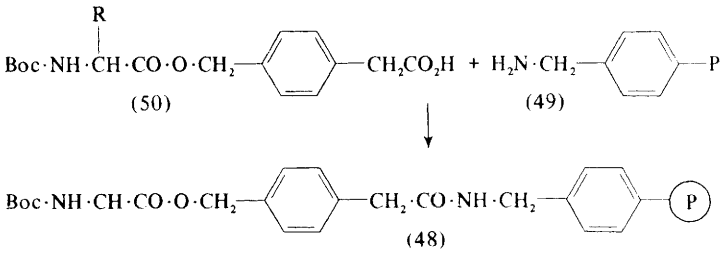
coupled with proline amide hydrochloride by the dicyclohexycarbondi-imide method in the presence of triethylamine, 35% D-histidine formation was observed using an enzyme digest method. With the $N\pi$-phenacyl derivative, however, only 2 % D-histidine was observed. It appears that the lone pair of electrons on the π nitrogen are implicated in the racemization of histidine peptides.

The use of additives in couplings mediated by dicyclohexylcarbodi-imide has prompted many investigations. A novel new approach has been the use of Lewis acids as additives[110] which cannot form reactive intermediates of the active ester type but still have a considerable ability to suppress racemization. The addition of two equivalents of zinc chloride gave the best results with an 80 % yield of optically pure product being obtained in the synthesis of the Anderson test peptide. Other Lewis acid additives also gave low racemization levels but frequently handling difficulties made their use impractical.

[110] H.D. Jakubke, Ch. Kleben, E. Berger, and K. Neubert, *Tetrahedron Letters*, 1978, 1497.

Repetitive Methods of Peptide Synthesis.—*Solid-phase synthesis.* A book entitled 'Aspects of the Merrifield peptide synthesis' has been written by Birr[111] and discusses solid-phase synthesis from a practical viewpoint. The contents include a discussion of the nature of the polymeric support, automation, and ends with a critical look at the applicability of Merrifield synthesis. A general review of insoluble polymeric supports has also appeared although only a relatively small section deals with peptide synthesis.[112]

In the period covered by this review development of new supports appears to have declined and more attention has been focused on the actual use of established solid-phase methods in the synthesis of peptides. The reasons for this may be two-fold; firstly as new analytical and preparative methods become available it is easier to check and maintain the quality of synthetic peptides, this being equally true whether the compounds are prepared by solid-phase or solution methods. Secondly for the synthesis of moderately sized peptides, *e.g.* the endorphins, a solid phase synthesis is generally much quicker and more economical although considerable attention must be directed towards checking the integrity of the final product. There is no doubt that the rapid exploration of many areas of fundamental biological importance would not have taken place if it were it not for the development of the solid-phase method.

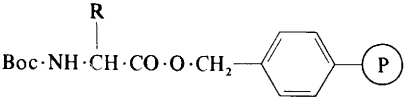


Reagents: i, DCCI

Scheme 15

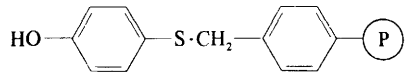
The use of *N*-t-butoxycarbonyl-4-oxymethylphenylacetamidomethyl polystyrene resin (48) in synthesis has been described in greater detail.[113] Scheme 15 shows the way in which the unit (48) may be prepared *via* the aminomethyl-polystyrene resin (49).[114] The intermediate (50) was obtained by reaction of the appropriate *N*-t-butoxycarbonyl amino-acid with 4-(bromomethyl)-phenylacetic acid phenacyl ester followed by removal of the phenacyl function by treatment with zinc in acetic acid. This benzylic ester linkage was 100-times more stable to refluxing trifluoroacetic acid than the conventional benzylic ester linkage; (51), this stability being due to the inductive effect of the

[111] C. Birr, 'Reactivity or Structure', Vol. 8, Aspects of the Merrifield peptide synthesis, Springer-Verlag, New York, 1978.
[112] C.C. Leznoff, *Accounts Chem. Res.*, 1978, **11**, 327.
[113] A.R. Mitchell, S.B.H. Kent, M. Engelhard, and R.B. Merrifield, *J. Org. Chem.*, 1978, **43**, 2845.
[114] A.R. Mitchell, S.B.H. Kent, B.W. Erickson, and R.B. Merrifield, *Tetrahedron Letters*, 1976, 3795.



(51)

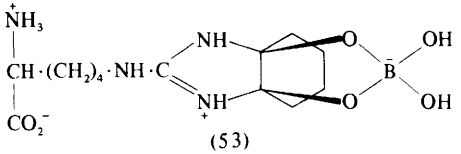
p-acetamidomethyl function. Cleavage may readily be achieved using anhydrous hydrogen fluoride–anisole (9 : 1) in 30 minutes at 0 °C. The utility of the resin was demonstrated by the synthesis of the 111–124 sequence of ribonuclease A. The authors suggested that similar linkages might be used in connection with polyamide based resins or with polethylene glycol in liquid, phase synthesis.



(52)

The resin (52) developed by Marshall and Liener[115] has been used in a partially automated synthesis of the 66—80 portion of horse heart cytochrome *c*.[116] The penultimate residue, N^{α}-t-butoxycarbonyl-N^{ε}-2-chlorobenzyl-oxycarbonyl-lysine, was coupled using dicylohexylcarbodi-imide and any remaining hydroxy-functions acylated with acetic anhydride. After addition of the remainder of the sequence the resin was oxidized with *m*-chloroperbenzoic acid and allowed to react with two equivalents of methionine sulphoxide-t-butoxycarbonylhydrazide, the activated resin ester linkage was cleaved giving an 85 % yield of the protected peptide. It is claimed that using the partially automated synthesizer the cost is dramatically reduced (compared to an automated synthesizer) and the risk of error is also significantly reduced.

The poly(trifluorochloroethylene-g-chloromethyl)styrene resin has been used in the synthesis of the 1—34 fragment of human parathyroid hormone.[117] This synthesis used a straightforward approach with benzylic-based side-chain protection, the imidazole group of histidine being protected as the tosyl derivative. The synthetic hormone fragment was modified at arginines 20 and 25 by reaction with cyclohexane-1,2-dione to give the borate stabilized



(53)

derivative shown (53). Using this modification it was shown that these arginine residues were important in the receptor binding interaction as only 16 % of the activity of the unmodified material was retained.

As described above (p. 336) the use of preformed symmetrical anhydrides probably provides the best means of acylating the extending peptide chain. It

[115] D.L. Marshall and I.E. Liener, *J. Org. Chem.*, 1970, **35**, 867.
[116] T.W. Kirby and P.K. Warme, *Analyt. Biochemistry*, 1978, **85**, 367.
[117] M. Rosenblatt, G.L. Shepard, A. Taylor, and J.T. Potts jun., *Biochemistry*, 1978, **17**, 3188.

has been found that using the preformed anhydride and polystyrene resins, a reaction time of only 45—75 minutes is required which is appreciably shorter than that required using the t-butoxycarbonylamino-acid with dicyclohexylcarbodi-imide.[118] The use of preformed symmetrical anhydrides used in conjunction with the basic Merrifield resin gave good crude yields of various apolipoprotein fragments but considerable purification was required. Losses from the resin by diketopiperazine formation constituted an appreciable problem in the preparation of several fragments, for the 147—168 fragment

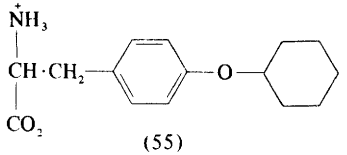
H-His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala-Pro-OH

(54)

(54) a loss of 0.029 mmol g^{-1} out of a total of 0.131 mmol g^{-1} was observed. The extent of this side-reaction was almost certainly due to the presence of proline at the *C*-terminus facilitating diketopiperazine formation.

The base used for the liberation of the free amino-group often needs careful consideration. In many examples such as in the syntheses of the apolipoprotein fragments[118] di-isopropylethylamine has been used in excess. Blake and Li,[119] however, in the synthesis of the *C*-terminal plasmin fragments of human somatrotropin (135—191, 140—191, and 145—191) found that better results were obtained by using *N*-methylmorpholine for the bulk of the neutralization and then completing it with di-isopropylethylamine. This procedure lessens the danger of base catalysed succinimide formation.

A more detailed account (see p. 330 of last year's report) of the acid-stability of several alkyl ethers of tyrosine has been published.[120] It concludes that

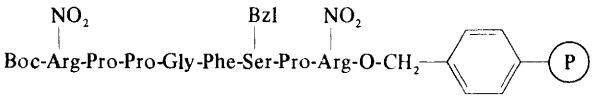


(55)

O-cyclohexyltyrosine (55) is the most useful derivative studied, as in a synthesis of angiotensin II less than 0.3 % of the rearranged 3-cyclohexyltyrosine was found in contrast to 2.6 % for the frequently used 2,6-dichlorobenzyltyrosine.

Additional information regarding the potassium cyanide catalysed transesterification of resin benzyl esters using methanol, ethanol, or benzyl alcohol has been reported.[121] As previously described, transesterification using the dry alcohol may be achieved in 24—48 h in the presence of 1 % potassium cyanide. No racemization and no α- to β-aspartyl rearrangement was observed, although it was found that with hindered alcohols the transesterification reaction was slow and some racemization was observed. With 95 % aqueous alcohol transesterification and subsequent hydrolysis occurs, side-chain ester functions were also transesterified but not hydrolysed.

[118] D.J. Kroon and E.T. Kaiser, *J. Org. Chem.*, 1978, **43**, 2107.
[119] J. Blake and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 315.
[120] M. Engelhard and R.B. Merrifield, *J. Amer. Chem. Soc.*, 1978, **100**, 3559.
[121] G. Moore and D. McMaster, *Internat. J. Peptide Protein Res.*, 1978, **11**, 140.

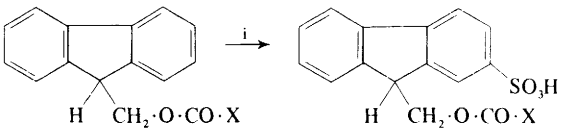


(56)

Catalytic transfer hydrogenolysis has been used to cleave protected resin-bound bradykinin (56).[122] The hydrogenolysis was carried out at atmospheric pressure at 70 °C, using palladium black generated *in situ*. A crude yield of 60 % was obtained after 4 h when cyclohexene was used as the hydrogen donor, under these conditions benzyloxycarbonyl, benzyl, and nitro functions are also removed.

The acidolytic removal of the t-butoxycarbonyl group during solid-phase synthesis has been studied[123] using the synthesis of porcine LHRH as a model system. The use of 0.5 M-HCl in formic acid (10-fold excess) was found to be most effective and scavengers were not required even in the presence of a tryptophan residue. However, during the build up of the peptide it was found that a greater loss from the resin was observed than when HCl in acetic acid was employed, although the latter required the use of 1 % mercaptoethanol as scavenger. Both deprotection methods gave identical products after final cleavage from the resin with hydrogen fluoride in the presence of phenol.

The 9-(2-sulpho)-fluorenylmethyloxycarbonyl (sulfmoc) group has been introduced by Merrifield and Bach[124] as an acidic handle by which peptides synthesized using solid phase methods may be purified. The group is introduced using the corresponding chloride or nitrophenyl ester, both of which may be obtained directly from the corresponding unsubstituted Fmoc



X = Cl or 4-nitrophenoxy

Reagent: i, $Cl.SO_3H$

Scheme 16

derivative by chlorosulphonation according to Scheme 16. The sulfmoc group is stable to hydrogen fluoride, hydrogen bromide, and pyridine but is easily removed by anhydrous morpholine or piperidine, alternatively aqueous triethylamine, sodium carbonate, or sodium hydroxide may be used. The group is attached as the last step in a solid-phase synthesis prior to deprotection, thus all terminated sequences will not have the acidic label. After cleavage and deblocking by anhydrous hydrogen fluoride the acidic labelled chain may be purified by ion exchange chromatography on a weakly basic

[122] S.A. Khan and K.M. Sivanandaiah, *Synthesis*, 1978, 750.
[123] H. Yonezawa, N. Takahashi, M. Ohno, and N. Izumiya, *Internat. J. Peptide Protein Res.*, 1978, **11**, 19.
[124] R.B. Merrifield and A.E. Bach, *J. Org. Chem.*, 1978, **43**, 4808.

resin. The product may be liberated from the resin by an ionic strength gradient, alternatively the sulfmoc group may be cleaved on the column and the free peptide eluted. The principle has been tested on small trial peptides and a real demonstration of its usefulness is awaited with interest.

Techniques for Monitoring Solid-phase Synthesis. A modified Edman degradation procedure has been developed whereby the resin-bound fully protected peptide may be analysed.[125] In addition to the usual phenylthiohydantoins which are well known those shown in Table 4 were also

Table 4 *PTH derivatives of side-chain protected amino-acids*[125]

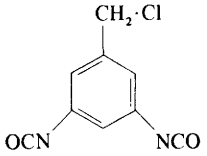
N^{ω}-nitro-arginine
β-benzylaspartic acid
γ-benzylglutamic acid
O-benzyl-serine,-threonine, and -tyrosine
2,4-dinitrophenylhistidine
N^{ϵ}-2-chlorobenzyloxycarbonyl-lysine
N^{ϵ}-benzyloxycarbonyl-lysine
S-acetamidomethylcysteine
S-benzylcysteine

prepared. The method used a peptide sequenator in combination with thin layer chromatography and gas liquid chromatography to identify the PTH derivatives; by this means protected 1—34 human parathyroid hormone and valine[8]-human calcitonin were sequenced.

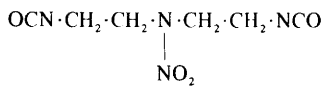
A method of quantitatively controlling a solid-phase synthesis by hydrazinolysis of small samples and subsequent chromatography on Sephadex G15 in dimethylformamide has been described.[126] Although small peptides differing by one amino-acid may be separated on Sephadex G15 it is clear that when the chain reaches a reasonable length little separation will be observed. Thus the method would probably not be of great value as a means of monitoring solid-phase syntheses unless an alternative method of separation was developed for larger peptide hydrazides.

Other Repetitive Methods. The liquid-phase method continues to attract considerable attention and several developments in terms of modified or new soluble supports have been made. The synthesis of soluble block co-polymers with functional groups at defined intervals has been described.[127] The bis-isocyanates (57) and (58) were used to form cross-links between polyoxyethylene chains of various lengths by reaction with the appropriate polyethlene glycol. The resulting co-polymers being soluble in chloroform, dichloromethane, dimethylformamide, and water. The tetrapeptide (59) was synthesized using symmetrical anhydrides in three-fold excess. The actual linkage to the support being formed through the compound (60), in the case of the polymer derived from (58) reduction of the nitro function was carried out to enable an amide linkage to be formed. Final cleavage from the soluble support

[125] D.J.P. Ranlais, P. Rivaille, B. Daguilhanes, G.J.G. Lefévre, and G. Milhaud, *J. Chem. Research* (M), 1978, **2**, 0267.
[126] K.D. Kaufmann and F. Raddatz, *J. prakt. Chem.*, 1978, **350**, 847.
[127] E. Bayer, I. Gatfield, H. Mutter, and M. Mutter, *Tetrahedron*, 1978, **34**, 1829.



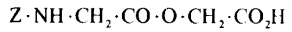
(57)



(58)

Z-Ile-Ala-Val-Gly-OH

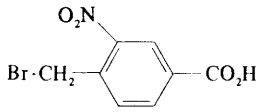
(59)



(60)

was achieved by alkaline hydrolysis which cleaved the ester linkage introduced by compound (60).

A photolabile support for use in liquid phase synthesis has been prepared[128] by esterification of the carboxylic acid (61) with polyethylene glycol (mol. wt.



(61)

6000), the coupling being mediated by dicyclohexylcarbodi-imide in the presence of hydroxybenzotriazole. The *C*-terminal residue was attached by heating in an appropriate solvent for five days in the presence of di-isopropylethylamine. Several small peptides were prepared as models of the double-stranded coil structure of tropomyosin using dicyclohexylcarbodi-imide couplings of t-butoxycarbonylamino-acids employing 50 % trifluoro-acetic acid in the deprotection steps. The final product was cleaved from the resin by photolysis at 350 nm in methanol. Up to 18 h may be required for complete cleavage depending on the hydrophobicity of the peptide. The resin linkage could also be cleaved by treatment with hydrazine should the protected peptide hydrazide be required.

Direct esterification of polyethylene glycol with amino-acids in the presence of *p*-toluene sulphonic acid has been reported.[129] A yield of up to 85 % may be achieved using an azeotropic distillation with benzene over 2—3 days to remove water. Similar conditions may be used to esterify hydroxymethyl-polystyrene but arginine, glycine, and histidine do not give satisfactory results.

$(BOC\text{-}Gly\text{-}Phe\text{-}O)_2PEG$

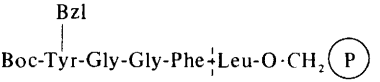
(62)

It is interesting to note that the ester linkage in (62) could be cleaved by chymotrypsin, thus introducing the possibility of an alternative method of cleavage. The conformation of peptide chains during stepwide synthesis on

[128] F.-S. Tjoeng, E.K. Tong, and R.S. Hodges, *J. Org. Chem.*, 1978, **43**, 4190.
[129] C.S. Pande and J.D. Glass, *Tetrahedron Letters*, 1978, 4745.

solubilizing polymeric supports has been investigated using ^{13}C n.m.r.[130] It was found that the length of the polyoxyethylene chain had a marked effect on the overall structure and at high resolution distinct differences between random-coil and α-helical conformations could be observed.

A soluble chloromethylpolystyrene has been prepared[131] by co-polymerization of *m*- and *p*-chloromethylstyrene (isomer ratio 7 : 3) with styrene in a molar ratio of 1 : 9. The polymer was soluble in dimethylformamide but was precipitated by pouring into methanol and adding brine. A study of fragment combination on this support was carried out using various reagents to prepare protected leucine enkephalin (63) coupling at the bond indicated, the coupling

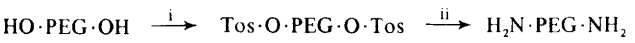


(63)

Table 5 *Yields of* (63) *using various coupling methods*[131]

Reagent	*Yield/%*
Dicyclohexylcarbodi-imide–hydroxybenzotriazole	96—97
Diethoxyphosphoryl cyanide	99
Triphenylphosphine–dipyridyldisulphide	86

yields being shown in Table 5. Using this soluble support the rate of acylation was of the same order as that found in normal solution synthesis, that is approximately 100-times faster than found in a conventional solid-phase synthesis. However the rate of acylation appears to be independent of chain-length, thus protected amino-acids coupled at a rate similar to that found for protected peptide fragments. The synthesis of polymeric reagents which remain in solution during the reaction but may be removed by precipitation during work up have been developed.[132] A derivative of polyethylene glycol in which the terminal hydroxy-functions have been replaced by amino-groups



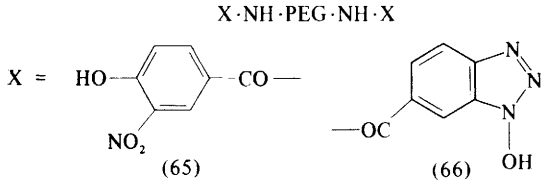
(64)

Reagents: i, Tos.Cl–pyridine ii, 3 eq. potassium phthalimide, $NH_2.NH_2$

Scheme 17

(64) has been prepared by the route shown in Scheme 17. This derivative was then converted into the polymeric intermediates (65), (66), and (67) which have obvious potential in peptide synthesis. The use of (65) in the synthesis of the tetrapeptide (68) has been demonstrated,[133] it was also found that the polymeric di-imide (67) was equal in activity to dicyclohexylcarbodi-imide giving the same coupling yields.

[130] D. Leibfritz, W. Mayr, R. Oekonomopulos, and G. Jung, *Tetrahedron*, 1978, **34**, 2045.
[131] M. Narita, *Bull. Chem. Soc. Japan*, 1978, **51**, 1477.
[132] M. Mutter, *Tetrahedron Letters*, 1978, 2839.
[133] M. Mutter, *Tetrahedron Letters*, 1978, 2843.

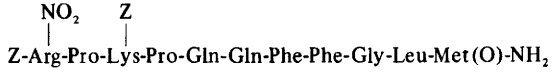


$Pr^i \cdot N{=}C{=}N \cdot PEG \cdot N{=}C{=}N \cdot Pr^i$

(67)

Boc-Val-Pro-Gly-Gly-OMe

(68)



(69)

A synthesis of protected substance P (69) using the repetitive mixed anhydride (REMA) method has been reported.[134] In this synthesis the t-butoxycarbonyl *C*-terminal heptapeptide was prepared stepwise by the REMA method using a 1.4-fold excess of the mixed anhydride formed using isobutyl chloroformate and *N*-methylmorpholine. The four remaining residues were added as two dipeptides with t-butoxycarbonyl for intermediate amino-protection. The free peptide was obtained by treatment with hydrogen fluoride in the presence of anisole, followed by removal of the sulphoxide function by treatment with mercaptoethanol.

Synthesis of Polymeric Models for Studies in Protein Chemistry.—Eighteen co-oligopeptides based on the unit (70) have been synthesized[135] using the mixed anhydride derived from isobutylchloroformate to link the monomeric

$$\text{Boc-Gly-Met}_n\text{-OMe} \quad n = 1-6$$

(70)

$$\text{H-(Tyr-Lys}_m)_n\text{-OH} \quad m = 1-3$$

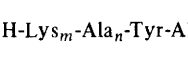
(71)

units. The products were disordered in hexafluoroacetonesesquihydrate but in trifluoroethanol secondary structure was observed when n was greater than 4. Polymers containing lysine and tyrosine of the type (71) have been prepared by polymerization of *N*-hydroxysuccinimide peptide monomers.[136] The di and tripeptide active ester monomers were prepared by coupling N^{ε}-benzyloxycarbonyl-lysine *N*-hydroxysuccinimide ester hydrochloride to the appropriate protected amino-acid or dipeptide by the mixed carbonic anhydride method. This eliminated the need to prepare the active esters in a separate step, thus avoiding the hydrolysis of alkyl esters and an intermediate activation step. The resulting polymers were utilized in a study of α-helix–random-coil transitions.

[134] E. Izeboud and H.C. Beyerman, *Rec. Trav. chim.*, 1978, **97**, 1.
[135] J. Champi, A.S. Steinfeld, F. Naider, and J.M. Becker, *Biopolymers*, 1978, **17**, 2199.
[136] S.St. Pierre, R.T. Ingwall, M.S. Verlander, and M. Goodman, *Biopolymers*, 1978, **17**, 1827.

$$\text{H-Lys}_m\text{-Ala}_n\text{-Tyr-Ala}_n\text{-Lys}_m\text{-OH} \quad n = 3,\ m = 3 \text{ or } 6$$

(72)



(73)

A series of model peptides (72) have been synthesized in order to investigate the binding of tyrosine with the aromatic bases of the nucleic acids.[137] In order to allow sufficient mobility of the tyrosyl residues alanine bridges were inserted. Fragments were assembled by dicyclohexylcarbodi-imide coupling and linked using isobutylchloroformate forming well defined block co-polymers. An interesting point of detail was that the protected tetrapeptide (73) could not be hydrolysed in acetone, dioxane, or dimethylformamide, but that when dimethylsulphoxide or dimethylsulphoxide–acetone was used the cleavage proceeded satisfactorily.

Boc-L-Leu-D-Leu-L-Leu-D-Leu-OH

(74)

The tetrapeptide (74) was prepared[138] using conventional coupling methods and polymerized by conversion to the pentachlorophenyl ester and subsequent removal of the *N*-t-butoxycarbonyl group. The 'molecular weights' of the resulting polymers were in excess of 34 000 and from optical rotation measurements in trifluoroacetic acid it was found that the polymers were highly syndiotactic, although a little racemization caused a small excess of L-leucine residues. From *X*-ray diffraction, n.m.r., i.r., and o.r.d. studies it was concluded that the syndiotactic polyleucine most probably exists in a π-helical conformation with a preferred screw sense in both the solid state and in chloroform solution.

As *N*-carboxyanhydrides are widely used to prepare homopolymers the effects of various solvents on the polymerization of these derivatives has been studied.[139] Polymerization in dipolar solvents of D,L-NCAs which are initiated by strong base were observed to give some stereoselelctivity on polymerization.[140] *N*-Substituted polypeptides have been prepared from the corresponding *N*-substituted NCAs although frequently steric hindrance prevents reaction, in these cases *N*-methylation using silver oxide and methyl iodide gave almost complete methylation in three days.

The peptide (75)[141] and several varied co-polymers of glycine, proline, and alanine[142] have been prepared as collagen models. Both groups of model compounds were able to form triple helices although *O*-acetylation of (75)

[137] R. Mayer, A. Caille, and G. Spach, *Biopolymers*, 1978, **17**, 325.
[138] J. Semen and H.G. Elias, *Macromolecules*, 1978, **179**, 579.
[139] Y. Hashimoto, Y. Imanishi, and T. Higashimura, *Biopolymers*, 1978, **17**, 2561.
[140] A. Cosani, M. Palumbo, M. Terbojevich, and E. Peggion, *Biopolymers*, 1978, **17**, 2519.
[141] R.W. Weber and H. Nitschmann, *Helv. Chim. Acta*, 1978, **61**, 701.
[142] Yu.A. Lazarev, V.M. Lobachov, B.A. Grishkovski, V.A. Shibnev, V.S. Grechishko, M.P. Finogenova, N.G. Esipova, and V.N. Rogulenkova, *Biopolymers*, 1978, **17**, 1215.

$$H\text{-}(Pro\text{-}Hyp\text{-}Gly)_{10}\text{-}OH$$
(75)

reduced the thermal stability of the helix. In the second group of model compounds triple helix formation appeared to depend on chain length, the position of repeating triplet units, and the composition of those units.

Purification Methods.—A method of purifying synthetic peptides has been developed which utilizes the covalent interaction between thiopropyl-Sepharose 6B and the thiol function of cysteine.[143] The sequence (76), which is

$$H\text{-}Cys\text{-}Met\text{-}\underset{33}{Val}\text{-}Gly\text{-}Leu\text{-}Asp\text{-}Ala\text{-}Phe\text{-}Ser\text{-}Ala\text{-}Asp\text{-}Glu\text{-}Leu\text{-}Lys\text{-}Lys\text{-}Leu\text{-}Phe\text{-}\underset{48}{Lys}\text{-}OH$$
(76)

the 33—48 allergen M from cod and has the dipeptide cysteinylmethionine added to the amino-terminus was synthesized by solid-phase. Cleavage from the resin with anhydrous hydrogen fluoride and treatment with dithioerythritol gave the fragment (76) which was passed down a column of thiopropyl-Sepharose 6B. Disulphide bonds rapidly formed and the column was washed free of other material using thiol-free buffer. The required product was then elated from the column with 0.1 M ammonium acetate containing dithioerythritol and the cysteinylmethionine fragment removed by treatment with cyanogen bromide. The method allows separation of terminated peptides and is related to that previously developed using organomercurial agarose[144] although thiopropyl-Sepharose 6B has a higher capacity than the organomercurial agarose. A similar principle has been demonstrated in the preparation of avidin–biotin affinity columns.[145] A derivative of $ACTH^{1-24}$ (25-biocytin)-$ACTH^{1-25}$ amide was prepared and was found to bind strongly to avidin bound to a column of Sepharose 6B.

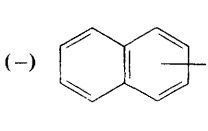
Ion exchange chromatography features in many papers and specific studies have been carried out to examine the purification of acidic[146] and neutral[147] peptides using appropriate resins. Continuous flow electrophoresis, although not widely applied, has been used to purify (1-β-mercaptopropionic acid, 8-D-arginine)-vasopressin (DDAVP) on a 10 gram scale[148] and ultimately more than 50 g of the compound was prepared.

Until recently gel filtration in organic solvents was restricted to the purification of protected peptides with relatively low molecular weights (up to about 3000). The molecular weight range has now been extended using Sephadex LH60, and using solvents such as dimethylformamide, *N*-methylpyrrolidone, or 2,2,2-trifluoroethanol much larger peptides may be purified[149] as the matrix gives high flow rates and good resolution. Although most

[143] G. Lindeberg, J. Tengborn, H. Bennich, and U. Ragnarsson, *J. Chromatog.*, 1978, **156**, 366.
[144] D.E. Krieger, B.W. Erickson, and R.B. Merrifield, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3160.
[145] K. Hofmann, F.M. Finn, and Y. Kiso, *J. Amer. Chem. Soc.*, 1978, **100**, 3585.
[146] P. Sørup, *J. Chromatog.*, 1978, **160**, 221.
[147] G. Oshima, H. Shimabakuro, and K. Nagasawa, *J. Chromatog.*, 1978, **152**, 579.
[148] M. Zaoral, M. Flegel, T. Barth, and A. Machová, *Coll. Czech. Chem. Comm.*, 1978, **43**, 511.
[149] I.J. Galpin, A.G. Jackson, G.W. Kenner, P. Noble, and R. Ramage, *J. Chromatog.*, 1978, **147**, 424.

protected peptides behaved satisfactorily, in some cases there was evidence of aggregation for the larger protected peptides. The use of h.p.l.c. and reverse phase h.p.l.c. in peptide synthesis has increased rapidly.[150—154] The major application has been in the analytical control of synthetic peptides although preparative separations have also been carried out. Using the reverse phase approach several groups[150, 152, 155] have been able to separate various diastereoisomers of several peptide hormones. An interesting cautionary note was indicated in the purification of D-histidine[2]-thyrotropin releasing hormone.[150] Previously[156] it had been found that this analogue had considerable activity, however, h.p.l.c. purification on a strongly acidic ion exchanger gave an inactive product and a small amount of the normal L-histidine containing product which must have been produced during the synthesis by racemization. The previously observed activity of the 'D-analogue' obviously was due to the small amount of L-contaminant.

As has been mentioned above[103, 105, 106] (p. 342) the use of glass capillary columns coated with a chiral phase has become a useful method of determining the enantiomeric populations in a hydrolytic mixture of amino-acids.[157] Frequently trifluoroacetyl derivatives are used but on some occasions a better separation has been obtained using heptafluorobutyryl derivatives.[158] The use of chiral reagents for the derivitization of amino-acids has also been



(77)

described.[159] Reaction of chiral naphthalene acetic acids (77) with amino-acid methyl esters in the presence of dicylclohexylcarbodi-imide yields diastereoisomers which may be separated using h.p.l.c. employing an optically inactive column.

Synthetic Operations with Peptides of Biological Origins.—Most of the semisynthetic studies which have been carried out have utilized peptides or proteins with particular properties which make them amenable to study. A

[150] W. Voelter, H. Bauer, S. Fuchs, and E. Pietrzik, *J. Chromatog.*, 1978, **153**, 433.
[151] E.P. Kroeff and D.J. Pietrzyk, *Analyt. Chem.*, 1978, **50**, 502.
[152] E. Lundanes and T. Greibrokk, *J. Chromatog.*, 1978, **149**, 241.
[153] C.E. Dunlap III, S. Gentleman, and L.I. Lowney, *J. Chromatog.*, 1978, **160**, 191.
[154] B. Larsen, V. Viswanatha, S.Y. Changa, V.J. Hruby, *J. Chromatog. Science*, 1978, **16**, 207.
[155] W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, M.T.W. Hearn, *Science*, 1978, **200**, 1168.
[156] D. Gupta and W. Voelter, 'Hypothalamic Hormones, Structure, Synthesis, and Biological Activity', Verlag Chemie, Weinheim, 1975.
[157] S.V. Vitt, M.A. Myagkova, M.B. Suporvskaya, S.B. Nikitina, and V.M. Belikov, *Bioorg. Khim.*, 1978, **4**, 154.
[158] C.F. Poole and M. Verzele, *J. Chromatog.*, 1978, **150**, 439.
[159] J. Goto, M. Hasegawa, S. Nakamura, K. Shimada, and T. Nambara, *J. Chromatog.*, 1978, **152**, 413.

short review has appeared which deals mainly with the interaction between ribonuclease *S*-peptide and protein.[160]

The reoxidation of reduced native and modified hen-egg-white lysozyme (HEL) has been studied.[161—163] Reoxidized 6,127-carboxymethylcysteinyl HEL showed 35 % of the activity of the intact enzyme[162] with the three disulphide linkages in the native form. Other non-native disulphide forms of 6,127-carboxymethylcysteinyl HEL were obtained by disulphide scrambling in the presence of mercaptoethanol. The results showed that the 6,127-carboxymethylcysteinyl HEL with the native arrangement of disulphide bonds was thermodynamically less stable than the native enzyme but that it was more stable than any of the forms with a non-native disulphide arrangement. In addition the native form has a lower hydrodynamic volume, as shown by gel filtration on Biogel P30, than the non-native forms. Thus the force responsible for the acquisition of three dimensional structure appears to still be operative even when one of the disulphide bonds cannot be formed. This conclusion was borne out by the reoxidation of reduced 1—123 HEL.[163] It was found that using carboxypeptidase the terminal hexapeptide, including Cys-127, could be removed from reduced HEL and that in the reduced state neither the native nor the 1—123 HEL had any three dimensional structure. On reoxidation, however, the reduced 1—123 HEL showed 65 % of the enzymic activity of the native protein (reduced 1—129 HEL gave 84 % under the same condition) but that it took longer to achieve 50 % of the final activity. A 15 minute oxidation of reduced 1—129 HEL gave 75 % activity whereas reduced 1—123 HEL had only reached 38 % activity. Thus the remainder of the polypeptide backbone is able to achieve the natural folding pattern even when the terminal hexapeptide is absent.

Studies have also been carried out[164, 165] on the reoxidation of hydrogen fluoride treated derivatives of HEL. 2-Chlorobenzyloxycarbonyl HEL, *S*-(4-methylbenzyl) and 2-chlorobenzyloxycarbonyl-*S*-4-methylbenzyl reduced HEL derivatives were prepared and treated with anhydrous hydrogen fluoride for 90 minutes of 0 °C. The yields of fully active lysozyme which were ultimately recovered were 23, 12, and 7.4 % respectively, thus although the authors claim that this permits a total synthesis of the enzyme it would be considerably more feasible if a milder acid treatment were envisaged.

Experiments examining the oxidation of reduced denatured seminal ribonuclease using Sephadex G75 chromatography, SDS gel electrophoresis, enzymic activity, and c.d. spectra have been carried out using the glutathione redox system.[166] In this case the native dimeric form is not regenerated during refolding, instead the major product is a monomer in which it appears that

[160] I.M. Chaike and A. Komoriya, *Trends in Biological Sciences*, 1978, 269.
[161] E.R. Johnson, W.L. Anderson, D.B. Welanfer, C.-L. Lee, and M.Z. Atassi, *J. Biol. Chem.*, 1978, **253**, 3408.
[162] A.S. Acharya and H. Taniuchi, *Biochemistry*, 1978, **17**, 3064.
[163] A. Vizet, J.P. Perrandin, J. Depreter, M. Deconinck, A.G. Schnek, and J. Leonis, *Experientia*, 1978, **34**, 1017.
[164] S. Aimoto and Y. Shimonishi, ref. 1, p. 65.
[165] S. Aimoto and Y. Shimonishi, *Bull. Chem. Soc. Japan*, 1978, **51**, 205.
[166] G.K. Smith, G. D'Alessio, and S.W. Shaffer, *Biochemistry*, 1978, **17**, 2633.

Cys-31 and -32 are blocked by intermolecular disulphide bond formation with glutathione. The c.d. spectra were different to those obtained for the native ribonuclease but were similar to those observed for pancreatic ribonuclease A.

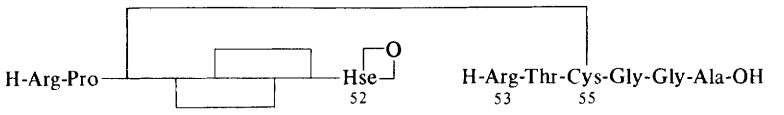
N^{α}-B1, N^{ε}-B29-Bis-t-butoxycarbonyl insulin has been synthesized by treatment of A1-trifluoroacetyl or A1-citraconyl insulin with t-butoxycarbonylazide, followed by subsequent removal of the A1 protecting group.[167] The bis-t-butoxycarbonyl derivative was then used to prepare des A1-glycine insulin (*via* Edman degradation) and a derivative in which A1-glycine had been amidated using 3,5-dimethylpyrazolformamidinium nitrate.

Des-(B-22—30) nonapeptide porcine insulin pentamethyl ester has been prepared by cleavage of the desoctapeptide derivative with carboxypeptidase B.[168] Lysine or alanine was inserted at B-22 in place of arginine giving the

Table 6 *Truncated semisynthetic insulin analogues with lysine or alanine at position B-22(X)*[168]

-X-Gly-OMe
-X-Gly-Phe-OMe
-X-Gly-Phe-Phe-OMe
-X-Gly-Phe-Phe-Tyr-OMe
-X-Gly-Phe-Phe-Tyr-Ala-OMe

group of modified hormones shown in Table 6 after coupling synthetic peptide fragments as their methyl esters. Following ester hydrolysis of the hexamethyl esters under carefully controlled conditions (pH 11.5, 15 h, 4 °C) and deprotection, the products were purified and tested for activity. The results indicated that the B-22 lysine and alanine analogues had similar low activity and that for significant activity B-22 arginine was required.



(78)

More details of manipulations on bovine pancreatic trypsin inhibitor (BPTI) have been reported.[169, 170] Treatment of the native peptide with cyanogen bromide cleaves at position-52 generating homoserine lactone (78). In neutral aqueous solution the amino-function of Arg-53 is held in close proximity and reforms the peptide bond between residues 52 and 53, the $t_{0.5}$ for this coupling being 29 h at pH 6.3. It is believed that the relative ease of formation is due to the cleaved product (78) retaining the conformation of the native protein. The 5—55 disulphide bond joining the two fragments could not be selectively reduced and facile spontaneous oxidation made it difficult to recover the fully reduced 1—52 cleaved material. Furthermore attempts at specific reoxidation

[167] H.-J. Friesen, V.K. Naithani, and H.G. Gattner, *Z. physiol. Chem.*, 1978, **359**, 103.
[168] G. Weitzel, F.-U. Bauer, and K. Eisele, *Z. physiol. Chem.*, 1978, **359**, 945.
[169] D.F. Dyckes, T.E. Creighton, and R.C. Sheppard, *Internat. J. Peptide Protein Res.*, 1978, **11**, 258.
[170] T.E. Creighton, D.F. Dyckes, and R.C. Sheppard, *J. Mol. Biol.*, 1978, **119**, 507.

even in the presence of a large excess of synthetic hexapeptide were not successful. Reduced BPTI in which the terminal hexapeptide had been removed by cyanogen bromide formed a rather random array of disulphide bonds on oxidation whereas BPTI itself folds quantitatively and correctly. Thus the *C*-terminal hexapeptide appears to be important in stabilizing long-range interactions which control the formation of the correct disulphide linkages. Interestingly, reduced 52-homoserine BPTI folds less readily than BPTI itself and the normal 30—51 disulphide is less stable by about 0.8 kcal $mole^{-1}$. This often results in the 5—30 disulphide being formed in preference to the usual 30—51 linkage; this change may possibly be explained by a decrease in hydrophobicity on changing from methionine to homoserine at position 52 as calculations indicate that the energy change resulting from this substitution should be of the order of 0.9 kcal $mole^{-1}$. The Met-52 of BPTI has been modified by reaction with [^{13}C]methyliodide[171] thus introducing a diastereotopic methyl group which could be used as a conformational probe. The denaturation profile indicated that the overall conformation was not disturbed by the introduction of the positively charged sulphonium group.

The Bowman–Birk soyabean trypsin inhibitor (BBI) has also been studied.[172] The overall approach was basically that used by Kowalski and Laskowski[173] (see Vol. 9, p. 352) in which after guanylation of the eight lysine residues and carbamoylation of the amino-terminus the Leu-43—Ser-44 bond was cleaved by limited proteolysis with chymotrypsin. One cycle of the Edman degradation was then used to remove the exposed Ser-44 which could then be replaced using various *N*-t-butoxycarbonylamino-acid hydroxysuccinimide active esters. In this way serine, alanine, threonine, valine, leucine, and glycine 44-substituted derivatives were obtained after removal of the *N*-t-butoxycarbonyl group with trifluoroacetic acid.

Considerable interest has been shown in semisynthetic studies on somatotropin (growth hormone). Non-covalent recombination of the two tryptic digest peptides 96—133 and the disulphide linked 1—95/151—191 fragment obtained from bovine growth hormone showed an activity greater than that exhibited by each constituent portion.[174] Following the observation that the *S*-carboxamidomethylated derivatives; [Cys(Cam)^{53}]-HGH-(1—134) and [Cys(Cam)^{165,182,189}]-HGH-(141—191) could be recombined by a non-covalent interaction to give a protein with the full activity of HGH[175, 176] two fully semisynthetic HGH analogues were prepared by non-covalent combination of natural [Cys(Cam)^{53}]-HGH-(1—134) with the carboxamidomethylated solid-phase synthesized[178] fragments (79) and (80). The recombinants had full growth promoting activity in the fibia test and showed nearly full immunoreactivity in a radioimmunoassay.

[171] B.M. Harina, D.F. Dyckes, M.R. Willcott III, and W.C. Jones jun., *J. Amer. Chem. Soc.*, 1978, **100**, 4897.
[172] S. Odani and T. Ikenaka, *J. Biochem. Japan*, 1978, **84**, 1.
[173] D. Kowalski and M. Laskowski jun., *Biochemistry*, 1976, **15**, 1300.
[174] K. Hara, C.-J.H. Chen, and M. Sonenberg, *Biochemistry*, 1978, **17**, 550.
[175] C.H. Li and T.A. Bewley, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 1476.
[176] C.H. Li, T. Hayashida, B.A. Doneen, and A.J. Rao, *Proc. Nat. Acad. Sci. U.S.A.* 1976, **73**, 3463.
[177] C.H. Li, J. Blake, and T. Hayashida, *Biochem. Biophys. Res. Comm.*, 1978, **82**, 217.
[178] J. Blake and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 315.

$Nle^{170}Ala^{165,182,189}HGH$ (140–191)

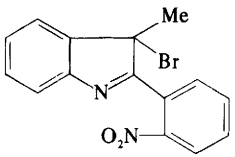
(79)

$Nle^{170}Ala^{165,182,189}HGH$ (145–191)

(80)

Human choriomammotropin (HCS), which has a 96 % sequence homology with HGH, has been cleaved by digestion with plasmin.[179] Cleavage of the Arg-134—Arg-135 and Lys-140—Glu-141 bonds gave two major inactive fragments 1—134 and 141—191. The high degree of sequence homology and similar cleavage pattern of HGH and HCS have prompted the combination of the amino-terminal fragment of HGH with the carboxyl fragment of HCS giving a protein with growth promoting activity.[180]

An alternative selective cleavage at the single Trp-86 of bovine growth hormone has been achieved[181] using 2-(2-nitrosulphenyl)-3-methyl-3′-bromoindolenine (81).



(81)

The 87—191 fragment was isolated as a monomer but the 1—86 portion could only be obtained in a highly, aggregated form. Both fragments had negligable biological activity.

The same reagent (81) was used to cleave ovine prolactin at tryptophans 91 and 150.[182] Neither of the resulting 92—150 or 1—91/151—199 disulphide linked fragments showed any immunological activity although an oxidized form of prolactin was obtained which did show immunological activity.

An ambitious project aimed at investigating a semisynthetic antibody has been described.[183] The antibody combining site of mouse myeloma protein 315 is composed of a variable region of light chain V_L and heavy chain V_H. A Merrifield type solid-phase synthesis of the 115 residue sequence of V_L of protein 315 was carried out and the synthetic protein removed from the resin by treatment with hydrogen fluoride. After formation of the single disulphide bond the product was purified by absorption on an anti-V_L Sepharose column. Ultimately, affinity purified V_L and native V_H were brought together and the product purified on Dnp-Lys-Sepharose eluting with Dnp-Gly. The binding analysis of the final product showed an affinity for Dnp-Lys which was similar to that of the native material. However only 7.1 % of the affinity purified synthetic V_L was suitable for combination with V_H representing 1.7 % of the total synthetic V_L. On these grounds it was concluded that chemical synthesis was not a viable proposition although clearly this approach does have

[179] C.H. Li and R. Houghton, *Internat. J. Peptide Protein Res.*, 1978, **12**, 114.
[180] C.H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 1700.
[181] R.A. Houghton and G.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 49.
[182] R.A. Houghton and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 345.
[183] M. Gavish, R. Zakut, M. Wilchek, and D. Givol, *Biochemistry*, 1978, **17**, 1345.

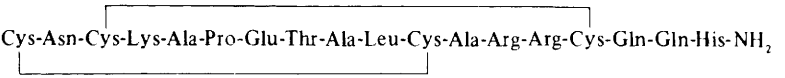
associated affinity purification methods which permit considerable purification of the synthetic product which is not available for the majority of solid-phase synthesized peptides.

3 Syntheses Achieved

Both solution methods and solid-phase synthesis have been used to prepare a large number of significant peptides and occasionally proteins. This section deals with many of the reported syntheses and relegation of any particular synthesis to Appendix 1 should not be interpreted as a judgment of the work but rather as a reflection of the interests of the reviewer. In addition generally analogues prepared by established routes have been placed in Appendix 1.

ACTH and MSH.—A synthesis of the dogfish (*Squalus acanthias*) complete ACTH has been reported.[184] The synthesis used benzyl derived protecting groups for most side-chains and *p*-methoxybenzenesulphonyl protection for arginine, all of which were removable by hydrogen fluoride treatment; tyrosine, serine, histidine, and methionine were unprotected. The molecule was assembled by dicyclobenzylcarbodi-imide–hydroxybenzotriazole fragment couplings, the constituent fragments being assembled using *o*-nitrophenyl esters or the azied method. In the final deprotection both anisole and ethanedithiol were used as scavengers.

The α-MSH[185] and β-MSH[186] from *Squalus acanthias* have also been prepared. The α-MSH derivatives oxidized at Met-4,Met-13, and at both positions have been prepared. In this synthesis the (2-methylsulphonyl)ethoxycarbonyl group was used for lysine side-chain protection, being rapidly removed by barium hydroxide along with the *C*-terminal methyl ester from the intermediate tridecapeptide. In the β-MSH synthesis, protecting groups removed by hydrogen fluoride were employed and a mixture of anisole, 2 % ethanethiol, and skatole was used to scavenge electophiles.



(82)

Apamin.—A solid phase-synthesis of the neurotoxin (82) has been described[187] using a benzhydrylamine resin as the support. The 2-phenylisopropoxycarbonyl group was used for N^{α}-protection and substantial protection of side-chains was employed [Gln(Mbh),His/Arg(Tos),Lys(Z), Glu(OBzl), Thr(Bzl), Cys(Acm)]. An improved yield was obtained by coupling in both dichloromethane and dimethylformamide. The yield in the oxidative disulphide bond forming step was approximately 20 % giving the fully active

[184] S. Shimamura, K. Okamoto, M. Nakamura, A. Tanaka, and H. Yajima, *Internat. J. Peptide Protein Res.*, 1978, **12**, 170.
[185] A. Eberle, Y.-S. Chang, and R. Schwyzer, *Helv. Chim. Acta*, 1978, **61**, 2360.
[186] H. Yajima, J. Iwai, K. Koyama, M. Nakamura, K. Miyata, A. Tanaka, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 571.
[187] B.E.B. Sandberg and U. Ragnarsson, *Internat. J. Peptide Protein Res.*, 1978, **11**, 238.

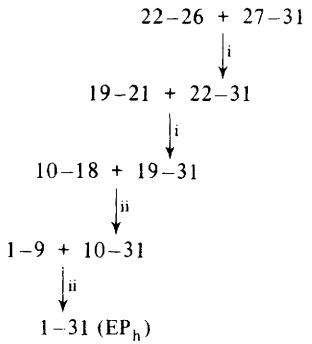
molecule. A series of analogues have also been prepared using a benzhydrylamine resin.[188] Replacement of one or both basic residues in the 13—14 arginylarginine sequence by lysine, N^{ε}-acetyl-lysine or homoarginine resulted in decreased potency. For 'lethal potency' a double positive charge is required including one arginine at either the 13 or 14 position. Interestingly, when the above replacements were made the yield on oxidation dropped considerably. Thus it appears that the precise sequence of amino-acid residues has a pronounced effect on disulphide bond formation.

Endorphins and Related Peptides.—In this highly active area attention has now moved from the pentapeptides leucine and methionine enkephalin to the larger endorphins[189—196] and ultimately to a total synthesis of the 91-residue precursor, β-Lipotropin (β-LPH).[197, 198]

H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-
Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Gln-OH

(83)

Human β-endorphin (83) has been prepared by two groups using solution methods; one synthesis[189] utilized the fragment coupling approach employing either the azide method or dicyclohexylcarbodi-imide–hydroxybenzotriazole to join the five protected fragments as indicated in Scheme 18. The benzyl-based side-chain protecting groups were finally removed using either



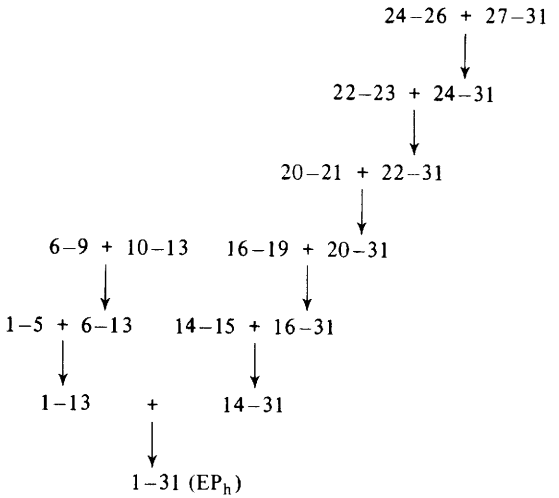
Coupling methods: i, azide; ii, DCCI–HOBt

Scheme 18

anhydrous hydrogen fluoride or sodium in liquid ammonia. The first method gave only 13% yield when anisole and diethyl sulphide were used as scavengers, an interesting result in view of the substantially higher yields previously reported for solid phase syntheses also using liquid hydrogen fluoride. The second method gave 27.5% of the purified β-endorphin. An

[188] C. Granier, E.P. Muller, and J. Van Rietschoten, *European J. Biochem.*, 1978, **82**, 293.
[189] C. Tzougraki, R.C. Makofske, T.F. Gabriel, S.-S. Wang, R. Kutny, J. Meienhofer, and C.H. Li, *J. Amer. Chem. Soc.*, 1978, **100**, 6248.

alternative synthesis has been described.[190, 191] which uses methane sulphonic acid as the final deprotecting agent, in this case methionine was protected as its sulphoxide in order to prevent side-reactions.[61] Again a fragment condensation approach using the same two coupling methods was employed according to the general route shown in Scheme 19. The final protected product was purified by



Coupling method: azide

Scheme 19

a batch washing procedure prior to deprotection; although this method may be satisfactory for small peptides it is difficult to see how a reasonable purification can be achieved for peptides of this size.

Syntheses of ovine β-endorphin, [D-Ala^2], [D-Ala^2,MePhe^4], and [D-Ala^2, MePhe^4, Met(O)^5] endorphins by the solid-phase method using a γ-glutamyl linked benzhydrylamine resin have been described.[192] In these syntheses preformed symmetrical anhydrides were used in the coupling steps and a refrigerated apparatus was used to avoid decomposition. As with many of the endorphin peptides, partition chromatography on Sephadex G50 was used in the purification of these analogues, permitting differentiation between each of the synthetic products.

Many other endorphin analogues have been prepared by the group of C. H. Li, these include; -camel β-endorphin analogues,[193] 2-and 5-position

[190] M. Kubota, T. Hirayama, O. Nagase, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 2132.

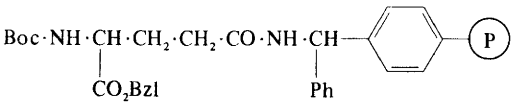
[191] M. Kubota, T. Hirayama, O. Nagase, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 2139.

[192] S. Lemaire, A. Bérubé, G. Derome, I. Lemaire, J. Magnan, D. Regoli, and S. St-Pierre, *J. Medicin. Chem.*, 1978, **21**, 1232.

[193] C.H. Li, D. Yamashiro, L.-F. Tseng, and H.H. Loh, *Internat. J. Peptide Protein Res.*, 1978, **11**, 154.

analogues,[194] human β-endorphins of various chain lengths[195] and 27- and 31-position analogues.[196] The number and variety of analogues prepared by the solid-phase method can leave no doubt as to the usefulness of the method when careful monitoring is carried out to check the homogeneity of the final products.

A solid-phase synthesis of the (41—91) fragment of ovine β-lipotropin[197] using a benzhydrylamine resin linked *via* a γ-glutamyl linkage (84) has been



(84)

reported. The product obtained in 5 % overall yield was purified by a variety of methods including partition chromatography on Sephadex G50 and agarose. In this synthesis an improved yield as compared to the synthesis of the 42—91 fragment[198] was obtained, this was claimed to result from the following factors: (*a*) use of the benzhydrylamine resin, (*b*) the addition of 2,2,2-trifluoroethanol in coupling steps which increased coupling efficiency, (*c*) removal of the final *N*-t-butoxycarbonyl group with TFA prior to treatment with hydrogen fluoride as this cuts down t-butylation of methionine,[199] and (*d*) using an improved method for the removal of the formyl protecting group from tryptophan[200] (pH 11.5, 1M-NaOH, 3 min).

A total solid-phase synthesis of ovine β-lipotropin using a bromostyrene (1 %) divinyl benzene resin has been described.[201] This resin which is between 15—50 times as stable as that containing normal benzylic linkage only results in the loss of 0.03 % per cycle. Symmetrical anhydrides were used in the couplings in the presence of 2,2,2-trifluoroethanol to enhance reactivity. A combination of *p*-halobenzyl and benzyl derived protecting groups were used for side-chain protection, being finally removed by hydrogen fluoride after removal of the last *N*-t-butoxycarbonyl group by TFA. The synthetic β-LPH, which was obtained in 0.66 % overall yield showed biological and physical properties indentical to those of the natural material. Although there were a few minor discrepancies in the amino-acid analysis the gel isoelectric focusing was very impressive, the natural and synthetic materials being indistinguishable. The immunoreactivity of both materials was similarly convincing although the presence of deletions cannot be excluded. The wide range of characterization methods used provided considerable evidence that the β-lipotropin had in fact been synthesized.

[194] D. Yamashiro, C.H. Li, L.-F. Tseng, and H.H. Loh, *Internat. J. Peptide Protein Res.*, 1978, **11**, 251.
[195] H.-W. Yeung, D. Yamashiro, W.-C. Chang, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **12**, 42.
[196] J. Blake, L.-F. Tseng, W.-C. Chang, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 323.
[197] S. Lemaire, D. Yamashiro, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 179.
[198] D. Yamashiro and C.H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4945.
[199] R.L. Noble, D. Yamashiro, and C.H. Li, *J. Amer. Chem. Soc.*, 1976, **98**, 2324.
[200] S. Lemaire, D. Yamashiro, and C.H. Li, *J. Medicin. Chem.*, 1976, **19**, 373.
[201] D. Yamashiro and C.H. Li, *J. Amer. Chem. Soc.*, 1978, **100**, 5174.

Glucagon.—The syntheses of turkey[202] and mammalian glucagon[203] have been reported. Both syntheses utilized dicyclohexylcarbodi-imide–*N*-hydroxy-5-norbornene-2,3-dicarboximide to couple six fragments which were themselves protected as indicated [Asp(OBzl), Arg(MBS), Met(O)]. Deprotection by hydrogen fluoride and methane sulphonic acid respectively, followed by treatment with 3 % aqueous thioglycollic acid gave the free peptides. A final treatment with ammonia was carried out in order to reverse any *N* to *O* acyl migration which might have occurred at serine or threonine.

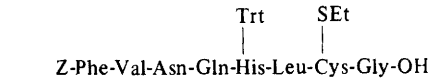
Duck glucagon has also been prepared[204, 205] using similar protecting groups, in this case however the fragments were combined by the azide method. The fully protected product was purified by a washing procedure and then deprotected using methane sulphonic acid in the presence of anisole. The purified crystalline product was homogeneous by disc electrophoresis at pH 2.3 and by isoelectric focusing. The biological activity was similar to that of porcine glucagon although in a radioimmunoassay it only showed a cross reactivity of 46 % when compared with the porcine hormone.

H-Lys-Arg-Asn-Asn-Lys-Asn-Ile-Ala-OH

(85)

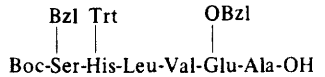
A brief report[206] of the synthesis of a proglucagon molecule containing 37 residues has appeared, the fragment (85) being joined to carboxy-terminus of the normal porcine or bovine hormones. An azide fragment condensation approach was used and a general policy of minimal protection was employed. The final product possessed one thousandth of the activity of the corresponding glucagon.

Insulin.—New syntheses of the B^{21-30} human insulin[207] and total B-chain *S*-sulphonate of bovine insulin[208] have been described. The first synthesis was carried out by fragment condensation of small peptides with side-chain t-butyl protection. An improved method of preparing bis-t-butyl threonine was reported which allowed a higher yield of this compound to be obtained than had been reported previously[209] with fewer impurities. The total B-chain synthesis was carried out by combination of the protected fragments (86)—(89)

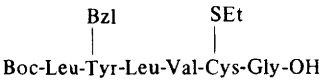


(86)

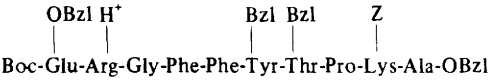
[202] M. Wakimasu and M. Fujino, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 1522.
[203] M. Fujino, M. Wakimasu, S. Shinagawa, C. Kituda, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 539.
[204] H. Ogawa and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 1540.
[205] H. Ogawa, M. Sugiura, H. Yajima, H. Sakurai, and K. Tsuda, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 1549.
[206] N. Yanaihara, T. Hiraiwa, M. Sakami, T. Hashimoto, and C. Yanaihara, ref. 1, p. 81.
[207] V.K. Naithani and J. Föhles, *Z. physiol. Chem.*, 1978, **359**, 1173.
[208] G. Losse, H. Strange, B. Schwenzer, and W. Naumann, *Tetrahedron*, 1978, **34**, 2687.
[209] E. Wünsch, F. Drees, and J. Jentsch, *Chem. Ber.*, 1965, **98**, 803.



(87)



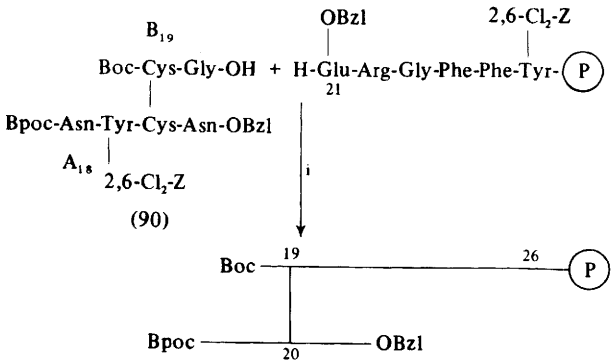
(88)



(89)

using dicyclohexylcarbodi-imide–hydroxybenzotriazole. The choice of protecting groups allows deprotection by hydrogen fluoride, hydrogen bromide in trifluoroacetic acid (in the presence of ethane thiol), and under special conditions by catalytic hydrogenolysis. The last two methods giving the highest yields and most homogenous products. The protected peptide was converted to the *S*-sulphonate in 30 % yield.

The synthesis of (A^1-L-alanine) and (A^1-D-alanine) insulin has been reported[210] as has the synthesis of an open chain asymmetrical cystine A^{18-21}—B^{18-26} bovine insulin fragment.[211] The A^{18-21} and B^{19-20} portions were synthesized by conventional solution methods and the *S*-tritylcysteine-20 activated by conversion to the ethoxycarbonylsulphenyl derivative. After conversion to the asymmetrical disulphide (90) the free, carboxy-function at B-20 was coupled with the resin bound peptide as shown in Scheme 20. Ultimately the asymmetrical cystine peptide was cleaved from the resin by the



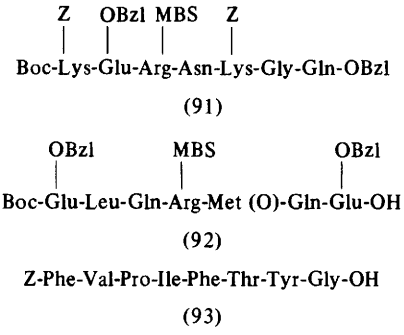
Reagents: i, DCCI–HONSu

Scheme 20

[210] A. Cosmatos, K. Cheng, Y. Okada, and P.G. Katsoyannis, *J. Biol. Chem.*, 1978, **253**, 6586.
[211] W. Kullmann and B. Gutte, *Internat. J. Peptide Protein Res.*, 1978, **12**, 17.

action of hydrogen fluoride. Thus there is a possibility that such an approach could be used in more complex syntheses related to the Ciba–Geigy solution synthesis of Insulin.

Motilin.—Syntheses of this peptide and its D-Phe[1] analogue [212, 213] have been reported along with the 8-L and 8-D-alanine analogues.[214] These preparations all employed the fragment condensation approach using[213] the protected fragments (91)—(93), the coupling being effected by dicyclohexylcarbodi-

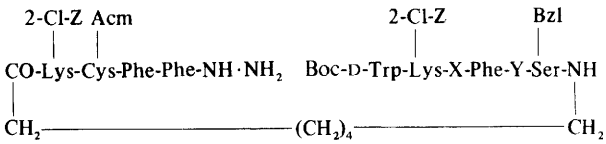


imide in the presence of *N*-hydroxy-5-norbornene-2,3-dicarboximide. The final products were deblocked with methane sulphonic acid in the presence of anisole and the methione sulphoxide reduced by the action of 3 % thioglycollic acid. The synthetic motilin was identical to authentic material when compared in the duodenal muscle contraction assay.

Somatostatin.—Considerable interest has been shown in the synthesis of 8-substituted somatostatins[215—218] using solid-phase methodology. Frequently the introduction of 8-D-tryptophan increases the inhibition of release of growth hormone, however the indole ring system appears to be important as 3-(1 or 2-naphthyl)-L-alanine analogues[215] at this position only show low biological activity. In the preparation of 8-halogenated tryptophan analogues[216] protected racemic tryptophan derivatives were used in the synthesis and the resulting diastereoisomeric peptides finally separated by preparative partition chromatography; overall homogenity was checked by reverse phase h.p.l.c.

Bicyclic analogues have also been prepared containing a hydrocarbon bridge between the amino and carboxy terminus in addition to the normal disulphide

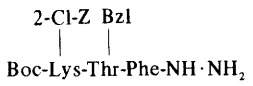
[212] M. Fujino, S. Shinagawa, M. Wakimasu, H. Yajima, and T. Segawa, ref. 1, p. 61.
[213] M. Fujino, S. Shinagawa, M. Wakimasu, C. Kitada, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 101.
[214] S. Shinagawa, M. Fujino, H. Yajima, T. Segawa, and Y. Okuma, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 880.
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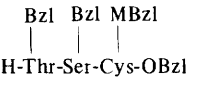
(*a*) X = Thr (Bzl); Y = Cys (Acm)
(*b*) X = Cys (Acm); Y = Thr (Bzl)

(94)

bond.[219] Generally peptides of the type (94) were assembled by the solid-phase method being cleaved from the resin by hydrazinolysis, the D-tryphophan being the last residue to be introduced in order to minimize degradative side-reactions occurring through repeated acidolysis. Azide cyclization, followed by deprotection with hydrogen fluoride, and subsequent iodine oxidation gave the bicyclic analogues. The bicyclic analogue des(Ala-Gly)-[Asu^{3,14}]-somatostatin was also prepared[220] using solution synthesis to build up the linear sequence. When the fragments (95) and (96) were coupled using the azide method



(95)



(96)

considerable racemization ensued, thus even under optimal conditions where no excess of triethylamine was present during the coupling, a level of 3 % racemization was observed. Purification by gel filtration on Sephadex LH20 was unsatisfactory but partition chromatography on Sephadex G25 eluting with n-BuOH–AcOH–H_2O (4 : 1 : 5) enabled the optical purity to be improved.

Other Syntheses.—The synthesis of 1—23 rat pancreatic ribonuclease by a fragment condensation approach has been reported.[221] Dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole was used to link five fragments which were side-chain protected with trifluoroacetic acid-labile protecting groups. The resulting 1—23 rat *S*-peptide was combined with bovine *S*-protein to yield a ribonuclease which was fully active against yeast RNA. This result indicated that residues 2,5,7,8,10,11,12,13, and 14 have a specific role in binding to the *S*-protein to generate activity also that the three 'extra' amino-terminal residues (97) of the rat enzyme do not have any influence on the activity.

H-Gly-Glu-Ser—

(97)

[218] J. Rivier, R. Kaiser, and R. Galyean, *Biopolymers*, 1978, **17**, 1927.
[219] D.F. Veber, F.W. Holly, W.J. Paleveda, R.F. Nutt, S.J. Bergstrand, M. Torchiana, M.S. Glitzer, R. Saperstein, and R. Hirschmann, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2636.
[220] J. Emura, T. Morikawa, T. Takaya, and S. Sakakibara, ref. 1, p. 36.
[221] I. Voskuyl-Holtkamp and C. Schattenkerk, *Internat. J. Peptide Protein Res.*, 1978, **11**, 218.

Synthetic [Ser^2,Val^8]-human calcitonin[222] has been prepared by azide condensation of five fragments. The strategy adopted was that of minimum protection, the subfragments being assembled by standard solution methods. It was hoped that the product would have a relatively high hypocalcemic activity comparable to that of eel or salmon calcitonin, however, the observed activity was approximately equal to that of relatively less active human calcitonin. The solid-phase synthesis of the locust adipokinetic hormone (98) and some

Glp-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2

(98)

analogues have been described.[223] The synthesis was straightforward except that the trans-xanthylation from Asp-7 to Trp-8 was observed in some cases during acidolytic deprotection. This side-reaction, which may also occur during solution synthesis, is facilitated when the residues are adjacent to each other.

H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH_2

(99)

H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2

(100)

The frog skin peptides granuliberin-R (99) and kassinin (100) were synthesized by fragment combination using dicyclohexylcarbodi-imide–hydroxybenzotrizole or the azide method. The fragments were assembled using a variety of standard methods employing *p*-methoxybenzyloxycarbonyl for N^{α}-amino protection with the following side-chain protection, [Arg(MBS), Asp(OBzl),Lys(Z), and Met(O)]. Deprotection of the fully protected peptides by treatment with methane sulphonic acid gave the required free peptides, the methionine being liberated from its sulphoxide by treatment with mercaptoethanol.

4 Appendix I: A List of Syntheses Reported during 1978

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. Abbreviations used in both appendices are collected in pp. xix—xxi.

Peptide	*Ref.*
Adipokinetic hormone	
Locust adipokinetic hormone and analogues	223
Adrenocorticotropic hormone (ACTH)	
Dogfish ACTH	184
ACTH-(1—25)-amide	145
ACTH-(5—24)-, -(6—24)-, -(7—24)- and	

[222] S. Nozaki, *Bull. Chem. Soc. Japan*, 1978, **51**, 2995.
[223] J.V. Stone, W. Mordue, C.E. Broomfield, and P.M. Hardy, *European J. Biochem.*, 1978, **89**, 195.

-(8—24)-peptides 224
ACTH-(7—10)-peptide 14
ACTH-(2—24)-peptide and [Lys[17], Lys[18]]-(2—18)-peptide 225
Dogfish corticotropin-like lobe peptide 226
ACTH precursor 227
Adrenododoxin
Bovine adrenodoxin-(50—63)- and -(90—103)-peptides 228
Allergen M
Cod allergen M-(33—48)-peptide 143
Angiotensin
Angiotensin II 120
[Asn[1], Val[5], Ile[8]]-Angiotensin II 229
Analogues of angiotensin II substituted in positions 4 and/or 8 230
[Asp[1], Ile[5]]-Angiotensin II *C*-terminal heptapeptide and [Ile[5]] hexapeptide 231
Halogenated analogues of angiotensin II 232
[(^{3}H)Tyr[4]]-Angiotensin II and [(^{3}H) Phe[8]]-angiotensin II 233
Apamin
Apamin synthesis, characterization Acm apamin 187
[Lys[13,14]]-Apamin, [Lys[13]] and [Lys[14]]-apamin 188
Synthesis of apamin 234
Apolipoprotein
Apolipoprotein-A-I-(158—168)-, -(156—168)-,
-(147—168)-, -(114—133)-, and apolipoprotein
-A-II-(22—31)-peptides 118
Apolipoprotein-A-I-(158—168)-, -(147—168)-,
-(114—133)-, and apolipoprotein-A-II-(22—31)-peptides 235
Bombesin
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Bombinin
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Bovine pancreatic trypsin inhibitor (BPTI)
Analogue with [Hse[52]] 169, 170
[^{13}C-*S*-MeMet[52]]-BPTI 171
Bowman–Birk inhibitor (BBI)
[Ser[44]]-BBI 172
Several analogues soybean BBI 238

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Several analogues 241
Bungarotoxin
α-bungarotoxin 157
α-bungarotoxin-(58—64)-, -(54—61)-, -(62—74)-, -(20—27)-, and -(11—19)-peptides 103
Calcitonin
[Ser^2, Val^8]-human calcitonin 222
[Val^8]-human calcitonin 125
Carbonic anhydrase
Human carbonic anhydrase B-(91—126)-peptide 242
Cardiotoxin
Snake venom cardiotoxin 243
Chemotactic peptide
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Cholecystokinin-pancreozymin (CCK-PZ)
C-terminal heptapeptide of CCK-PZ 245
Immunological studies of CCK-PZ 246
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Fragment of human choriomammotropin 180
Cytochrome *c*
Horse heart cytochrome *c*-(66—80)-peptide 116
Distal retinal pigment hormone (DRPH)
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Enantio-ferrichrome
Enantiomer of natural sideramine ferrichrome 248
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Human-β-endorphin-(14—31)-peptide 190
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Four camel-β-endorphin analogues modified at position 2 and four human-β-endorphin analogues modified at position 2 or 5 194
Human-β-endorphin-(1—15)-, -(1—21)-,

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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. Derivatives of D-amino-acids fall naturally into the second group. Abbreviations used in the tables are collected on pp. xix–xxi.

Coded Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$*1	*Conc.*	*Solvent*	*Ref.*
Alanine					
Ala-CH_2CCl_3,HBr	237 dec.	−20.5	—	DMSO	37
Ala-OCH_2CCl_3	239—241	−10.2	5	DMF	394
Ala-OCH_2CCl_3,tosylate	160—161	−5.7	4.5	DMF	37
Aox-Ala	219—220	−27.2*2	1	MeOH	34
$(Boc\text{-}Ala)_2O$	95—97	−16.9	2	$CHCl_3$	75
Boc-Ala-SBu^t	116—118	−2.12	1.08	$CHCl_3$	36
Cox-Ala	199—200	−27.9*2	1	MeOH	34
2,4-Dnps-Ala	117—118	+35.8	2	MeOH	29
Nmps-Ala,Dcha	153—155	−50.7	2	MeOH	29
Ppt-Ala	128	−13.5	1	EtOH	24
Tfa-Ala-SBu^t	140	+17.14	0.2	$CHCl_3$	36
Z(OMe)-Ala-NHNH,Troc	142—145	−33.8	0.9	MeOH	204
$(Z\text{-}Ala)_2O$	119—121	−16.4	2	$CHCl_3$	75
Z-Ala-SBu^t	88—89	−16.09	1.5	$CHCl_3$	36

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*1 $[\alpha]_D$ recorded at 589nm unless indicated by superscripts: ^{a}546nm ^{b}578nm, and were measured at 20—25 °C *2 $[\alpha]_D$ measured at 16 °C *3 $[\alpha]_D$ measured at 26 °C *4 $[\alpha]_D$ measured at 27 °C *5 $[\alpha]_D$ measured at 19 °C *6m.p. and $[\alpha]_D$ dependent on method of preparation

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Arginine					
Arg(Tos)-OBu^t,HCl	108—109	–4.2	0.5	EtOH	272
Boc-Arg(NO_2)-CHN_2	150—151	–57.2	—	DMF	293
Boc-Arg(NO_2)-OPcp	127—129	–20.6	1.1	DMF	83
Boc-Arg(NO_2)-OPfp	104–105	–24.8	1.05	EtOAc	83
2,4-Dnps-Arg(NO_2),Dcha	124—125	–7.9	2	MeOH	29
Nps-Arg(NO_2),Dcha	149—151	+10.0	2	MeOH	29
Ppt-Arg, tosylate	199—201	+4.9	1	DMF	24
Z-Arg-ONp,nitrate	123—125	–25.2*[3]	2.7	HOAc	42
Z-Arg-ONp,picrate	—	–18.6*[3]	2.4	DMF	42
Z(OMe)-Arg(Mbs),Cha	110—114	+25.1	0.6	MeOH	269
Asparagine					
Boc-Asn(Xan)	183—184	+2.1*[3]	6	DMF	223
Ppt-Asn	163—164	–5.0	1	MeOH	24
Aspartic acid					
Boc-Asp(OBu^t)-ONSu	103—105	–25.6	1	Dioxan	264
(Boc-Asp[OBzl])$_2$O	oil	–4.0	2	$CHCl_3$	75
Ppt-Asp(OBzl),Dcha	162	–13.7	1	EtOH	24
(Z-Asp[OBzl])$_2$O					75
Z-Asp(OBu^t)-OPcp	128–129	–24.4	1	EtOH	264
Cysteine					
Box-Cys(SZ),Dcha	122—125	–27.0	1.01	$CHCl_3$	50
Cys(Ad)	227—228	–14.5*[3]	0.51	HOAc	49
Cys (Bu^t)	244—245	–15.4*[3]	0.57	HOAc	49
Cys(Bzl)	156—156.5	–64.7	4.5	DMF	37
Cys(Me)	148—149	–6.0	4.5	DMF	37
Cys(MBzl)-OBu^t, L-Tartrate	100—102	–3.2*[4]	0.53	DMF	49
2,4-Dnps-Cys(Bzl)	138—139	–96.4	2	MeOH	29
Ppoc-Cys(Acm)	43—45	–30.4	1	MeOH	187
Ppt-Cys(Bzl),Dcha	170—171	+22.5	1	MeOH	24
Z-Cys(SCB)-OMe	oil	+44.2	1.55	$CHCl_3$	50
Z-Cys(SZ)-OMe	57.5	+17.7	1.02	$CHCl_3$	50
Z(NO_2)-Cys(MBzl)	163—165	–51.2	0.61	DMF	49
Glutamic acid					
(Boc-Glu[OBzl])$_2$O	70—72	–27.0	2	$CHCl_3$	75
Nps-Glu(OBu^t)-ONSu	118—121	–50.1	0.3	DMF	313
(Z-Glu[OBzl])$_2$O					75
Glutamine					
Ppt-Gln,Dcha	172—174	+8.7	1	EtOH	24
Glycine					
Aox-Gly	170—172	—	—	—	34
Aox-Gly-ONp	125—126	—	—	—	34
(Boc-Gly)$_2$O	82—83	—	—	—	75

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D^{*1}$	*Conc.*	*Solvent*	*Ref.*
Gly-OCH_2CCl_3,tosylate	149—149.5	—	—	—	37
Ppt-Gly	118—119	—	—	—	24
Tfa-Gly-SBu^t	104—105	—	—	—	36
Voc-Gly	90—91	—	—	—	23
$(Z\text{-Gly})_2O$	118—119	—	—	—	75
Z-Gly-SBu^t	oil	—	—	—	36
Histidine					
Boc-His(Boc)	88—90	—	—	—	395
Boc-His(Z),Dcha	122—124	+32	0.5	EtOH	119
Isoleucine					
$(Boc\text{-Ile})_2O$	68—69	−32.8	2	$CHCl_3$	75
Phthaloyl-Ile	124—126	−45.4	2	EtOH	396
Ppt-Ile,Cha	187—188	−7.5	1	EtOH	24
$(Z\text{-Ile})_2O$	oil	−39.0	2	$CHCl_3$	75
Leucine					
Aox-Leu	169—170	−22.4	1	MeOH	34
$(Boc\text{-Leu})_2)O$	oil	−16.6	2	$CHCl_3$	75
Cox-Leu	119—120	−23.4	1	MeOH	34
Leu-OCH_2CCl_3,tosylate	216—218	+1.9	4.5	DMF	37
Leu-Opop,HCl	142—142	−2.7	1	AcOH	369
Nps-Leu-Opop	76—78	−117.9[a]	1	$CHCl_3$	369
Ppt-Leu,Dcha	137—138	−15.0	1	EtOH	24
$(Z\text{-Leu})_2O$	oil	−3.0	2	$CHCl_3$	75
Lysine					
Boc-Lys(Me_2)	156—158	+6.7	2	H_2O	397
Box-Lys(Pms)	94—95	−1.5	0.5	MeOH	45
Boc-Lys(^iPr)	243	+6.8	2	H_2O	397
Boc-Lys(2,4,-Cl_2-Z),Dcha	63—67	+5.9	1	$CHCl_3$	369
Lys(HCO)	no data	+13.9	2	sat. $NaHCO_3$	398
Lys(Pms)	261 dec.	+15.5	0.5	HOAc	45
Nps-Lys(2,4-Cl_2-Z),Dcha	174—177	−32.0	1	$CHCl_3$	369
Nps-Lys(Z)-NHEt	126—127	+23.7[a]	1	DMF	137
Nps-Lys(Z)-ONp	77—78	−123.5[a]	1	$CHCl_3$	369
Nps-Lys(Z)-OPcp	110—111	−59.4[a]	1	DMF	137
Lys(Tos)-ONb,HBr	153—156	−2.5	0.5	MeOH	351
Lys(Z)-NHEt,HCl	151—152	+25.0[a]	1	DMF	137
Lys(Z)-ONp,HCl	153—155	+6.3[a]	1	HOAc	369

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Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Z(OMe)-Lys(Z)-NHNH,Troc	101—105	−30.9	1	MeOH	205
Z-Lys(Tos)-ONb	108—109	−8.3	0.5	DMF	351
Methionine					
Boc-Met(O)*[6]	116—118	−13.9	—	DMF	16
$(Boc\text{-}Met)_2O$	76—78	−1.8	2	$CHCl_3$	75
Boc-Met-NH_2	119—121	−7.0	1	MeOH	134
Boc-Met(O)-NH_2	97—99	+8.0	1	MeOH	134
Met-OCH_2CCl_3, tosylate	176—177	+1.3	4.5	DMF	37
Met(O_2)-OMe,HCl	164	+23.2	1	0.1M-HCl	185
		+14.5	1	DMF	185
Met-NH_2,HCl	206—208	+16.4	1	0.1M-HCl	185
		+25.5	1	DMF	185
Met(O)-NH_2,HCl	144—145	+20.3	1	0.1M-HCl	185
		+21.2	1	DMF	185
Met(O_2)-NH_2,HCl	170—172	+19.2	1	0.1M-HCl	185
		−9.5	1	DMF	185
Ppt-Met,Dcha	145—146	−1.2	1	MeOH	24
Z-Met(O)*[6]	100—104	+2.5	—	AcOH	16
$(Z\text{-}Met)_2O$	—		—		75
Z-(OMe)-Met(O)*[6]	97—100	+8.4	—	AcOH	16
Z(OMe)-Met(O)-NH_2	194—196	+93.8*[4]	0.2	MeOH	292
Phenylalanine					
Aox-Phe	201—203	−147.0	1	MeOH	34
$(Boc\text{-}Phe)_2O$	95—97	+19.6	2	$CHCl_3$	75
Cox-Phe	224—225	−170*[2]	1	MeOH	34
Phe-CH_2CCl_3,HBr	193	+0.9	—	DMSO	37
Phe-OCH_2CCl_3 (Tosylate)	181.5—182	+7.2	4.5	DMF	37
Phe-NH_2,HBr	249—250	+19.7	1	DMF	263
Ppt-Phe,Dcha	190—191	+8.7	1	EtOH	24
Tfa-Phe-SBu^t	164—166	+85	1.02	CH_3CN	36
Z-Phe-NHNH,Boc	104—107	−24.4[b]	1	DMF	399
$(Z\text{-}Phe)_2O$	128—129	+27.3	2	$CHCl_3$	75
Z-Phe-OBu^t	120—125	—	—	—	69
Z-Phe-SBu^t	55—57	−7.24	1.27	$CHCl_3$	35
Proline					
$(Boc\text{-}Pro)_2O$	oil	−60.8	2	$CHCl_3$	75
Boc-Pro-SBu^t	oil	−74.78	1.8	$CHCl_3$	36
Ppt-Pro	128—130	−15.0	1	EtOH	24
Tfa-Pro-SBu^t	100–102	+29.6	0.5	$CHCl_3$	36

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Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
$(Z\text{-Pro})_2O$	oil	−71.0	2	$CHCl_3$	75
Z-Pro-SBu^t	oil	−78.77	2.28	$CHCl_3$	36
Serine					
Boc-Ser(Bzl),Dcha	133—134	−23.6	2.28	MeOH	400
$(Boc\text{-Ser[Bzl]})_2O$	oil	−3.5	2	$CHCl_3$	75
2,4-Dnps-Ser(Bu^t),Dcha	127—128	+40.0	2	MeOH	29
Nps-Ser(Bu^t),Dcha	159—161	−16.5	2	MeOH	29
Ppt-Ser,Dcha	157—158	−5.0	1	EtOH	24
Ppt-Ser(Bzl),Dcha	138–139	+5.0	1	EtOH	24
Tfa-Ser-OBzl	74—75	—	—	—	401
Z(OMe)-Ser(Bzl),Cha	134—137	+19.7*[5]	1.1	MeOH	269
Z(OMe)-Ser(Bzl)-NH_2	120—123	+34.1*[3]	0.7	MeOH	269
$(Z\text{-Ser[Bzl]})_2O$	—	—	—	—	75
Z(OMe)-Ser(Bzl)-OBzl	50—52	−8.9*[3]	0.7	DMF	269
Threonine					
Boc-Thr(Bzl)-OPfp	90—92	−30.6	0.5	EtOH	272
Ppt-Thr,Dcha	147—149	−10.0	1	EtOH	24
Thr-OBzl,HCl	127—128	—	—	—	326
Z-Thr(Bu^t)	34—36	+4.4	1	MeOH	221
Z(NO_2)-Thr,Dcha	186—188	−3.8	0.67	DMF	49
Tryptophan					
Z(NO_2)-Trp	108—115	−43.5	0.65	DMF	49
Tyrosine					
Boc-Tyr(Bzl)-ONSu	149—150	−6.1	1	Dioxan	136
$(Boc\text{-Tyr[Bzl]})_2O$	75—77	+8.1	2	$CHCl_3$	75
Boc-Tyr(cyclohexyl), Dcha	142	+36.5	1	MeOH	120
Ppt-Tyr,Dcha	192—193	−1.2	1	MeOH	24
Tyr(^{i}Bor)	185	−6	1	HOAc	120
Tyr(cyclohexyl)	195 dec.	+5	1	HOAc	120
3-Cyclohexyl-Tyr	197	—	—	—	120
Z-Tyr(Et)	76—78	−34.2	1	DMF	353
Z-Tyr(Bu^n)	91—93	−33.6	1	DMF	353
Z-Tyr(Bu^n)-ONp	138—139	−12.5	2	DMF	353
$(Z\text{-Tyr[Bzl]})_2O$	—	—	—	—	75
Z-Tyr(Pr^n)	87—89	−31.3	1	DMF	353
Z-Tyr(Et)-ONp	122—123	−12.9	2	DMF	353
Z-Tyr(n-hexyl)-ONp	129—130	−11.3	2	DMF	353
Z-Tyr(n-hexyl)	80—81	−31.1	1	DMF	353
Z-Tyr(Pr^n)-ONp	142—143	−12.8	1	DMF	353
Valine					
$(Boc\text{-Val})_2O$	84—85	−4.0	2	$CHCl_3$	75

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Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Boc-Val-SBu^t	43—45	−14.32	1.8	$CHCl_3$	36
Phthaloyl-Val	115–117	−68.0	2	EtOH	396
Ppt-Val	112—114	−17.5	1	EtOH	24
Tfa-Val-SBu^t	148—150	−30.63	1.08	$CHCl_3$	36
Val-CH_2CCl_3,HBr	182.5—183	+3.3	—	DMSO	37
Val-OCH_2CCl_3 (Tosylate)	199—200	+8.5	4.5	DMF	37
Val-NH_2,HCl	281—282	+34.1	1	MeOH	92
$(Z\text{-Val})_2O$	99—101	+17.3	2	$CHCl_3$	75
Z-Val-SBu^t	oil	−6.8	3.2	$CHCl_3$	36
Z-Val-6-chloro-hydroxybenzotriazolyl ester	171—172	—	—	—	69

Other Amino-acids

D-Alanine					
Z-D-Ala-pNP ester	79	+38*[3]	1.5	EtOAc	210
α-Aminodecanoic acid					
Boc-D-aminodecanoic acid Pfp ester	45—46	+19.0	1	Dioxan	263
Boc-L-aminodecanoic acid Pfp ester	45—46	−19.5	1	Dioxan	263
α-Amino heptenoic acid					
D,L-2-amino-6-heptenoic acid	260 dec.	—	—	—	402
Boc-D,L-2-amino-6-heptenoic acid	65	—	—	—	402
D-Arginine					
Boc-D-Arg(NO_2)-CHN_2	150—151	+50.7	—	DMF	293
4-chlorophenylglycine					
L-4-chlorophenylglycine-NH_2,HBr	275 dec.	+29.0	1	DMF	264
Z-L-4-chloro-phenylglycine	133—134	+116.0	1	EtOH	264
Z-L-4-chloro-phenylglycine-NH_2	175—176	+32.8	1	DMF	264
Cyclohexylglycine					
D-Cyclohexylglycine-NH_2,HBr	268—270	−63.8	1	DMF	264
L-cyclohexylglycine-NH_2,HBr	268—270	+63.8	1	DMF	264
Z-D-cyclohexylglycine	114—115	−12.5	1	EtOH	264
Z-L-cyclohexylglycine	115—116	+12.4	1	EtOH	264
Z-D-cyclohexylglycine-NH_2	240—241	−19.0	1	DMF	264
Z-L-cyclohexylglycine-NH_2	240—241	+19.0	1	DMF	264

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Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
3,4-Dihydroxyphenylalanine					
O,O'-di-acetyl-L-Dopa,HCl	199—200	+12.2	1	MeOH	360
O,O'-di-acetyl-L-Dopa,NCA	139—141	—	—	—	360
4-Guanidino-phenylalanine					
4-Guanidino-Phe	141—143	+6	0.5	50 % HOAc	403
Tos-4-guanidino-Phe	275—276	+2	1	80 % HOAc	403
Tos-4-guanidino-Phe-OMe amorphous		+35	1	MeOH	403
ε-Hydroxynorleucine					
N-Boc-*O*-acetyl-ε-hydroxynorleucine	54—56	−25.8	1.1	EtOAc	245
ε-Hydroxynorleucine	234—241	+19.6	2	6M-HCl	245
Hydroxyproline					
Boc-Hyp(Bzl)	oil	−37.2	1.7	EtOH	141
Hyp(Bzl),HCl	183—184	—	—	—	141
Hyp(Bzl)-OMe,HCl	135	—	—	—	141
D-Lysine					
D-Lys(Z)	—	−16.4	1	1.5M-HCl	398
Formyl-D-Lys(HCO)	119—121	+3.3	2	H_2O	398
Formyl-D-Lys(Z)	93—95	−10.7	2	EtOH	398
N-Methylcysteine					
Bpoc-(Me)Cys(Bzl);Dcha	146—148	−53.9 ± 1.0	1	MeOH	404
(Me)Cys-(Bzl)OBu^t,HCl	153—153.5	−11.9 ± 0.6	1	MeOH	404
N-Methylvaline					
Bpoc-(Me)Val	138.5—139	−60.1 ± 1.0	1	MeOH	404
3-(1 or 2-naphthyl)-L-alanine					
Boc-Nal(1)	94—95	−55.1	0.8	MeOH	215
Boc-Nal(2)	89—91	+32.9	0.88	MeOH	215
4-Nitrophenylalanine					
Boc-4-NO_2-Phe-NH_2	187—188	−8	1	DMF	405
Boc-4-NO_2-Phe-ONp	187—188	−40	1	DMF	405
Norleucine					
2-NO_2-5-azidobenzoyl-Nle	155—156	—	—	—	231
2-NO_2-5-azidobenzoyl-Nle-OSu	141—143	—	—	—	231
Boc-Nle,Dcha	137—138	+9.4	1	MeOH	263
Boc-Nle,Dcha	144—145	+11.4	1	MeOH	263
Boc-Nle-OPfp	55—57	−26.8	1	dioxan	263
Nle-OMe,HCl	139—140	+14.4	1	O.1M-HCl	357
Norvaline					
Boc-Nva-OPfp	60—62	−32.3	1	dioxan	263
Ornithine					
Boc-Orn(Me_2)	169—170	+8.9	2	H_2O	397

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Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$*[1]	*Conc.*	*Solvent*	*Ref.*
Boc-Orn(Pr^i)	225	+7.3	2	H_2O	397
Boc-Orn(Tfa)	oil	+18.9	1	dichloroacetic acid	303
Boc-Orn(Z)-OSu	80—82	−18.44	2	EtOAc	406
Tfa-Orn	247—249	+13.3	0.2	3M-HCl	303
Pentafluorophenylalanine					
Boc-L-pentafluoro-phenylalanine	106	−5.9*[3]	1.1	MeOH	253
Phenylglycine					
D-phenylglycine-NH_2, HBr	263—264	−75.6	1	DMF	264
L-phenylglycine-NH_2, HBr	263—264	+76.5	1	DMF	264
Z-D-phenylglycine-NH_2	171—173	−76.8	1	DMF	264
Z-L-phenylglycine-NH_2	172—173	+80.7	1	DMF	264
D-Proline					
D-Pro-NHMe	oil b.p. 85° 0.01 torr	+104	1.196	$CHCl_3$	407
D-Serine					
Bpoc-D-Ser,Dcha	152—154	−4.3 ±0.5	1	MeOH	404
Bpoc-D-Ser,H_2O	76—77	+7.6 ±0.5	1	MeOH	404
1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid					
Boc-Tca	>270	+87.0	0.96	MeOH	215
3,5-Dichlorotyrosine					
3,5-Cl_2-Tyr(Me)	218—220	+6.7	1.064	3M-HCl	408
Ac-3.5-Cl_2-Tyr-OMe	119—119.5	+58.3	1.56	dioxan	408
Z-3,5-Cl_2-Tyr(Me)-OMe	103—104	−47.7	1.54	DMF	408
Z-3,5-Cl_2-D-Tyr(Me)-OMe	—	+37.1	1.454	dioxan	408

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4

Chemical Structure and Biological Activity of Hormones and Related Compounds

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1 Hypothalamic Releasing Hormones

Contributed by D. H. Coy

Progress in the search for new hypothalamic releasing and inhibiting factors has been the subject of a number of recent and helpful review articles.[1] Of the three established hormones, thyrotophin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH), and somatostatin, published reports on structure–activity relationships have been limited to the last two peptides during the 1977—78 period examined here.

Luteinizing Hormone-Releasing Hormone.—Recent studies on the LH-RH decapeptide (Figure 1) continue to illustrate[2,3] the probability of the His-Trp sequence representing the active centre of the molecule and the importance of positions 1, 6, and 10 in preserving high binding-affinity for pituitary receptors.

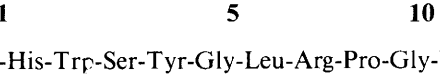


Figure 1 *Amino-acid sequence of LH-RH*

A major consequence of this has been the development of LH-RH antagonists by modification of the second and third positions, the discovery of very active agonists resulting from changes to positions 6 and 10, and the improvement of antagonist activities by incorporation of beneficial changes in positions 1 and 6.

There are now many analogues which are vastly more effective than LH-RH in releasing the gonadotrophins and their pharmacology has become extremely interesting in view of reported anti-fertility effects,[4] reduction of ovarian luteinizing hormone receptors,[5] and regression of mammary tumours in the rat[6] after prolonged administration of very small quantities. The clinical

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[6] E.S. Johnson, J.H. Seely, W.F. White, and E.R. DeSombre, *Science*, 1976, **194**, 329.

necessity for increased activity over existing compounds is debatable and reports on new highly active agonists have been limited to a series of analogues containing aza-glycine in either positions 6, 10, or both.[7,8] The most potent peptide was [D-Ser(Bu^t)^6,azaGly^{10}]-LH-RH which was several times more effective than the highly active and related [D-Ser(Bu^t)^6,desGly^{10}]-LH-RH ethylamide. Incorporation of aza-glycine for glycine in position 6 generally resulted in considerable loss of activity.

It seems reasonably well accepted that the principal reason for the beneficial effects of D-amino-acids in place of glycine in position 6 is the stabilization of a receptor-binding conformation rather than increased resistance to endopeptidases. More controversial are the functions of the ends of the chain, particularly the reason for the increased activities of the desGly^{10}-ethylamides. An examination[9] of the Glp at the *N*-terminus highlighted the importance of the pyrrolidone-ring carbonyl group and it was suggested that it could be undergoing hydrogen-bonding with some other part of the chain or even the receptor. Indeed, a close proximity between the ends of the molecule seems possible in view of the significant activities of two analogues cyclized *via* their *N*- and *C*-termini, despite lack of activity of the linear precursors.[10] It is tempting to argue[11] that positions 1, 6, and 10 are important to the preservation of the same favourable receptor-binding conformation, however, firm evidence for this is lacking.

LH-RH Antagonists. Only substitutions for histidine in position 2 or tryptophan in position 3 have resulted in analogues with extremely low gonadotrophin releasing activities which are still able to bind to LH-RH receptors. The most effective single substitution continues to be D-phenylalanine in place[12] of histidine, and efforts to improve on this by modifications to the benzene ring[3] have thus far been unsuccessful. An enormous amount of effort has gone into improving the binding affinities of peptides containing this modification, the most effective way being incorporation of D-aromatic amino-acids in position 6. In contrast to the agonists, the desGly^{10}-alkylamide alterations are of no value when present in inhibitory structures.

The differences in effects of modifications at the termini of the chain on the properties of the agonists and antagonists are intriguing. For instance, [D-Ala^{10}]-LH-RH has less than 1 % gonadotrophin releasing activity and yet the D-Ala^{10}-antagonists retain much of their activity.[11] [D-Glp^1]-LH-RH is only 10 % active, however, the D-Glp^1-antagonists have increased inhibitory

[7] A.S. Dutta, B.J.A. Farr, M.B. Giles, B. Valcaccia, and A.L. Walpole, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 382.

[8] A.S. Dutta, B.J.A. Farr, M.B. Giles, and B. Valcaccia, *J. Med. Chem.*, 1978, **21**, 1018.

[9] K. Nikolics, D.H. Coy, J.A. Vilchez-Martinez, E.J. Coy, and A.V. Schally, *Internat. J. Peptide Protein Res.*, 1977, **9**, 57.

[10] J. Seprodi, D.H. Coy, J.A. Vilchez-Martinez, E. Pedroza, W.Y. Huang, and A.V. Schally, *J. Med. Chem.*, 1978, **21**, 993.

[11] D.H. Coy, J. Seprodi, J.A. Vilchez-Martinez, E. Pedroza, J. Gardner, and A.V. Schally, Central Nervous System Effects of Hypothalamic Hormones and Other Peptides, ed. R. Collu, A. Barbeau, J.R. Ducharme, and J.G. Rochefort, Raven Press, New York, 1979, 317.

[12] R.W.A. Rees, T.J. Foell, S.Y. Chai, and N. Grant, *J. Med. Chem.*, 1974, **17**, 1016.

activity.[13] The azaGly[10]-modification is equally effective in both the agonists and antagonists.[7, 14]

Tryptophan in position 3 can be replaced by phenylalanine with retention of inhibitory effects and by non-aromatic amino-acids with some loss of activity, although proline actually increases potency.[15] The most effective substitution still appears to be D-tryptophan which approximately doubles antagonist activity,[16] despite virtually destroying gonadotrophin-releasing activity when present in LH-RH itself.

An unusual approach[11, 17] in the design of both antagonists and agonists has been the preparation of a number of analogues with a variety of peptide chains attached *via* the ε-amino-group of a D-lysine residue in position 6. Initially, [D-Phe[2],D-Trp[3], N^{ε}-(Glp-D-Phe-D-Trp-Ser-Tyr)-D-Lys[6]]-LH-RH was found to have much increased inhibitory activity despite its increased molecular weight. Extension of the experiment to full dimers of [D-Phe[2],D-Trp[3],D-Lys[6]]-LH-RH, conjugated through the free amino-group by the use of several dicarboxylic acid active esters, gave some extemely potent compounds and there was some possibility of these analogues binding to more than one receptor site simultaneously. Trimeric molecules made along the same lines were inactive probably due to steric problems. Interestingly, similar peptides derived from the extremely potent agonist, [D-Lys[6], desGly[10]]-LH-RH ethylamide, generally had diminished activities in an inverse trend to that of the inhibitor family.

Table 1 *Anti-ovulatory activities of some representative LH-RH antagonists in increasing order of effectiveness*

Peptide	*Dose/mg*	*% Blockade of ovulation*
[D-Phe[2],D-Leu[6]]-LH-RH	3 × 2	83
[D-Phe[2],Phe[3],D-Phe[6]]-LH-RH	1.5	86
[D-Phe[2],D-Trp[3],D-Phe[6]]-LH-RH	1.0	90
Isophthaloyl-bis[D-Phe[2],D-Trp[3],D-Lys[6]-LH-RH]	0.5	60
[D-Glu[1],D-Phe[2],D-Trp[3],D-Phe[6]]-LH-RH	0.5	80
Isophthaloyl-bis[D-Glp[1],D-Phe[2], D-Trp[3],D-Lys[6]-LH-RH]	0.25	100

To a considerable degree, success in developing increasingly powerful antagonists has been tied to the availability of the rapid and reliable rat anti-ovulation assay. In this test, peptides are used to block the pre-ovulatory surge of LH and FSH in cycling rats which results in inhibition of ovulation. As can be seen in the Table, weaker compounds available several years ago had to be given in multiple injections to be completely effective. With the more recent

[13] J.E. Rivier and W. Vale, *Life Sci.*, 1978, **23**, 869.
[14] A.S. Dutta, B.J.A. Farr, and M.B. Giles, Peptides: Proceedings of the 5th American Peptide Symposium, ed. M. Goodman and J. Meienhofer, J. Wiley and Son, New York, 1977, 189.
[15] J. Humphries, Y.P. Wan, K. Folkers, and C.Y. Bowers, *Biochem. Biophys, Res. Comm.*, 1976, **72**, 939.
[16] D.H. Coy, J.A. Vilchez-Martinez, and A.V. Schally, Peptides 1976: Proceedings of the 14th European Peptide Symposium, ed.A. Loffet, Editions de l'Université de Bruxelles, 1977, 463.
[17] J. Seprodi, D.H. Coy, J.A. Vilchez-Martinez, Pedroza, and A.V. Schally, *J. Med. Chem.*, 1978, **21**, 276.

peptides, single injections are quite sufficient and minimum effective doses have fallen to about 100μg per animal. A few of the more potent inhibitors have been demonstrated to be effective in species other than the rat, including rabbits,[18] baboons,[19] and humans.[20] However, doses required in man must be considerably lowered before LH-RH antagonists can be fully evaluated for anti-fertility effects.

Somatostatin.—Subsequent to the isolation of the growth hormone-release inhibiting hormone, somatostatin, from hypothalamic extracts, it came as a series of major suprises to find that a brain peptide could have such a variety of peripheral activities, particularly on the gut. Now, of course, this no longer appears unusual since many brain peptides have been shown to exert gastrointestinal effects, the reverse being true for gut hormones and peptides. Clearly, somatostatin has great potential as a drug in treating a number of diseases including excess release of growth hormone, gastric acid, pancreatic enzymes, and glucagon. However, at present this broad spectrum of activities severely limits its use for the treatment of one disorder at a time and attempts are being made to develop analogues with more selective activites. As with all natural peptides, the short biological half-life of somatostatin is a problem from a therapeutic standpoint and analogues are being sought with increased durations of action.

Unlike structure–activity studies with LH-RH, the overall picture with respect to somatostatin is a good deal less coherent. The molecule can certainly not at present be divided into residues primarily responsible for binding or for mechanism of action, however, somatostatin has been under investigation for considerably less time and its structure (Figure 2) and biology are considerably more complex than for LH-RH.

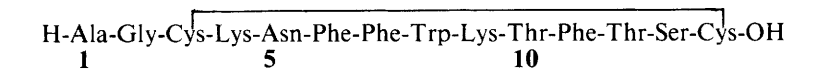


Figure 2 *Amino-acid sequence of somatostatin*

Certainly, replacement of each residue by simple amino-acids such as alanine has revealed[2] those side-chains which are more sensitive to alteration. For instance, [Ala^2]- and [Ala^5]-somatostatin are fully active whereas most of the other alanine analogues have negligible activities except for changes to positions 4, 10, 12, and 13 which caused a less drastic reduction in biological activity. Similarly, each amino-acid in somatostatin has been replaced by its D-isomer to give[2, 21] predominantly inactive or only slightly active analogues. The notable exceptions are [D-Trp^8]-somatostatin which is 6—8 times more active, and [D-Cys^{14}]-somatostatin[22, 23] which is equipotent for

[18] C.P. Phelps, D.H. Coy, A.V. Schally, and C.H. Sawyer, *Endocrinology*, 1977, **100**, 1526.
[19] N. Hagino, D.H. Coy, A.V. Schally, and A. Arimura, *Horm. Metab. Res.*, 1977, **9**, 247.
[20] D. Conzalez-Barcena, A.J. Kastin, D.H. Coy, K. Nikolics, and A.V. Schally, *Lancet*, 1977, 997.
[21] M.P. Brown, D.H. Coy, A. Gomez-Pan, B.H. Hirst, M. Hunter, C. Meyers, J.D. Reed, A.V. Schally, and B. Shaw, *J. Physiol.*, 1978, **277**, 1.
[22] C.A. Meyers, A. Arimura, A. Gordin, R. Fernandez-Durango, D.H. Coy, A.V. Schally, J. Drouin, L. Ferland, M. Beaulieu, and F. Labrie, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 630.
[23] M. Brown, J. Rivier, and W. Vale, *Science*, 1977, **196**, 1467.

inhibition of GH release and selective for glucagon inhibition in some assay systems. Unfortunately, none of the D-amino-acid analogues are long-acting despite the blocking of specific enzyme cleavage points.

Efforts to further increase the potency of the molecule have capitalized on the D-Trp[8]-modification. Other substitutions made in conjuction with this have produced additive effects, for instance [*N*-Tyr,D-Trp[8]]- and [D-Ala[2], D-Trp[8]]-somatostatin are around 10 times more active than somatostatin in suppressing GH release *in vitro*.[24] Even more beneficial results were obtained by incorporation of D- and L-halo-tryptophans in position 8.[25] The D- and L-stereoisomers of [5-fluoro-Trp[8]]-, [6-fluoro-Trp[8]]-, and [5-bromo-Trp[8]]-somatostatin are each more active for inhibition of *in vitro* GH release and *in vivo* gastric acid secretion[26] and [D-5-bromo-Trp[8]]- and [D-5-fluoro-Trp[8]]-somatostatin are 30 and 25 times more active in the GH assay. The reason for the increased activities of the halogenated compounds remains uncertain, although biological activity seems dependent on the degree of resonance in the tryptophan indole nucleus.

Selective biological actions have been observed for a fair number of somatostatin analogues beginning with desAsn[5]-peptides[27] which inhibit insulin and GH, but not glucagon release. The deletion of asparagine in combination with the D-Trp[8]-modification resulted in even greater specificity.[20] This approach was then used with the D-Cys[14]-analogues which are more GH and glucagon specific,[22, 23] selectivity again being enhanced by incorporation of D-tryptophan in position 8. More recently, [des (Ala[1], Gly[2]), His[4,5], D-Trp[8]]-somatostatin was also reported[28] to be a more potent inhibitor of GH and glucagon over insulin release. [Phe[4]]-Somatostatin has been reported[24] to be more active than somatostatin in blocking GH release whereas non-aromatic substitutions for this lysine residue drastically reduce activity. The Phe[4]-peptide was considerably less active than somatostatin for inhibition of insulin and glucagon secretion.

Dissociated activities have not been observed only in the GH, insulin, and glucagon systems. Many very active position 8 analogues have much reduced inhibitory effects on gastric acid compared to other secretions.[21, 26] A series of conformationally restricted cyclic analogues[29] had from 10 times less to no detectable gastric acid inhibitory activity compared to effects on GH levels. [D-Cys[3]]-Somatostatin[21] appeared to be more active on gastric acid than GH release.

It has been known almost from the beginning of somatostatin studies that the non-cyclic Ala-Gly portion of the molecule is not essential for full activity. Apparently, several of the residues within the ring are dispensible since a cyclic

[24] D.H. Coy, C. Meyers, A. Arimura, A.V. Schally, and T.W. Redding, *Metabolism*, 1978, **27**, suppl. 1, 1407.
[25] C.A. Meyers, D.H. Coy, W.Y. Huang, A.V. Schally, and T.W. Redding, *Biochemistry*, 1978, **17**, 2326.
[26] J.D. Reed, B.H. Hirst, A. Gomez-Pan, D.H. Coy, A.V. Schally, and C. Meyers, *Metabolism*, 1978, **27**, suppl. 1, 1411.
[27] S. Effendic, R. Luft, and H. Sievertsson, *F.E.B.S. Letters*, 1975, **58**, 302.
[28] D. Sarantakis, J. Teichman, R. Fenichel, and E. Lien, *F.E.B.S. Letters*, 1978, **92**, 153,
[29] D.F. Veber, F.W. Holly, W.J. Paleveda, R.F. Nutt, S.J. Bergstrand, M. Torchiana, M.S. Glitzer, R. Saperstein, and R. Hirschman, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2636.

analogue containing only 8 amino-acids has been reported[30] to have some activity and to be long-acting after the injection of large amounts. Several peptides containing one extra residue within the ring retained[24,31] high activities, again demonstrating the considerable tolerance of the molecule to quite drastic changes.

Predictions of possible preferred binding conformations for somatostatin are, of course, of great interest and possible aids to analogue design. An ingenious study along these lines has been reported by Veber *et al.*[29] who synthesized several bicyclic analogues, thus introducing more conformational restraints into the molecule. The introduction of disulphide linkages between positions 5 and 12, and 6 and 11 in dicarba-analogues resulted in peptides with much increased activities in all but a gastric acid system. The explanation of this involved the presence of a type II β-turn from Phe^{7} to Thr^{10} and an anti-parallel β-pleated sheet structure including Phe^{6}-Phe^{7} and Thr^{10}-Phe^{11}. Their model explains the high activity of [D-Trp^{8}]-somatostatin and predicts that the side-chains of Asn^{5} and Thr^{12}, and Phe^{6} and Phe^{11} are close, allowing hydrophobic interactions to occur between the latter. Stacking of the aromatic rings previously proposed by Holladay *et al.*[32] cannot take place in this conformation.

It must be assumed that the structural requirements for binding to receptors in different tissues differ considerably. The criteria remain obscure, but there appear to be good prospects for developing extremely specific compounds. An area which has not received much reported attention is the development of competitive inhibitors of somatostatin which should have very interesting biological properties. Studies on peptide antagonists tend to proceed slowly at first until modifications are found which remove activity but not binding affinity. Thus far, inactive analogues which have been screened[26] have also been devoid of inhibitory effects.

2 Posterior Pituitary Peptides

Contributed by M. Manning, M. Kruszynski, and W. H. Sawyer

Structure–activity studies on oxytocin and vasopressin show no signs of letting up. In our last report (see Vol. 10, p. 418) which covered the literature for two years, *i.e.* from early 1976 to early 1978, we listed over 140 newly synthesized analogues. In this review which covers the literature for only one year, *i.e.* from early 1978 to early 1979, we report the syntheses of over 90 new or known analogues. These fall into the following categories as presented in Table 1—6.

1. Antagonists of the oxytocic response to oxytocin.
2. Antagonists of the vasopressor response to arginine vasopressin (AVP) and of the antidiuretic response to lysine vasopressin (LVP).

[30] W. Vale, J. Rivier, N. Ling, and M. Brown, *Metabolism*, 1978, **27**, suppl. 1, 1391.
[31] C.A. Meyers and D.H. Coy, *Endocrinology*, 1978, **102**, 239A.
[32] L.A. Holladay, J. Rivier, and D. Puet, *Biochemistry*, 1977, **16**, 4895.

Table 1 *Antagonists of the oxytocic response to oxytocin*

Peptide	Antioxytocic, pA_2[a] *Isolated rat uterus* (in vitro) No Mg	0.5 mM Mg	*Rat uterus* in situ (in vivo)	*Antivasopressor* pA_2[a]	*Ref.*
dPOT	7.14 ± 0.05(31)[b,c] (6.94[f], 7.04[g])	5.63 ± 0.17(4)[d,e]	—	6.27[f]	
dPTOT	7.52 ± 0.04(68)[c]	6.23 ± 0.11(30)[d,e]	6.31 ± 0.05(6)[d,e]	6.67 ± 0.09(7)	1*a*
dPTyr	7.76 ± 0.12(16)	7.80 ± 0.12(17)	6.68 ± 0.12(11)	7.59 ± 0.05(9)	1*a*
dP Tyr(Me)TOT	7.64 ± 0.14(16)	7.79 ± 0.09(11)	6.84 ± 0.10(8)	7.32 ± 0.04(8)	1*a*
dEt_2OT	7.55 ± 0.14(8) (7.24[f], 7.41[g])	6.28 ± 0.11(10)	6.20 ± 0.12(5)	6.83 ± 0.08(7)	1*a*
dEt_2TOT	7.72 ± 0.11(18)	7.36 ± 0.09(17)	6.47 ± 0.11(17)	6.30 ± 0.13(5)	1*a*
$d(CH_2)_5OT$	7.61 ± 0.08(14) (7.43[f], 7.53[g])	7.15 ± 0.10(17)	6.65 ± 0.06(11)	weak[f]	1*a*
$d(CH_2)_5TOT$	7.91 ± 0.13(25)	7.81 ± 0.09(30)	6.94 ± 0.11(12)	5.86 ± 0.03(10)	1*a*
[1,6-N^{ε}-carbonyl-L-Lys]OT[h]	'agonist'[i,j]	—	—	—	1*b*
Pen[1],Leu[2]OT	7.14(12)	—	—	—	1*c*
2-D-Trp OT	6.87	—	—	—	1*d*
2-D-Phe OT	6.00	—	—	—	1*d*
2-D-Leu OT	5.23	—	—	—	1*d*
d,2-Phe(4-Br-Ch_2-CO-NH) OT[l,m]	6.73	—	—	—	1*e*
d,2-Phe(4-Et-CO-NH) OT[m]	6.65 ± 0.68(3)	—	—	—	1*e*

(*a*) pA_2 represents the negative logarithm to the base 10 of the average molar concentration (M) of an antagonist which will reduce the specific biological response to 2X units of an agonist to the level of response to X units of the agonist[1i]; (*b*) means ± S.E. number of assay groups in parentheses; (*c*) data from Manning *et al.*[1f]; (*d*) data from Sawyer *et al.*[1g]; (*e*) also act as partial agonists; (*f*) from Nestor *et al.*[1h]; (*g*) calculated from present data by the method of Nestor *et al.*[1h]; (*h*) $-CH_2-S-S-CH_2-$ bridge-in-ring of oxytocin was replaced by the $-(CH_2)_4-$ group of lysine, while the ε-NH_2 group of the same residue took the place of the α-CH of cysteine-I; (*i*) has a rat uterotonic acitivity *in vitro* of 3.9 ± 0.3(6) units mg^{-1} and less than 0.5(4) unit mg^{-1} of rat antidiuretic activity; (*j*) anti-avian vasodepressor $pA_2 = 7.27$; (*k*) no oxytocic activity; inhibitor of oxytocin in the milk-ejection assay; (*l*) 'irreversible' antagonist of oxytocin (long persistence of inhibitory effect, slow wash-out from the tissue); (*m*) does not give an inhibition of the blood pressure response to LVP in single doses given concomitantly with LVP (23 rats).

The abbreviations and their full names are as follow: dPOT-[1-deaminopenicillamine]oxytocin; dPTOT-[1-deaminopenicillamine,4-threonine]oxytocin; dP Tyr(Me)OT-[1-deaminopenicillamine, 2-*O*-methyltyrosine]oxytocin; dP Tyr(Me)TOT-[1-deaminopenicillamine,2-*O*-methyltyrosine,4-threonine]oxytocin; d Et_2OT-[1-(β-mercapto β,β-diethylpropionic acid)]oxytocin; dEt_2TOT-[1-(β-mercapto-β,β-diethylpropionic acid),4-threonine]oxytocin; d$(CH_2)_5$ OT-[β- mercapto-β, β-cyclopentamethylenepropionic acid)]oxytocin; d$(CH_2)_5$TOT-[1-(β-mercapto-β, β-cyclopentamethylenepropionic acid), 4-threonine]oxytocin; Pen, Leu^2OT-[1-Penicillamine,2-Leucine]oxytocin; d,2-Phe(4-Br-CH_2CO-NH)OT-[1-deamino, 2-(*p*-bromoacetylamino)phenylalanine]oxytocin; d,2-Phe(4-CH_3CH_2 CONH)-OT-[1-deamino, 2-(*p*-propionylamino)phenylalanine]oxytocin;

1*a* J. Lowbridge, M. Manning, J. Seto, J. Haldar, and W.H. Sawyer, *J. Med. Chem.*, in the press.
1*b* M. Bodanszky, D.T. Fagan, R. Walter, and C.W. Smith, *J. Med. Chem.*, 1978, **21**, 306.
1*c* V.J. Hruby, K.K. Deb, D.M. Yamamoto, M.E. Hadley, and W.Y. Chan, *J. Med. Chem.*, 1979, **22**, 7.
1*d* D.A. Kaurov, Ya.D. Mikhailov, and M.P. Smirnova, *Bioorg. Khim.*, 1978, **4**, 619
1*e* V. Pliska, and P. Marbach, *European J. Pharmacol.*, 1978, **49**, 213.
1*f* M. Manning, J. Lowbridge, J. Seto, J. Haldar, and W.H. Sawyer, *J. Med. Chem.*, 1978, **21**, 179.
1*g* W.H. Sawyer, J. Haldar, D. Gazis, J. Seto, K. Bankowski, J. Lowbridge, A. Turan, and M. Manning, *Endocrinology*, 1980, **106**, 81.
1*h* J.J. Nestor, jun., M.F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, 1975, **18**, 284.
1*i* H.O. Schild, *Brit. J. Pharmacol.*, 1947, **2**, 189.

3. Agonistic analogues of oxytocin.
4. Agonistic analogues of AVP.
5. Deuteriated and ^{13}C enriched analogues of oxytocin and AVP
6. New syntheses of known analogues of oxytocin and of AVP.

Analogues of oxytocin and vasopressin possessing highly active and selective potencies highlighted the studies presented in our last report. With one exception (Table 4) no new dramatic advances have been made in this area during the past year. The focus has shifted to the development of potentially useful antagonists of oxytocin and vasopressin (Tables 1 and 2). The new agonistic analogues of oxytocin presented in Tables 3 and 4 are of interest in some cases because of the chemical methodology involved in their synthesis as well as for the insights provided for structure–activity studies. Many of the analogues in Tables 5 and 6 were made for conformational studies and have helped to yield significant new information in this area. The application of h.p.l.c. to the purification of analogues of oxytocin, (Tables 5 and 6), has been one of the real highlights of this past year.

Antagonists of the Oxytocic Response to Oxytocin (Table 1).—A number of promising analogues possessing potent antagonism *in vivo* have been synthesized. dP Tyr(Me)OT*, dP Tyr(Me)TOT, and $d(CH_2)_5TOT$ fall into this category.[1a] Also, investigation of the *in vivo* antagonistic properties of the known *in vitro* antagonist, $d(CH_2)_5OT$[1a 1b] revealed that it also is a potent antagonist of oxytocic responses to oxytocin in the rat.

Antagonistic activities were measured on the rat uterus (*a*) in the absence of Mg^{2+}, (*b*) in the presence of Mg^{2+}, and (*c*) *in situ* and are expressed as pA_2 values.[1e] Thr[4] substitution in oxytocin analogues with dialkyl substitutions in position 1 led to an enhancement of antagonistic potency in all three assay systems. Of particular interest is the enhancement of antagonistic potency *in vivo* in the β,β-diethyl and β,β-cyclopentamethylene analogue series. Thus, dPOT is a partial agonist, the diethyl analogue has a pA_2 of 6.20, and the cyclopentamethylene analogue has a pA_2 of 6.65. Comparable figures for the Thr[4] analogues are 6.31, 6.47, and 6.94. Thus, increasing the size of the alkyl substituents on the β-carbon at position 1 leads to enhanced *in vivo* anti-oxytocic potency and Thr[4] substitution leads to a further enhancement in each case.

Substitutions at position 2 of oxytocin alone and in combination with changes at position 1 continue to be a very fruitful approach to the design of antagonists. Thus the substitution of D-Trp, D-Phe, and D-Leu at position 2 in oxytocin gave rise to compounds possessing low to moderate *in vitro* antagonistic potency. Leu substitution at position 2 in dPOT did not substantially increase antagonsitic potency *in vitro.* However the substitution of *O*-methyltyrosine [Tyr(Me)] at position 2 in dPOT and in dPTOT brought about striking enhancements in antagonistic potency in all three assay systems. With *in vivo* pA_2 values of 6.86, 6.84, and 6.94, dPTyr(Me)OT, dP Tyr(Me)TOT, and $d(CH_2)_5TOT$ are the most potent *in vivo* antagonists of

*Abbreviations are defined in the footnotes to the Tables.

oxytocic responses to oxytocin reported to date. These findings have obvious implications for the design and synthesis of even more potent *in vivo* antagonists.

Bodanszky and co-workers reported the synthesis of one of the most intriguing analogues of oxytocin reported to date *i.e.* [1,6-N^{ε}-carbonyl-L-lysine]-oxytocin.[1b] This analogue was synthesized by treating Tyr-Ile-Gln-Asn-Lys-Pro-Leu-Gly-NH_2 with di-*p*-nitrophenyl carbonate. In this peptide the side-chain of lysine *i.e.* $-(CH_2)_4NH-$ replaces the $-CH_2SSCH_2CH_2-$ bridging portion of deamino-oxytocin. It differs from deamino dicarba-oxytocin in having an –NH– group in place of the α-CH_2– group at position 1. This compound has weak *in vitro* uterotonic activity and is an effective antagonist of the vasodepressor response of oxytocin in chickens, with a pA_2 of 7.27 in this assay.

Antagonists of the Vasopressor Response to AVP and of the Antidiuretic Response to LVP (Table 2).—*Vasopressor Antagonists.* At the present time antagonists of the pressor response to AVP are of value for studies to determine what role, if any, AVP plays in regulating blood pressure under normal and pathophysiological conditions (see below under 'Other Studies'). In the future they may thus find clinical use as diagnostic and/or therapeutic tools. A number of the antagonists shown in Table 2 resulted from a rational approach to design following the serendipitous discovery that dPVDAVP was a potent antivasopressor substance (See Vol. 10, p. 423). Thus Tyr(Me) substitution at position 2 in AVP brought about a reduction in pressor activity. The substitution of the deamino-penicillamine (dP) residue at position 1 in AVP gave rise to an analogue, dPAVP, possessing potent antivasopressor properties. The combination of the dP and Tyr(Me)[2] substitutions produced a peptide, dPTyr(Me)[2] AVP, possessing the highest antivasopressor potency reported to date.[2a] The incorporation of the β,β-cyclopentamethylene substitution in place of dP at position one in dPVDAVP, although reducing the antivasopressor potency slightly, greatly increased the antivasopressor–antidiuretic selectivity.[2b] Thus $d(CH_2)_5$VDAVP has less than 1/1000th the antidiuretic potency of dPVDAVP. Furthermore $d(CH_2)_5$VDAVP has been found to behave like a competitive inhibitor of vasopressin-induced renal adenylate cyclase activation.[2c] These findings offer useful clues to the design of more potent antagonists of the vasopressor response to AVP and may also in combination with information from the following section lead to the design of effective antagonists of the antidiuretic response to AVP.

Antidiuretic Antagonists. An effective antagonist of the antidiuretic response to AVP would be extremely valuable for the treatment of the syndrome of inappropriate secretion of AVP. The three Tyr[2]-*O*-alkylated analogues of [deaminolysine]vasopressin (dLVP) synthesized by Larsson and co-workers may represent promising leads towards such an antagonist.[2d] They possess mixed agonist–antagonist properties. All three analogues exhibited weak antidiuretic activity at high doses. Also, none of the analogues inhibited the antidiuretic action of LVP when the two substances were given together in a single injection. However, when administered in low subthreshold doses they

Table 2 *Antagonists of the vasopressor response to AVP and of the antidiuretic response to LVP*

Peptide	*Antivasopressor* pA_2[a]	*Antioxytocic* pA_2[a]	*Anti-antidiuretic (% inhibition)*	*Antidiuretic activity*[b]	*Pressor activity*[b]	*Ref.*
dP Tyr(Me)AVP	7.96 ± 0.05(14)	7.61 ± 0.14(14)	—	3.5 ± 0.5	anti	*2a*
$d(CH_2)_5$VDAVP	7.68 ± 0.05	6.62 ± 0.07	—	0.10 ± 0.02	anti	*2b*, *2c*
dPAVP	7.45 ± 0.11(8)	6.93 ± 0.10(14)	—	42 ± 3	anti	*2a*
Tyr(Me)AVP	—	7.44 ± 0.12(18)	—	386 ± 36	9.7 ± 0.5	*2a*
d Tyr(Et)LVP[c,d]	—	—	(74 ± 16)[e]	0.2 – 2.0[g]	0.5 – 3.0[f]	*2d*
d Tyr(Pr^n)LVP[d]	—	—	(47 ± 18)[e]	0.2 – 2.0[g]	0.5 – 3.0[f]	*2d*
d Tyr(Bu^n)LVP[d]	—	—	(36)[e]	0.2 – 2.0[g]	0.5 – 3.0[f]	*2d*

(*a*) as in Table 1; (*b*) U mg^{-1}; (*c*) when administered in low subthreshold doses complete inhibition was obtained; (*d*) inhibition of the LVP induced antidiuretic effects was demonstrated when LVP was administered by a permanent infusion. The antagonistic effect was not observed when LVP and the analogue were injected simultaneously. Agonistic activity was shown with potencies in the range 0.2—2.0 I.U. μmol^{-1}; (*e*) inhibition expressed % ± S.E; (*f*) no antagonistic effects, either in an infusion–injection or injection–injection experiment; activity expressed in I.U. μmol.

The abbreviations and their full names are as follows:dP Tyr(Me)AVP-[1-deaminopenicillamine, 2-*O*-Tyr(Me), 8-Arg]vasopressin; $d(CH_2)_5$VDAVP-[1 (β-mercapto-β,β-cyclopentamethylenepropionic acid, 4-Val, 8-D-Arg]vasopressin; dPAVP-[1-deaminopenicillamine, 8-Arg]vasopressin; Tyr(Me)AVP-[2-*O*-Tyr(Me), 8-Arg]-vasopressin; Tyr(Me)AVP [2-*O*-Tyr(Me), 8-Arg]vasopressin; d Tyr(Et)LVP-[1-deamino, 2-*O*-Tyr(Et), 8-Lys]vasopressin; d Tyr(Pr^n)LVP-[1-deamino, 2-*O*-Tyr(Pr^n), 8-Lys]vasopressin; d Tyr(Bu^n)LVP-[1-deamino, 2-*O*-Tyr(Bu^n), 8-Lys]vasopressin.

2a K. Bankowski, M. Manning, J. Haldar, and W.H. Sawyer, *J. Med. Chem.*, 1978, **21**, 850.
2b J. Lowbridge, M. Manning, J. Haldar, and W.H. Sawyer, *J. Med. Chem.*, 1978, **21**, 3132.
2c D. Butlen, G. Guillon, R.M. Rajerison, S. Jard, W.H. Sawyer, and M. Manning, *Molecular Pharmacol.*, 1978, **14**, 1006.
2d L.E. Larsson, G. Lindeberg, P. Melin, and V. Pliska, *J. Med. Chem.*, 1978, **21**, 352.

suppressed the antidiuresis induced by a continuous infusion of LVP. Complete inhibition of the effect of infused LVP was observed when dTyr(Et)[2]LVP was infused simultaneously. The antagonistic potency decreased with increasing size of the alkyl substituent: the Tyr(hexyl)[2] derivative possessed no antagonistic activity (see Table 4). These analogues did not behave as typical partial agonists in this assay in that they showed weak antagonistic activity over a limited range of doses. This suggests that the antagonism may have been 'physiological' rather than pharmacological, in that low doses might have reversed submaximal antidiuresis by increasing glomerular filtration rate, free water formation or solute excretion, or by any combination of these effects. This illustrates the difficulties in interpreting results of assays based on such a complicated response as the rate of urine formation in an intact animal. Considering the potential importance of specific antagonists of antidiuretic hormones the actions of these peptides in adenylate cyclase activation and receptor-displacement assays on isolated renal tubular membrane preparations would be very informative.

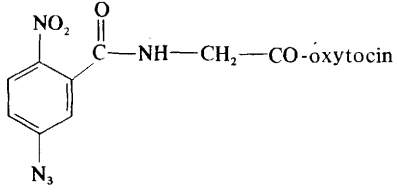
Agonistic Analogues of Oxytocin (Table 3).—Although none of the analogues in this category are more active than oxytocin, they are all of interest for a variety of other reasons. Replacement of the sulphur atoms at positions 1 or 6 in oxytocin by methylene groups, to give carba-1 and carba-6 derivatives respectively, can alone and in combination with deamination at position 1 give rise to analogues of oxytocin with strikingly enhanced oxytocic potency relative to oxytocin.[1—4] (This information was inadvertently omitted from the list of structural modifications of oxytocin which enhance activity given in our review in Vol. 10.) The activities obtained provided more evidence for the non-equivalence of the sulphur atoms at these two positions. Thus deaminocarba-1-oxytocin[2] has twice the oxytocic potency (1899 U mg^{-1}) of deaminocarba-6-oxytocin (929 U mg^{-1}). When these analogues were converted to their respective sulphoxides[3b] the oxytocic potencies obtained represent a striking reversal. The potencies of both peptides were reduced but to markedly different degrees. The deaminocarba-6-sulphoxide retained 50 % of the oxytocic potency of its parent whereas the deaminocarba-1-sulphoxide retained less than 1 % of the oxytocic activity of its parent. The resulting activities afforded a ratio of 35 : 1 in favour of the deaminocarba-6-sulphoxide. These findings further substantiate the non-equivalence of the sulphur atoms at positions 1 and 6 and point to a critical steric requirement for the sulphur atom position at position 6 in facilitating receptor binding and possibly receptor activation. Further evidence for a steric specificity at this position is shown by the further oxidation of the deaminocarba-1-sulphoxide to the deaminocarba-1-sulphone. Thus conversion of the –SO– group to an $-SO_2-$ group at position 6 virtually abolished biological activity. The

[1] J. Rudinger and K. Jošt, *Experientia*, 1964, **20**, 570.
[2] K. Jošt, *Coll. Czech. Chem. Comm.*, 1971, **32**, 218.
[3] K. Jošt, T. Barth, I. Krejčí, L. Fruhaufova, Z. Prochazka, and F. Šorm, *Coll. Czech. Chem. Comm.*, 1973, **38**, 1073.
[4] K. Jošt and F. Šorm, *Coll. Czech. Chem. Comm.*, 1971, **36**, 234.

Table 3 *Agonistic analogues of oxytocin*

Peptide	*Biological activities*[a] *Rat uterus oxytocic* (O)*	*Rat milk ejection* (M.E.)	*Rat antidiuretic* (A)	*Rat pressor* (P)	*Ref.*
Oxytocin	520 ± 12 486 ± 15(Mg^{2+})	474 ± 16[b] 533 ± 62[c]	4.0 ± 0.8	4.3 ± 0.12	3*a*
Sulphoxide of 1-deamino-6-carba-oxytocin	454.4	216.2	—	—	3*b*
Sulphoxide of 1-deamino-1-carba-oxytocin	13.1	91.0	—	—	3*b*
Sulphoxide of 1-carba-oxytocin	0.26	0.02	—	—	3*b*
Sulphone of 1-deamino-1-carba-oxytocin	0.05	0.03	—	—	3*b*
5-Asp-oxytocin[d]	20.3 ± 0.8	—	0.14 ± 0.02		3*c*
[2-D-(*p*-Ethoxy)Phe]oxytocin	2.59	—	—	—	3*d*
[2-Trp]oxytocin	0.24	—	—	—	3*d*
[2-Ph-Gly]oxytocin	0.01	—	—	—	3*d*
NAB-Gly-oxytocin[e]		—	—	—	3*e*
[2-L-m-Tyr]oxytocin[f]	—	—	—	—	3*f*
[4-Glu-1-deamino-1-carba]oxytocin	]	—	—	—	3*g*
Cyclo-oxytocin[g]	*i*	—	—	—	3*h*
[8-Met]oxytocin[h]	200 ± 4	—	0.4 ± 0.2	27 ± 0.8	3*i*

(*a*) Activities expressed in I.U. mg^{-1} ± S.E.; (*b*) rabbit; (*c*) rat-mammary strip; (*d*) avian vasodepressor, 41 ± 2 U mg^{-1}; (*e*) [$^{\alpha}$*N*-(2-nitro-5-azidobenzoylglycyl]oxytocin:



(*f*) [2-L-*m*-Ho-Phe]oxytocin; (*g*) in uterotonic, pressor, and milk ejection assays *cyclo*-oxytocin was inactive both as agonist and inhibitor up to a dose of 5×10^{-2} mg; (*h*) avian vasodepressor, 193 ± 7 U mg^{-1}.

*Mg^{2+} indicates that assays were carried out in the presence of 0.5 mM Mg^{2+}.

3*a* M. Manning, E.J. Coy, and W.H. Sawyer, *Experientia*, 971, **27,** 1372.
3*b* M. Lebl, T. Barth, and K. Jošt, *Coll. Czech. Chem. Comm.*, 1978, **43,** 1538.
3*c* R. Walter, G. Skala, and C.W. Smith, *J. Amer. Chem. Soc.*, 1978, **100,** 972.
3*d* Ref. (1*d*) from Table 1.
3*e* J.M. Stadel, D.B.P. Goodman, R.E. Galardy, and H. Rasmussen, *Biochemistry*, 1978, **17,** 1403.
3*f* K. Midura-Nowaczek and S. Drabarek, *Polish J. Chem.*, 1978, **52,** 341.
3*g* M. Lebl and K. Jošt, *Coll. Czech. Chem. Comm.*, 1978, **43,** 523.
3*h* M. Zaoral and V. Krchňák, *Coll. Czech. Chem. Comm.*, 1977, **42,** 3500.
3*i* M. Bodanszky, N. Chandramouli, J. Martinez, and R. Walter, *J. Med. Chem.*, 1979, **22,** 270.

deaminocarba-1-sulphoxide was obtained by two routes (*a*) by oxidation of deaminocarba-1-oxytocin using periodate[5] and (*b*) by stepwise synthesis in solution. Reduction of the deaminocarba-1-sulphoxide with hydrogen bromide and acetone[6] brought about regeneration of the fully active deaminocarba-1-oxytocin.

[5-Aspartic acid]-oxytocin[3c] is the first 5-position oxytocin analogue to retain a high degree of oxytocin-like potencies. It retains a high affinity for oxytocin receptors and was also shown to exhibit an intrinsic activity identical with that of oxytocin under various experimental conditions. This finding has been interpreted in terms of a proposed conformational model of oxytocin (see Vol 5, p. 409; Vol. 6, p. 444), a model which heretofore has stressed the essential role of the asparagine residue at position 5 for activity, and was predicted on the basis that aspartic acid could retain much of the hydrophilicity, appropriate steric requirements, and hydrogen-bonding capabilities of asparagine.

2-Nitro-5-azidobenzoylglycyloxytocin (NAB-Gly-oxytocin) is a photo affinity labelling peptide.[3e] It was synthesized to facilitate the isolation and characterization of neurohypophysial hormone receptors from toad urinary bladder. It was shown to retain a high affinity for these receptors and was found to be a potent agonist for the stimulation of osmotic water-flow and [^{14}C]urea diffusion. Photolysis of the toad bladder in the presence of NAB-Gly-oxytocin led to a permanent inhibition of the oxytocin-enhanced change in [^{14}C]urea permeability. This inhibition was shown to be due to the covalent attachment of the photo-activated analogue to the hormone receptors. It is proposed to incorporate tritium into NAB-Gly-oxytocin. With a high radiospecific activity ($\geq$ 30 Ci mmol^{-1}) this would be a very useful tool with which to isolate and possibly characterize neurohypophysial hormone receptors from the toad bladder and, possibly, other tissues.

[Deamino-1-carba-4-glutamic acid] oxytocin[3g] was prepared for possible use in the isolation of oxytocin receptors from uterine tissue as previously described[7] for [deamino-4-glutamic acid]-oxytocin. By coupling the analogue *via* the free carboxy-group to an insoluble support it was hoped that it could be utilized as an affinity-type ligand. Since mono substituted carba analogues are more active, have greater metabolic and chemical stability than their disulphide counterparts, they could thus offer advantages for such an affinity support. However this analogue is very inactive and is in fact the first monocarba analogue of deamino-oxytocin with lower activity than the parent disulphide (d-Glu^4-oxytocin has 13 U mg^{-1} of utertonic activity[7]). More detailed pharmacology will be presented elsewhere.

[2-L-*m*-Tyrosine]-oxytocin[3f] represents the first reported synthesis of a peptide analogue in which tyrosine is replaced by L-3-hydroxyphenylalanine. Its pharmacological activities have not yet been reported. This information

[5] K.J. Wynne and I. Haiduc, in 'Methodieum Chimicum' Vol. 7, ed. H. Zimmer and K. Niedenzu, Thieme, Stuttgard, 1976, p. 693.
[6] B. Iselin, *Helv. Chim Acta.* 1961, **44**, 61.
[7] B.M. Ferrier and L.A. Branda, *Canad. J. Biochem.*, 1975, **53**, 21.

will provide fresh insight into the precise role played by the position of the hydroxy-group at position 2 in oxytocin.

Cyclo-oxytocin[3h] represents yet another unusual and intriguing analogue of oxytocin in which the *C*-terminal carboxy-group of the glycine of the side-chain of oxytocinoic acid was cyclized as the *p*-nitrophenyl ester with the α-NH_2 group of the cysteine residue at position 1. *Cyclo*-oxytocin thus has two rings; the 20 membered oxytocin ring and a new 17-membered ring formed by joining the side-chain, the so-called 'tail', and the 'head' of the molecule together. The 17-membered ring was formed first, followed by deprotection and oxidative cyclization of the 20-membered ring. The yield of pure product from this latter stage was only 4 % which contrasts with yields of 40—70 % reported for non-sterically hindered analogues.[8] This suggests a marked steric and/or constraining effect by the preformed 17-membered ring. As might have been anticipated from known structure–activity studies, the formation of the 17-membered ring completely abolished all the known activities associated with oxytocin. The loss of activity could be due to the lack of the *C*-terminal amide, the blocking of the *N*-terminal α-NH_2 group, the increased strain and decreased flexibility suggested by the low yield on forming the S–S bridge or to combinations of all of these. Enlargement of the 17-membered ring to a 20-membered or larger ring, the incorporation of Asn as the *C*-terminal amino-acid for cyclization thus retaining the required –$CONH_2$ group, might alone or in combination help to differentiate between the roles played by these three factors.

The synthesis of [8-methionine]oxytocin was motivated by a variety of reasons.[3i] Thus it could serve as a model compound for the synthesis of methionine containing peptides; it has relevance to studies on the phylogeny of the neurohypophysial peptides[9] and it would provide more insight into structure–activity relationships of position-8 analogues of oxytocin.[10] The synthesis was accomplished by coupling the partially protected tocinoic ring compound[11,12] with the *C*-terminal Pro-Met-Gly-NH_2. These were obtained using stepwise *p*-nitrophenyl ester couplings[13] catalyzed by 1-hydroxy-benzotriazole.[14] Two interesting and useful approaches were used to eliminate side reactions: acetyl-DL-methionine-n-butyl ester was used as a scavenger in the trifluoroacetic acid (TFA) deprotection steps to suppress *S*-alkylation of the methionine residue. The use of 2,4-dinitrophenol or pentachlorophenol as additives during coupling of the tyrosine residue prevented *O*-acylation and allowed the tyrosine residue to be incorporated into the peptide chain with its phenol hydroxy-group unprotected. Boc-[8-methionine]oxytocin was obtained in 50 % yield from the 'ring' and the 'tail' coupling step carried out with dicyclohexylcarbodi-imide in the presence of 1-hydroxy-benzotriazole[14]. Deprotection with TFA gave [8-methionine]oxytocin.

[8] M. Manning, L. Balaspiri, M. Acosta, and W.H. Sawyer, *J. Med. Chem.*, 1973, **16**, 975.
[9] W.H. Sawyer, *Fed. Proc. Fed. Amer. Soc. Exp. Biol.*, 1977, **36**, 1842.
[10] J.W.M. Baxter, M. Manning, and W.H. Sawyer, *Biochemistry*, 1969, **8**, 3592.
[11] M.F. Ferger, W.C. Jones, D.F. Dyckes, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1972, **94**, 982.
[12] M. Mühlmann, M.I. Titov, R. Schwyzer, and J. Rudinger, *Helv. Chim. Acta*, 1972, **55**, 2854.
[13] M. Bodanszky, *Nature*, 1955, **175**, 685.
[14] W. König and R. Geiger, *Chem. Ber.*, 1973, **106**, 3626.

The pharmacological properties of this material show that substitution of methionine for leucine at position 8 resulted in the retention of almost 50 % of the uterotonic activity of oxytocin. The increase in pressor activity is also quite striking. In this respect the effects of methionine substitution are analogous to those previously reported for [8-valine]oxytocin[15] and [8-glutamine]oxytocin[16] analogues which were shown to exhibit enhanced vasopressor activities (17 and 34 units mg^{-1} respectively) relative to oxytocin (4 units mg^{-1}).

Nine known naturally occurring variants of the neurohypophyseal peptides have been discovered to date.[9] They can be classified into neutral oxytocin-like peptides and basic vasopressin-like peptides. The former group comprises oxytocin, mesotocin, isotocin, glumitocin, aspartocin and valitocin. The latter group consists of arginine vasotocin, arginine vasopressin, and lysine vasopressin. Position 3,4, and 8 are the known variable positions with position 8 being the most variable. Six amino-acids: Leu, Ile, Gln, Val, Arg, and Lys occupy this position in the known peptides. These amino-acids are related to each other by single point changes in their respective codons. Methionine substitution at position 8 could also take place by a single point mutation. Therefore, this relationship coupled with the similarity in structures between methionine and the other known amino-acids at this position suggests the possibility that [8-methionine]oxytocin may exist in nature.

Agonistic Analogues of Arginine Vasopressin (Table 4).—These analogues were synthesized for a variety of reasons: to increase antidiuretic potency, to act possibly as antagonists of the antidiuretic response to LVP, to shed light on the biosynthesis of AVP, and to modify and prolong the activities of known peptides by forming hormonogen derivatives.

The carba derivatives of oxytocin and vasopressin[1—4] continue to be a gold mine of synthetic challenges and of fascinating biological characteristics. Deamino-carba-6-AVP was found to be over three times more potent than AVP as an antidiuretic agent.[4g] Its reported antidiuretic activity is 1569 units mg^{-1}. More recently deamino-carba[1] AVP[4f] has been found to be an extraordinarily potent antidiuretic substance. Based on the preliminary data for its potency reported by Procházka and co-workers[4f] its activity is in the range of 16 000—24 000 units mg^{-1}. If this value is confirmed, this would make this analogue the most potent antidiuretic analogue reported to date (see Vol. 10, p. 422). The 10—15 fold differences in antidiuretic potencies of these deamino-carba-6 and deamino-carba-1 AVP analogues are in keeping with the previously observed difference for other monocarba analogues[1—4, 4f] and again points to the critical role of the sulphur atom at position 6 for receptor binding and activation. This analogue was also found to have a prolonged antidiuretic effect. N^{α}-$(Gly)_3$des-9-glycinamide-lysine-vasopressin[4b] acts as a hormogen to release des-9-glycinamide-LVP[17,18] which has been shown to delay extinction of learned avoidance behaviour in rats.[19] It was synthesized with the prolong-

[15] see ref. 10
[16] J.W.M. Baxter, T.C. Wuu, M. Manning, and W.H. Sawyer, *Experientia*, 1969, **25**, 1127.
[17] V. du Vigneaud, M.F. Bartlett, and A. Johl, *J. Amer. Chem. Soc.*, 1957, **79**, 5572.
[18] S. Lande, A. Witter, and D. de Wied, *J. Biol. Chem.*, 1971, **246**, 2058.
[19] D. de Wied, *Nature*, 1971, **232**, 58.

Table 4 *Agonistic analogues of arginine vasopressin*

Peptide	*Biological activities*[a] (A)	(P)	(O)	(M.E.)	*Ref.*
Arginine vasopressin	332 ± 20	376 ± 6	15 ± 1	—	4*a*
$^{\alpha}N$-$(Gly)_3$,8-Lys[des-9-glycineamide]vasopressin	0.22	10	<0.1[b]	no effect	4*b*
$^{\alpha}N$-$(Gly)_3$,8-Orn vasopressin	0.9[b]	5.25[b]	<[b]	—	4*c*
d Tyr(Hex^n)LVP[c]	0.2 – 2.0[e]	0.5—3.0[e]	—	—	4*d*
Tyr(Bu^n)LVP[d]	0.2 – 2.0[e]	0.5—3.0[e]	—	—	4*d*
$^{\alpha}N$-Pro-Arg-Val-Arg-vasopressin	—	0.3 ± 0.01[f]	—	—	4*e*
[8-Arg]vasopressinoyl-Ala-Met-Ala-NH_2	—	0.2 ± 0.01[e]	—	—	4*e*
Deamino-carba′-Arg-vasopressin	16 000—24 000[g]	~550	34	65	4*f*

(*a*) Activities expressed I.U. mg^{-1} ± S.E.; (*b*) in reference 4*c* the authors gave results relative to earlier reported values for $^{\alpha}N$-$(Gly)_3$, 8-Lys-vasopressin[4*h*]); (*c*) [1-deamino, 2(*O*-n-hexyl^n)Tyr, 8-Lys]vasopressin; (*d*) 2-(*O*-Bu^n)Tyr, 8-Lys]vasopressin; (*e*) partial agonist; (*f*) the log (dose)-time response curve 4*i*-was parallel to that obtained for AVP, indicating that the observed pressor activity originated from an intrinsic activity of the peptide rather than conversion *in vitro* into AVP[*j*], (*g*) this potency range is based on the observation that this analogue is 10—15 times more potent than [deamino carba 6]-AVP which was reported to have antidiuretic activity of 1569 units mg^{-1}.[4*g*]

4*a* M. Manning, L. Balaspiri, M. Acosta, and W.H. Sawyer, *J. Med. Chem.*, 1973, **16**, 975.
4*b* Z. Procházka, T. Krejčí, B. Kupkowá, J. Slaninova, V. Bojanovská, Z. Prusik, J.A. Vosekalna, P. Maloň, T. Barth, J. Fric, K. Bláha, and K. Jošt, *Coll. Czech. Chem. Comm.*, 1978, **43**, 1285.
4*c* M. Lebl, T. Barth, and K. Jošt, *Coll. Czech. Chem. Comm.*, 1978, **43**, 1300.
4*d* Ref. (2*c*) in Table 2.
4*e* G. Moore, *Biochem. J.*, 1978, **173**, 403.
4*f* Z. Procházka, T. Barth, J.J. Cort, K. Jošt, and F. Šorm, *Coll. Czech. Chem Comm.*, 1978, **43**, 655.
4*g* K. Jošt, Z. Procházka, J.H. Cort, T. Barth, J. Skopkova, Z. Prusik, and F. Šorm, *Coll. Czech. Chem. Comm.*, 1974, **39**, 1290.
4*h* J. Kynch, K. Rezabek, E. Kasafirek, V. Pliska, and J. Rudinger, *European J. Pharmacol.*, 1974, **28**, 294.
4*i* V. Pliška, *European J. Pharmacol.* 1969, **5**, 253.
4*j* J. Rudinger, V. Pliška, and I. Krejči, *Recent. Prog. Horm. Res.*, 1972, **28**, 131.

ation of such effects in mind. N^{α}-$(Gly)_3$LVP and N^{α}-$(Gly)_3$ des-9-glycinamide LVP both prolong learned avoidance behaviour in rats and there is a suggestion that these hormonogens may act longer than LVP or des-9-glycinamide LVP but direct comparisons were not made. The hormonogens were also reported to reduce amnesia induced by halothane.

How oxytocin and AVP are biosynthesized still remains a puzzle. There is considerable evidence that AVP is synthesized as part of a larger prohormone[20] and recent studies indicate that AVP and oxytocin and their respective neurophysins derive from common precursors of about 22 000 daltons.[21] It is assumed that the linear nonapeptide sequences are synthesized on ribosomes as part of the larger sequences and that a conformationally induced cyclization to the respective disulphides takes place off the ribosome *prior* to the liberation of the free peptides *via* enzymatic hydrolysis. By studying the relative ease of cyclization of linear AVP sequences containing additional amino-acids at the *N*-terminal and *C*-terminal ends respectively, Moore[4e] hoped to obtain clues to the possible position of the linear AVP sequence within a putative precursor molecule. The linear sequences of N^{α}-Pro-Arg-Val-AVP, AVP-Ala-Met-Ala-NH_2, AVP, arginine vasotocin, oxytocin, oxypressin, pressinamide, and tocinamide were synthesized and following deprotection cyclized to the corresponding disulphides. N^{α}-Pro-Arg-Val-AVP and AVP-Ala-Met-Ala-NH_2 represent model pro-hormones simulating covalent attachment of AVP to the carboxy and amino termini respectively of bovine neurophysin II. The products obtained were shown to depend on the length of the peptide chain: (i) nonapeptides gave monomer and dimer species, (ii) hexapeptides produced mixtures containing higher polymers, and (iii) dodecapetides gave predominantly monomer with some dimerized material. It was thus concluded that the tripeptide tail facilitated the formation of the disulphide bridge. Extension of the peptide chain from either the *N*- or the *C*-terminal end further facilitated intramolecular bridge formation. (These studies have an obvious bearing on the low yield obtained in the cyclization of the greatly constrained *cyclo*-oxytocin which was discussed above under Table 3.)

With regard to the biosynthesis of AVP it was cautiously concluded from these studies that the linear AVP sequence may well have a *central* position in a putative precursor. A similar study with linear sequences of AVP simultaneously extended at both ends might add further support to this hypothesis.

Deuteriated and ^{13}C-Enriched Analogues of Oxytocin and Vasopressin (Table 5).—Twenty deuteriated analogues and six ^{13}C-enriched analogues have been prepared.[5a—e] All of these analogues were prepared for studies dealing with their conformation and/or binding properties as part of current investigations of the relationships of the conformations of oxytocin and vasopressin to their biological properties (see Vols 5, p. 409, Vol. 6, p. 444, Vol. 10, p. 431). The development of reverse phase h.p.l.c. for analytical[5c] and preparative[6j] separation of diastereoisomers of the neurohypophysial peptides greatly facilitated the synthesis of a number of these. Thus the deuteriated analogues of

[20] H. Sachs and Y. Takabatake, *Endocrinology*, 1964, **75**, 943.
[21] H. Gainer, Y. Sarne, and M.J. Brownstein, *J. Cell. Biol.*, 1977, **73**, 366.

Table 5 *Deuteriated and ^{13}C-enriched analogues of oxytocin and vasopressin*

Oxytocin analogue	*Ref.*
[Leu^2,Pro^3,(α,α-$^2H_2$)Gly^4]	5*a*
[9-(α,α-$^2H_2$)glycinamide]	5*b*
[1-hemi(α,β,β-$^2H_3$)cystine]	5*b*
[6-hemi(α-^{2}H)cystine]	5*b*, 5*e*
[6-hemi(β,β-$^2H_2$)cystine]	5*b*
[6-hemi-D-(α-^{2}H)cystine]	5*b*
[1-hemi(α-^{2}H)cystine]	5*b*
[1-hemi-D-(α-^{2}H)cystine]	5*b*,5*e*
[7-(α,β,β,γ,γ,δ,δ-$^2H_7$)Pro]	5*b*
[2-DL-(α-^{2}H)Tyr][a]	5*b*,5*e*
[2-DL-(β,β-$^2H_2$)Tyr][a]	5*b*
[1-hemi-D-(α,β,β-$^2H_3$)cystine]	5*c*2
[2-DL-(2-^{13}C)Tyr][a,b]	5*c*
[1-hemi(3-^{13}C)cystine]	5*d*
[1-hemi-D-(3-^{13}C)cystine]	5*d*
[2-(2-^{13}C)Tyr]Arg[b]	5*c*
[3-DL-(2-^{13}C)Leu][a]	5*e*
[8-DL-(2-^{13}C)Leu][a]	5*e*
Vasopressin analogue	
[1-hemi(α-^{2}H)cystine]Arg	5*b*
[1-hemi-D-(α-^{2}H)cystine]Arg	5*b*
[1-hemi(β,β-$^2H_2$)cystine]Arg	5*b*
[2-(α-^{2}H)Tyr]Arg	5*b*
[2-D-(α-^{2}H)Tyr]Arg	5*b*
[2-(α,β,β-$^2H_3$)Tyr]Arg	5*b*
[3-(α-^{2}H)Phe]Arg	5*b*
[9-(α,α-$^2H_2$(glycinamide]Arg	5*b*

(*a*) The resolution of diastereoisomers is also described; (*b*) 90 % ^{13}C-enrichment of [2-^{13}C]Tyr.

5*a* R. Deslauriers, I.C.P. Smith, G.C. Levy, R. Orlowski, and R. Walter, *J. Amer. Chem. Soc.*, 1978, **100**, 3912.

5*b* V.J. Hruby, K.K. Deb, A.F. Spatola, D.A. Upson, and D. Yamamoto, *J. Amer. Chem. Soc.*, 1979, **101**, 202.

5*c* M. Blumenstein, V.J. Hruby, and D.M. Yamamoto, *Biochemistry*, 1978, **17**, 4971.

5*d* R. Deslauriers, I.C.P. Smith, G.L. Stahl, and R. Walter, *Internat. J. Peptide Protein Res.*, 1979, **13**, 78.

5*e* B. Larsen, V. Viswanatha, S.Y. Chang, and V.J. Hruby, *J. Chromatog. Sci.*, 1978, **16**, 207.

the 1 and 6-hemi D- and L-cystine-oxytocins, of 2-D-and 2-L-tyrosine oxytocin, and the ^{13}C-enriched analogues of 8 D- and L-leucine oxytocin were obtained from their respective DL mixtures by analytical h.p.l.c.[5c]

Deslauriers and co-workers[5a] studied the ^{13}C n.m.r. characteristics of [Pro^3, Gly^4]oxytocin, an analogue possessing a sterically restricted peptide backbone. Assignments were also made for [Pro^3]oxytocin and for [Leu^2, Pro^3, (α,α^2H_2) Gly^4]oxytocin. The introduction of a prolyl residue at position 3 was shown to cause conformational heterogeneity as a consequence of *cis–trans* isomerism about the Tyr-Pro peptide bond. They also found that the more sterically crowded [Pro^3]oxytocin possessed more of the *cis* isomers (45 %) as compared to [Pro^3, Gly^4]oxytocin (33 %).

Hruby and co-workers have performed the herculean task of synthesizing twenty-nine analogues of the neurohypophysial peptides.[5b] The deuteriated

and ^{13}C-enriched ones are presented in Table 5, the remainder are given in Table 6. These were used (*a*) to make unequivocal assignments of most of the α and β carbon atoms of oxytocin and (*b*) to obtain and compare the ^{13}C n.m.r. spectra of six of the known naturally occurring neurohypophysial peptides: 1. oxytocin, 2. AVP, 3. mesotocin, 4. isotocin, 5. aspartocin, and 6. glumitocin with a view to possible correlations of their conformations with their biological activities. The ^{13}C n.m.r. spectra of a number of inhibitors of oxytocin as well as a number of diastereoisomeric peptide pairs were also examined. The assignments for oxytocin generally agree with those previously reported by Walter and co-workers.[22, 23]

These studies have, in conjunction with previous studies,[22—24] helped to firmly establish the ^{13}C n.m.r. chemical shift assignments for essentially all of the carbon atoms in oxytocin and AVP except for the carbonyl carbons.

The ^{13}C chemical shifts for mesotocin, isotocin, aspartocin, and glumitocin were found to be nearly identical with those of oxytocin except for specific residue changes, indicating that, despite major differences in biological properties, their conformations are very similar, with any differences being undetectable by ^{13}n.m.r. techniques. The antagonists showed few changes in some instances while in others, significant changes in ^{13}C n.m.r. chemical shifts were observed. With respect to the oxytocin and AVP diastereoisomers, extensive changes in chemical shifts were observed both at the modified residue and in other parts of the molecules. These latter changes appear to reflect specific conformational perturbations. These authors urge caution in the interpretation of ^{13}C n.m.r. chemical shifts of neurohypophysial peptides in terms of correlating these with changes in their biological properties.

Previous studies on the binding of oxytocin and vasopressin to their carrier molecules, the neurophysins,[24—31] carried out in different laboratories by different techniques, are in substantial agreement that the *N*-terminal tripeptide portions of the respective rings are critical for binding. There has, however, been disagreement over the magnitude of the dissociation rate constant of the hormone–protein complex.[26, 27, 29, 30, 31] Using suitable controls Blumenstein *et al.*[5c] have repeated their earlier ^{1}H n.m.r. studies,[29] carried out new ^{13}C n.m.r. studies, and have reconfirmed that the dissociation rate constant for oxytocin–NP binding at or near neutral pH is very slow $< 10\ s^{-1}$. Their studies reaffirm the importance of the tyrosine residue at position 2 in the binding process in both oxytocin and vasopressin, and show the similarity in the binding of oxytocin and AVP to the bovine neurophysins I and II at a 1 : 1

[22] R. Walter, K.U.M. Prasad, R. Deslauriers, and I.C.P. Smith, *Proc. Nat.. Acad. Sci. U.S.A.*, 1973, **70**, 2086.
[23] R. Deslauriers, R. Walter, and I.C.P. Smith, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 265.
[24] P. Balarum, A. Bothnerby, and E. Breslow, *Biochemistry*, 1973, **12**, 4695.
[25] E. Breslow, *Adv. Enzymol.*, 1974, **40**, 271.
[26] J.A. Glasel, V.J. Hruby, J.F. McKelvy, and A.F. Spatola, *J. Mol. Biol.*, 1973, **79**, 555.
[27] R. Alazard, P. Cohen, J.S. Cohen, and J.H. Griffin, *J. Biol. Chem.*, 1974, **249**, 6895.
[28] V.J. Hruby, D.M. Yamamato, Y.C.S. Yang, and M. Blumenstein, in 'Peptides', Proceedings of the 5th American Symposium, Sand Diego, 1977, ed. M. Goodman and J. Meienhofer, Academic Press, New York, 1977, p. 179.
[29] M. Blumenstein, V.J. Hruby, D. Yamamato, Y. Yang, *F.E.B.S. Letters*, 1977, **81**, 347.
[30] J.H. Griffin, C. Dibello, R. Alazard, P. Nicolas, and P. Cohen, *Biochemistry*, 1977, **16**, 4104.
[31] A.F. Pearlmutter and C. McMains, *Biochemistry*, 1977, **16**, 628.

mole ratio. Evidence for conformational changes in the peptides upon binding to the protein was also obtained.

Using ^{13}C n.m.r. techniques[5d] Deslauriers and co-workers have shown that the binding of [1-hemi(3^{13}C)cystine]oxytocin with bovine neurophysin II takes place over a wide pH range and that the rate of exchange is a function of pH.

New Synthesis of Known Analogues of Oxytocin and AVP (Table 6).—These analogues served as models in a number of interesting investigations of synthetic and purification methods as well as for conformational studies. The highlights of these studies were (*a*) the development of a h.p.l.c. procedure[6j] for the preparative separation of a series of diastereoisomeric analogues of oxytocin and (*b*) reports of two new approaches for the synthesis of arginine

Table 6 *New syntheses of oxytocin, of arginine vasopressin, and of their analogues*

Peptide	*Ref*
Oxytocin[a]	6*j* 6*a*,6*b*
Mesotocin	6*c*
Isotocin	6*c*
Aspartocin	6*c*
Glumitocin	6*c*
Oxytocin	
[des-4-glutamine]	6*c*
[4-Leu]	6*c*
[2,4-di-Leu]	6*c*
[2-Ile,4-Leu]	6*c*
[2-Phe,4-Leu]	6*c*
[4-Gly]	6*a*
[1-Penicillamine][b]	6*a*
[1-Penicillamine-2,-Leu][a]	6*a*
[1-desamino][a,c]	6*b*
[4-Thr][a,d]	6*b*
[3-Pro,4-Gly]	6*h*
[3-Leu]	6*i*
[2-D-Tyr]	6*i*
Arginine vasopressin	6*c*
Oxypressin	6*c*
DDAVP[e]	6*d*
Arginine vasotocin[f]	6*e*
[2-(*O*-Et)Tyr,8-Lys]vasopressin	6*f*
Pressinamide	6*g*
Oxypressin[g]	6*g*
Tocinamide	6*g*
Oxytocin	
[1-hemi-D-cystine]	6*j*
[2-D-Tyr]	6*j*
[4-D-glutamine]	6*j*
[5-D-Asp]	6*j*
[7-D-Pro]	6*j*
[8-D-Leu]	6*j*
[6-hemi-D-cystine]	6*j*

(*a*) Synthesized by solid-phase method using active esters in the presence of 1-hydroxybenzotriazole;[6b] (*b*) antagonist of oxytocin; (*c*) oxytocic activity [O] = 837 U mg^{-1}; (*d*) [O] = 899 U mg^{-1}; (*e*) [1-β-mercaptopropionic acid, 8-D-Arg]vasopressin: more than 50 g of DDAVP was prepared by purification on a continuous free-flow electrophoresis. Some biological data were given as a percentage of standard DDAVP (= 100 %): antidiuretic activity [A] 79.8—128.0 %; vasopressor activity [P] 0.3—191 %: in antidiuretic and blood pressor assays, the concentration of DDAVP samples was expressed by an arithmetic mean of the concentration calculated from the nitrogen content and determined by polarographically; (*f*) the nitro-group from nitroarginyl peptides was selectively reduced by titanium(III); (*g*) [P] = 2·8 ± 0·2; [O] = 59 ± 3 (Mg^{2+}).

6*a* V.J. Hruby, K.K. Deb, J. Fox, J. Bjarnason, and A.T. Tu, *J. Biol. Chem.*, 1978, **253**, 6060.
6*b* S.A. Khan and K.M. Sivanandaiah, *Internat. J. Peptide Protein Res.*, 1978, **12**, 164.
6*c* Ref. (5*b*) in Table 5.
6*d* M. Zaoral, M. Flegel, T. Barth, and A. Machova, *Coll. Czech. Chem. Comm.*, 1978, **43**, 511.
6*e* R.M. Freidinger, R. Hirschmann, and D.F. Veber, *J. Org. Chem.*, 1978, **43**, 4800.
6*f* Ref. (2*d*) from Table 2.
6*g* Ref. (4*e*) from Table 4.
6*h* A. Ballardin, A.J. Fischman, W.A. Gibbons, J. Roy, I.L. Schwartz, C.W. Smith, R. Walter, and H.R. Wyssbrod, *Biochemistry*, 1978, **17**, 4443.
6*i* Ref. (5*e*) in Table 5.
6*j* B. Larsen, B.L. Fox, M.F. Burke, and V.J. Hruby, *Internat. J. Peptide Protein Res.*, 1979, **13**, 12.
6*k* A.T. Tu, J.B. Bjharnason, and V. Hruby, *Biochim. Biophys. Acta*, 1978, **533**, 530.

containing peptides[6e] and of oxytocin analogues[6b] respectively. With the adaptation of their analytical h.p.l.c. method[5e] for the preparative separation of diastereoisomers of oxytocin, Larsen and co-workers have made available a new and very powerful tool for obtaining such desired peptides in the highest degree of purity.[6j] Oxytocin and seven of its diastereoisomers were examined. Procedures which provide separations from oxytocin and from each other were reported. The effects of pH, solvent, and salt concentrations on chromatographic behaviour were also examined and the results obtained discussed and interpreted in terms of the proposed solution conformations of some of the diastereoisomers. The resolving power obtained (as illustrated in Figure 1) is most impressive. A new synthesis of arginine vasotocin (AVT) was described.[6e] This peptide was obtained in a high degree of purity suitable for the development of a radioimmunoassay for AVT. A very novel feature of this synthesis was the use of titanium trichloride for the selective deprotection of the nitro-group from nitro-arginine. New syntheses of oxytocin,[32] deamino-oxytocin,[33] and [4-threonine]oxytocin[34] which employed the diphenylmethyl (DPM) group[35] for protection of the cysteine residues have been reported.[6b] Active esters in the presence of hydroxybenzotriazole[14] were used to mediate the coupling steps. The values for the oxytocic activities of these materials are similar to those previously described.

Oxytocin,[6k] [4-glycine]oxytocin,[6a] and the 1-penicillamine analogues of

[32] V. du Vigneaud, C. Ressler, J.M. Swan, C.W. Roberts, and P.G. Katsyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.
[33] D.B. Hope, V.V.S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, 1962, **237**, 1563.
[34] M. Manning, E. Coy, and W.H. Sawyer, *Biochemistry*, 1970, **9**, 3925.
[35] I. Photaki, V. Bardakos, A.W. Lake, and G. Lowe, *J. Chem. Soc. (C)*, 1968, 1860.

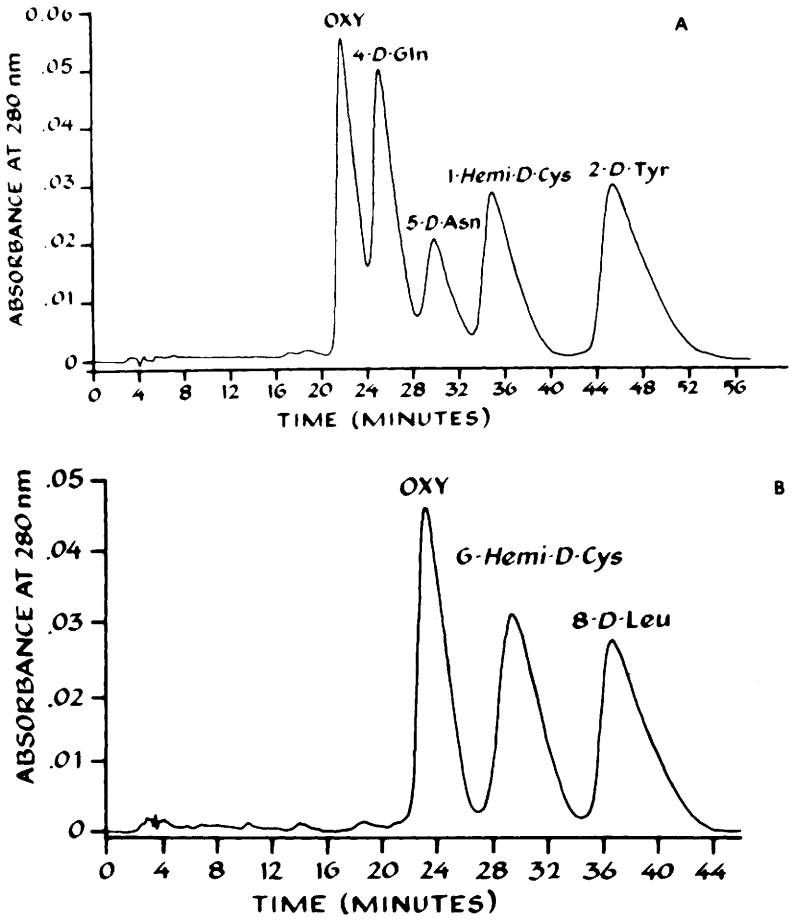


Figure 1 *A. H.p.l.c. of a mixture of oxytocin, [4-D-Glu]oxytocin, [5-D-Asp]oxytocin, [1-hemi-D-cystine]oxytocin and [2-D-Tyr]oxytocin on* μBondapak C_{18} 0.39 × 60 cm, 0.05 M-NH_4OAc, pH 4.0/THF[90 : 10 V/V], 1.6 ml min^{-1}, 0.1 AFS *at* 280 nm. *B. H.p.l.c. of a mixture of oxytocin, [6-hemi-D-cystine]oxytocin and [8-D-Leu]oxytocin on* μBondapak C_{18} 0.39 × 60 cm, 0.01 M-NH_4OAc, pH 4.0/MeCN [82 : 18 V/V], 2.0 ml min^{-1}, 0.1 AFS *at* 280 nm.

(Reproduced by permission from *Internat. J. Peptide Protein Res.*, 1979, **13**, 16)

oxytocin and of [2-leucine] oxytocin[6a] were prepared for laser Raman (LR) and circular dichroism (c.d.) spectroscopy studies of their conformations relative to that of oxytocin. The Raman spectra and the c.d. spectra of the two agonists were similar to each other but differed from those of the two antagonists both of which were also similar. The data furthermore indicated that the penicillamine analogues are less flexible than oxytocin and its [4-glycine] analogue. These results are in agreement with the results of previous n.m.r. studies on oxytocin and [1-penicillamine]oxytocin in aqueous solution (see Vol 10, p. 423).

Use of Synthetic Analogues in Other Studies.—Synthetic analogues of oxytocin and AVP are finding increasing usefulness in a variety of other investigations. Examples of these are studies which seek (*a*) to clarify the role(s) of AVP and/or oxytocin in learning and memory,[36—41] in pain processes,[42] and in the regulation of blood pressure[43—46] and (*b*) to determine how oxytocin and AVP are metabolized.[47—50] Due to space considerations only brief summaries of the most interesting aspects of these studies are presented.

Learning and Memory.—The recent report by Legros *et al.*[36] that LVP appeared to enhance performance by men aged 50—65 on psychometric tests of attention, immediate memory, and learning has sparked immense interest throughout the world. An accompanying, somewhat anecdotal, account reports improvement of memory in several patients with traumatic amnesia during LVP treatment.[37] Further careful studies are clearly indicated. LVP or DDAVP could be used but these preparations suffer from several serious drawbacks. The other effects of LVP, including headache, vasoconstriction, abdominal cramps, and antidiuresis, make truly double-blind testing impossible. DDAVP would not produce any of these symptoms except antidiuresis but this analogue is only about 3 % as active as AVP when tested on conditioned avoidance behaviour in rats.[38] The effects of either LVP or DDAVP on water excretion make them unsuitable for chronic use except under close clinical scrutiny. Des-9-glycinamide-LVP[17, 18] has about 1 % or less of the vasopressor and antidiuretic activities of LVP, but it appears about as potent in preventing loss of conditioned avoidance behaviour in rats.[38] It would appear to offer a more promising candidate for clinical testing. Large doses would have to be administered, however, and its antidiuretic activity of about 3 U mg^{-1} cannot be ignored.[39]

The effects of vasopressin analogues on learned behaviour in rats, at least, can be clearly dissociated from their other pharmacological effects. This situation should provide a challenge calling for the design of even more specific analogues devoid of antidiuretic activity. Much smaller doses of LVP or des-9-glycinamide-LVP are effective when injected into the cerebral ventricles of rats than when injected systemically.[40] It is possible that structural analogues could be designed that could gain access to the brain more readily, perhaps by increasing their lipophilic properties. These could be more effective after systemic administration.

Vasopressin and the Regulation of Blood Pressure.—Although AVP is the most potent known vasopressor agent in mammals it has long been believed that it only participated in cardiovascular regulation when released in massive

[36] J.J. Legros, P. Gilot, X. Seron, J. Claessens, A. Adam, J.M. Moeglen, A. Audibert, and P. Berchier, *Lancet*, 1978, 41.
[37] J.C. Oliveros, M.K. Jandali, M. Timsit-Berthier, R. Remy, A. Benghezal, A. Audibert, and M.J. Moeglen, *Lancet*, 1978, 42.
[38] R. Walter, J.M. Van Ree, and D. de Wied, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2493.
[39] D. deWied, H.M. Greven, S. Lande, and A. Witter, *Brit. J. Pharmacol.*, 1972, **45**, 118.
[40] D. de Wied, and W.H. Gispen, in 'Peptides in Biology', ed. H. Gainer, New York, Plenum Press, 1977, p. 397.
[41] B. Bohus, G.L. Kovacs, and D. deWied, *Brain Research*, 1978, **157**, 414.
[42] K.S. Kendler, R.E. Weitzman, and D.A. Fisher, *Clinical Endocrinology*, 1978, **8**, 89.

quantities after severe blood loss. Studies in dogs in which all compensatory reflexes were blocked, however, reveal that doses of AVP in the antidiuretic range could cause marked systemic vasoconstriction.[43] Recent experiments using specific antisera to AVP show that plasma levels of AVP that would not raise blood pressure in normal rats do contribute to the maintenance of hypertension in rats made hypertensive by chronic administration of deoxycorticosterone and salt[44] and to the vasopressor response by normal rats to injection of angiotensin II into the cerebral ventricles.[45] Antagonistic analogues of AVP also lower blood pressure in deoxycorticosterone- and salt-treated rats.[46] More specific and potent antagonists of the vasopressor action of AVP should represent a promising area for further development. These may not only serve as useful pharmacological probes of the possible importance of vasopressin in cardiovascular regulation in physiological and patholphysiological states but also may have potential clinical uses.

Metabolism.—Structural analogues of the neurohypophysial peptides can be useful in investigations on mechanisms involved in the elimination of these hormones. Weitzman and Fisher[47] report that monoiodinated AVP is eliminated less rapidly than AVP or tritiated AVP and occupies a smaller apparent volume of distribution in dogs. Since iodinated AVP is biologically inert, they suggest that a substantial proportion of the AVP is eliminated by a receptor-mediated and saturable pathway. An alternative possibility is that iodinated AVP resists hydrolysis by an enzyme system with a high affinity for AVP and a limited capacity. Gazis and Sawyer[48] found that deamino-AVP infused into rats is eliminated slowly and, essentially, only by renal excretion, whereas only about 6 % of infused AVP is excreted intact. This emphasizes the importance of aminopeptidases, such as those previously reported in rat-kidney extracts, in the metabolism of AVP by rats. If iodination of the 2-tyrosine inhibits inactivation of AVP by such enzymes, as does deamination, this could explain the delayed elimination of iodinated AVP.

Studies on metabolism of structural analogues of the neurohypophysial hormones are complicated by the lack of available radioimmunoassays and labelled peptides. Gazis and Sawyer[49] reported a simple method for analysing vasopressor dose–response curves to obtain estimates of plasma half-lifes of analogues. With this method Gazis[50] further confirmed that deamination, or substitution of a hydroxyl for the free amino-group, prolongs the half-life of AVP but not of oxytocin. Oxytocin half-life is prolonged by certain substitutions for the 8-leucine. This suggests that enzymatic cleavage at a site in the tripeptide 'tail' is relatively more important than aminopeptidase activity in the metabolism of oxytocin in rats. Synthetic peptides containing isotopic labels on appropriate amino-acid residues would be highly useful in more definitive studies on the metabolism of neurohypophysial hormones.

[43] A.W. Cowley, jun., E. Monos, and A.C. Guyton, *Circ. Res.*, 1974, **34**, 505.
[44] J. Mohring, B. Mohring, M. Petri, and D. Haack, *Amer. J. Physiol.*, 1977, **232**, F260.
[45] D. Haack and J. Mohring, *Pflugers Archiv.*, 1978, **373**, 167.
[46] J.T. Crofton, L. Share, R.E. Shade, W.J. Lee-Kwon, M. Manning, and W.H. Sawyer, *Hypertension*, 1979, **1**, 38.
[47] R.E. Weitzman and D.A. Fisher, *Amer. J. Physiol.*, 1978, **235**, E591.
[48] D. Gazis and W.H. Sawyer, *J. Endocrin.*, 1978, **78**, 179.
[49] D. Gazis and W.H. Sawyer, *Proc. Soc. Exp. Biol. Med.*, 1978, **157**, 584.
[50] D. Gazis, *Proc. Soc. Exp. Biol. Med.*, 1978, **158**, 663.

3 Pancreatic Hormones

Contributed by D. Brandenburg, D. Saunders, and B.-E. Rudolph

The proceedings of the 9th Congress of the International Diabetes Federation[1] contain several papers of interest in the context of this chapter: synthetic C-peptides and proinsulin,[2] the structure of insulin,[3] chemical modification and structure–function studies,[4] receptors and insulin action,[5,6] and its degradation,[7—10] as well as a section on somatostatin, gut hormones, and metabolism. Other reviews deal with insulin and glucagon,[11] the newer insulins,[12] the role of proinsulin C-peptide,[13] hormone–receptor interactions,[14—16] and insulin receptors and the site of its action.[17] A variety of papers on different aspects of hormone (particularly insulin and glucagon) binding and action can be found in further Proceedings.[18—20] Ectopic insulin production has been reviewed.[21] The roles of intuition, luck, and misfortune in diabetes research have been discussed.[22] The Proceedings of the Symposium 'Proinsulin and C-peptide' have been published.[23] The Proceedings of the international meeting on protein semi-synthesis[24] deserves special interest. Insulin clearly dominates the scene, and the nine papers present a fine review of the state of the art, and also contain a good deal of experimental detail.[25—33]

[1] 'Diabetes', Proceedings of the IX Congress of the International Diabetes Federation, New Delhi, 1976, ed. J.S. Bajaj, Excerpta Medica, Amsterdam–Oxford, 1977.
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Insulin.—*Synthesis, Semisynthesis, and Chemical Modification.* Gene technology directed towards the synthesis of insulin has made impressive progress. The mRNA from a rat insulinoma was cloned in *E. coli* and gave a fused protein with both insulin and penicillase immunoreactivity. Previous DNA-sequence analysis had suggested that this would have had the following amino-acid sequence: penicillase (24—182)-$(Gly)_6$-proinsulin(rat I)(4—86).[34] The approach of Crea *et al.*[35] consists of chemical synthesis of the separate

Table 1. *Analogues and derivatives of insulin*[a]

Compound number	*Structure*[b]	*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
1*a*	*Insulin*	bf			F	36
b		ca		81	C,*f*	37
c		c		250	F	38
d		ch		23	F	38
e		g		51	F	38
f		gp			F	36
				1.2	C	39
g		hg		3.3	C	39
					X	3
				3.7	E	39
				6.9	F	38
					F	36
					g	40
h		h		99	C,*f*	27
					f	29
i		m			F	36
j		rI		51	C,*g*	41
				89	F,*h*	41
k		rII		71	C,*g*	41
				70	F,*h*	41
l		s		19,27	F	38
m		ss		54	F	38

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Table 1. *(Cont.)*

Compound number	*Structure*[b]		*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
A-chain modifications							
2	Ac	A1	b		40	C	39
					33	F	38
				+	82	G	42,66
3	Aceto-Ac	A1	b		27	C	39
					68	G	42
					24	F	36,38
4	Boc	A1			10	F	5
5	Carbamoyl	A1			34	C	39
				+	91	G	42,66
6	Guanyl	A1			88	C	43
7	3-Oxobutyl	A1	b	100		A	44
8	Phthaloyl	A1	b			*i*	45
9	Suc	A1		+	17	C	39
10	Thiaz	A1			18	C	39
					21	F	36,38
11	[Ala^{A_1}]		b	20	12	B,C	25
				34	9.4	A,D	46
					12.5	H	46
					12	F	46
			p		53	F	47
12	[D-Ala^{A_1}]		b	100		B	25
				47		A	46
					66	C	39
			b		63	C	25
					95	D	46
					100	H	46
					100	F	46
					79	F	47
13	[β-Ala^{A_1}]			45	48	B,C	25
			p		90	F	47
14	[$Acap^{A_1}$]		b	28	35	B,C	25
15*	[D-Glu^{A_1}]		b	72[j]	71[j]	B,C	25
16*	[D-Leu^{A_1}]		b	68[j]	109[j]	B,C	25
17	[D-Nle^{A_1}]		p	50		B	26
18	[D-Lys^{A_1}]		b	43[j]	68[j]	B,C	25
			p	100	84	B,C	26
19*	[D-Phe^{A_1}]		b	52	54	B,C	25
20	des-Gly^{A_1}				2	C	43
21*	[Ala^{A_4}]		b	60			25
22*	[$Phe^{A_{19}}$]				23	C,*h*	48

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Table 1. *(Cont.)*

Compound number	*Structure*[b]		*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
23	[Asp^{A21}](A21-des-amido)		b	66		A,*g*	49
						F	36
B-chain modifications							
24	Aceto-Ac	B1			86	C	39
				+	[81]	G	42,66
						F	36
25	Carbamoyl	B1			116	C	39
				+	[85]	G	42,66
26*	Cys(O_3H)	B1			70	C	4
27*	I_2Hpp	B1			29	C	4
28	Phtaloyl	B1	b			*i*	45
29*	[desamino-Phe^{B1}]		b		82	C	4
30*	[desamino-Tyr^{B1}]		b		73	C	4
31*	[I_2-Hpp^{B1}]		b		69	C	4
32*	[I_2-Tyr^{B1}]				109	C	39
				+		G	66
33*	[Lys^{B1}]		b		83	C	37
34*	[Met^{B1}]		b		90[j]	C	4
35*	[D-Phe^{B1}]		b		61	C	4
36*	[Tyr^{B1}]		b		102	C	4
37*	[Tyr(Bu^t)^{B1}]		b		92	C	4
38	des-Phe^{B1}		b	97		B	25
						i	50
			p		98	C	37
					92	F	5,36

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Table 1. *(Cont.)*

Compound number	*Structure*[b]	*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
		p			*i*	50
39*	[Ala^{B_1},Ala^{B_2}]	b		88	C	37
40*	des-Phe^{B_1}[Ala^{B_2}]	b		76	C	37
41*	des-Phe^{B_1}[Lys^{B_2}]	b		80	C	37
42	des-(B1–2)	b	95		B	25
				88	C	37
43*	[Lys^{B_3}]	b	70		B	25
44*	des-(B1—2)[Lys^{B_3}]	b		74	C	37
45	des-(B1—3)[Glp^{B_4}]	b	70		B,*i*	25
				74	C	37
46*	des-(B1—3)[Lys^{B_4}]	b		44	C	37
47a	des-(B1—4)	b	65		B,*i*	25
b*				42	C	37
		h	54		A,*i*	51
48*	[Arg^{B_5}]	b	10		A	52
49*	[Gly^{B_5}]	b	30		A	52
50a	des-(B1—5)	b	15		B	25
		b		9	C	37
b*		h		5	D,*i*	51
51*	[Gly^{B_6}]	b	27		A	52
52*	des-(B1—6)	b		9	C	37
53*	[$Ala^{B_{16}}$]	p	50		A	53
54	des-(B23—30)			1	F	36,54
					i	56
		p		0.25	F	55
55a	des-(B24—30)	b	10		A,*i*	52
55b		p	3—10		A,*i*	57
56*	des-B24—30)[$Ala^{B_{22}}$]	b	2.5		A,B	58
57*	des-(B24—30)[$Lys^{B_{22}}$]	b	2.5		A,B	58
58*	des-(B25—30)[$Ala^{B_{22}}$]	b	9		A,B	58
59*	des-(B25—30)[$Lys^{B_{22}}$]	b	10		A,B	58
60	des-(B26—30)				F	36
		b	13.7		F	5
61	des-(B26—30)[$Ala^{B_{22}}$]	b	10		A,B	28,58
62	des-(B26—30)[$Lys^{B_{22}}$]	b	13		A,B	28,58
63*	des-(B27—30)[$Ala^{B_{22}}$]	b	20		A,B	58
64*	des-(B27—30)[$Lys^{B_{22}}$]	b	21		A,B	58
65*	des-(B27—30)[des-carboxy-$Tyr^{B_{26}}$]		50		B	29
66*	des(B28—30)[$Ala^{B_{22}}$,$Ala^{B_{27}}$]	b	25		A,B	58
67*	des-(B28—30)[$Lys^{B_{22}}$,$Ala^{B_{27}}$]	b	24		A,B	58
68	Ac B29			87	C	39
			+	[74]	G	42,66
69*	Biotinyl B29			100	C	59
70*	Ftc B29			~30	C	4
71*	Msc B29			73	C	4
72*	Rtc B29			75	D	60
				89	F	61
73	Suc B29			80	C	39
			+		G	66
74*	Trp B29			50	C	4

Table 1. *(Cont.)*

Compound number	*Structure*[b]		*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
75*	[Arg^{B29}]des-Ala^{B30}			>80		B	29
76	des-Ala^{B30}		p		100	C	62
			b			F	36
Modifications in A and B chains							
77	Ac_2	A1,B29			29	C	39
					24	E	39
					25	F	5,36
				+	[111]	G	42,66
						G	63
78	Boc_2des-(B23—30)	A1,B1				*i*	56
79	Boc_3	A1,B1,B29				*i*	56
80	$(Carbamoyl)_3$	A1,B1,B29			10	C	39
			b			*i*	45
81	$(Citraconyl)_2$Mib	A1,B1,B29	b				60
82	$(Maleyl)_3$	A1,B1,B29	b			F,*i*	36,45
83*a*	$(Phthaloyl)_3$	A1,B1,B29	b			*i*	45
83*b*	$(Phthaloyl)_3$	^{125}I	b			*i*	45
84	$(Succinyl)_3$	A1,B1,B29	b			*i*	45
85	des-Gly^{A1},des-Phe^{B1}				0.4	F	5
86	[Ala^{A4},Glp^{B4}]des-(B1—3)						25
87*	[Cys^{A7}—^{A11},Cys^{A6}—^{B7}]		h	37	14	D,B,*i*	64
				29	28	A,E	64
88*	[Cys^{A6}—^{A7},Cys^{A11}—^{B7}]			21	17	D,B,*i*	64
					26	E	64
89*a*	des-Asn^{A21},des-Ala^{B30}				1	F	54,65
89*b*	des-Asn^{A21},des-Ala^{B30}[^{125}I]				10	F	65
90	des-(B23—30)$(OMe)_6$					*i*	56
91*	des-Ala^{B30}$(OMe)_5$						27
92	$(NO_2)_4$Tyr					F	36
93	$(OMe)_6$						56
Crosslinked, monomers							
94	Oxalyl	A1–B29			5.1	C	39
				+	[9]	G	42,66,67
95	Succinyl	A1–B29			8.1	C	39
96*	Chd	A1–B29			0.5	C	67
97	Adipoyl	A1–B29			4.2	C	39
						G	63
98*	A_2Pim	A1–B29	b	45	4.4	B,C	67
99	$CO(Met)_2$	A1–B29				F	36
100	Suberoyl	A1–B29			5.4	C	39
					3.6	E	39
				+	[8]	G	66,42
					[42]	G	67
101*	Suberoyl,des-B1	A1–B29			6.3	C	39
102	A_2Sub	A1–B1			2.3	C	39
103		A1–B29			6.1	C	39
						G	3

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Table 1. *(Cont.)*

Compound number	*Structure*[b]		*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
104*	$(Cys)_2$	A1–B29		32	2—3	B,C	67
					3.1	C	39
105	Boc_2-Cys				2.2	C	39
106	Dodecane-dioyl	A1–B29			6.8	C	39
				+	4.6	E	39
					[23]	G	66,42
107*	$Ad(Lys)_2$	A1–B29			8—10	C	67
108	$(Z\text{-}Lys)_2(Ad)$	A1–B1			0.4	C	39
109*		A1–B29			3—4	C	67
		A1–B29			3.7	C	39
Crosslinked, dimers							
110*	A_2Sub	A1–A1′		0.5	15	B,C	67
111*	A_2Sub	B1–B1′		25	15	B,C	67
112*	Suberoyl	B1–B29′			38	C,E	68
				25		B	68
				[40]		G	68
					20[k]	F	68
					103[l]	F	68
113*	Sebacoyl	B29–B29′			5	C	67
114*	Suberoyl	B29–B29′			2	C,E	68
					5	B	68
				5		G	68
				10[k]		F	68
				76[e]		F	68
Heteroconjugates and complexes							
115	Rtc-lactalbumin B29				8.3	F	60
						F	61
					1.2	D	60
116	Polyvinylpyrrolidone B29				0.5—7	C,*i*	69
117	Co-poly[*N*-(2-hydroxypropyl) methacrylamide–*N*-metha-cryloyldiglycyl]			10		B,*i*	70

[a]New compounds are marked by an asterisk. [b]Abbreviations: Acap = ε-aminocaproyl, Ad = adipoyl, $Cys(O_3^-)$ = cysteic acid, I_2Hpp = 3,5-di-iodo-, 4-hydroxy-phenylacetyl, Rtc = tetramethylrhodamine thiocarbamoyl, Suc = succinyl-, Ftc = fluorescein-, Mib = *m*-maleimidobenzoyl, Chd = cyclohexene-*cis*-1,2-dioyl. [c]bovine = b, bonito = bf, camel = ca, chicken = c, chinchilla = ch, guinea-pig = gp, goosefish = g, hagfish = hg, human = h, mouse = m, pork = p, rat = r, rI rII, rI + II, shark = s, sea scorpion = ss, [d]% potency based on insulin = 100 (porcine, bovine), % degradation in brackets, also based on insulin = 100. [e]The following tests are indicated: A = mouse convulsion assay, B = *in vivo* blood glucose assay, C = lipogenesis in isolated fat cells, D = *in vitro* glucose oxidation, E = antilipolysis in isolated fat cells, F = receptor binding, G = enzymatic degradation, H = glucose transport, *f* = semisynthesis, *h* = c.d.-studies, *i* = immunological studies, *j* = corrected, *k* = hepatocytes, *l* = adipocytes, *x* = *X*-ray studies, [f]General: % activity ( ) *in vivo*, % degradation [ ].

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Table 1 (*Cont.*)

Compound number	*Structure*[b]	*Species*[c]	*Biological activity* in vivo	in vitro	*Test*[e]	*Ref.*
118	Avidin–biotinyl–insulin–complex B29			5	C,D	59
119	Poly-(*N*-thiolated)					71
Photoaffinity labels						
120	4-Azidobenzoyl ^{125}I				F	72
121	Azidoaryl	B1			F	73
122		B29			F	73

genes for A- and B- chain of human insulin, fusion to the β-galactosidase gene, and eventual production in separate clones of *E. coli*. It will be necessary to develop efficient procedures to obtain the pure hormone from a single precursor chain or the separate A- and B-chains. As A1-B29-crosslinked chains give high yields of insulin, the recent finding of Carpenter,[31] that A- and B-chain *S*-sulphonates can be linked with carbonyl(bismethionine *p*-nitrophenyl ester) in a yield of 50—60 %, is promising.

New analogues and derivatives which have been described in 1978 are listed in Table 1. Compounds of already known structure are included if new data and/or further details have been published. Expression of potency in percent (insulin = 100 %) appeared to be more appropriate than the ranges given before. The preparation and extensive characterization of rat insulins I and II,[41] as well as carp insulin,[74] have been reported. Several analogues (11,12,22,47—51,53) have been obtained through random combination of a synthetic chain analogue and its natural partner-chain. The first two isomers with unnatural disulphide bridges (87,88) have been synthesized, using the peptide A(1—13) with either a pre-formed (7—11) or (6—7) cystine bond as intermediate.[64]

Chemical substitution at primary amino-groups and enzymatic hydrolysis in the *C*-terminal B-chain region remain the principal pathways for modifying the native molecule.

Acylation of insulin with a number of reagents has been studied in detail and conditions for optimal mono-substitution are presented.[30] In aqueous solution, alkylation with methyl ethyl ketone gave improved yields of (7) in the presence of zinc ions.

Tetramethylguanidine and 1,2,4-triazole are effective additives in acylations with Boc-N_3.[76] Replacement of A1-glycine followed previously described

[71] M. Mahbouba and H.J. Smith, Protein Crosslinking, ed. M. Friedman *Adv. Exp. Med. Biol.*, 1977, **86A**, 247.
[72] C.C. Yip, C.W.R. Yeung, and M.L. Moule, *J. Biol. Chem.*, 1978, **253**, 1743.
[73] M.H. Wisher, A. Khalili-Shirazi, M. Tatnell, R.H. Jones, P. Thamm. and D. Brandenburg. *Diabetologia*, 1978, **15**, 213.
[74] T.A. Raroport, K.D. Kohnert, S. Knospe, A. Lukowsky, S. Prehn, H. Schäfer, S. Schmidt, and M. Ziegler, *Acta Biol. Med. Ger.*, 1978, **37**, 1153.
[75] G. Losse, *Z. Chem.*, 1978, **18**, 330.
[76] D. Keglević, S. Vuksan, B. Mulac, B. Ladesić, S. Iskrić, and S. Kveder, *Croatica Chemica Acta*, 1978, **51**, 167.

procedures with Z-Met[26] or Msc-groups[25] to block B-chain amino-groups. A third route, employing Boc-groups, has now been published in detail[43] and gave des-Gly-insulin (20) as well as (6). Repetitive Edman degradation and coupling of a synthetic tetrapeptide was used for the preparation of the Ala^{A4}-analogue (21).[25] A21-Desamido-insulin (23) was obtained through mild acid treatment of insulin and was thoroughly characterized.[49]

A1,B29-Msc_2-insulin[77] proved to be a particularly valuable intermediate for acylations at B1-phenylalanine,[4] sequential Edman degradation[25] up to the A7-B7-cystine bond,[37] replacement of phenylalanine by one of several amino-acids,[4] and for further semisynthetic alterations in positions 2—4[25, 37] (compounds 26—47,50,52).

A1,B1-Boc_2-des-nonapeptide (B22—30)$(OMe)_5$-insulin served as intermediate for preparing two series of truncated analogues in which Arg^{B22} was replaced by Lys or Ala,[58] Orn, Cit, or Gly.[28] Several des-hexapeptide-insulins had been prepared previously by similar methods, and have now been reported.[78] From the data, it appears that this approach is useful for screening purposes, but needs refinement. An alternative semisynthesis, this time without carboxyl protection,[29] could be used for the preparation of the Arg^{B29}-analogue (75) and the tyramide^{B26}-analogue (65), but was less satisfactory in attempts to introduce D-Ala^{B23} and Phe^{B26}. Coupling of peptides to Boc_2-des-pentapeptide (26—30) insulin also indicated influences of sequence and side-chains; the yield of human insulin was as low as 1 %.[27]

Selective cleavage of Ala^{B30} with carboxypeptidase A can be achieved in ammonium hydrogen carbonate instead of Tris buffer.[62] Substitutions at B29-lysine were either carried out directly,[59] or after protection of *N*-terminal groups,[60] usually by citraconyl residues.

Intramolecularly (94 — 109) and intermolecularly (110) cross-linked derivatives have been prepared from unprotected insulin by previously described methods.[67] The B1—B1′ -dimer was synthesized *via* A1,B29-Boc_2-insulin, the B29—B29′ -dimers from A1,B1-Tfa-insulin.

The fluorescent derivative 115 was obtained[60] by cross-linking the sulphydryl groups of partially reduced rhodamine-labelled lactalbumin to the B29 amino-group of citraconylated insulin using the intermediate 81.

Partial hydrolysis of polyvinylpyrrolidone K15 was followed by activation of the generated carboxy-groups with DCC–HONSu, and reaction with citraconylated insulin. An incorporation of 7 moles insulin per average mol. wt. of 10 000 (compound 116) was achieved. Tests for leakage using ^{125}I-insulin were negative.[69]

A different soluble carrier-bound insulin was prepared by treating insulin with a co-polymer prepared from poly *N*-(2-hydroxypropyl)methacrylamide and methacroylated diglycine-*p*-nitrophenyl ester.[70]

A 1 : 1 complex of B29-biotinyl-insulin and avidin was obtained by allowing the former to bind to $(biotinyl)_{3.85}$-avidin. Calculation shows that 13.4 % of the avidin molecules would have only one free binding site, and only 0.8 % two or more sites.

[77] R. Geiger, R. Obermeier, and G.I. Tesser, *Chem. Ber.*, 1975, **6**, 2758.
[78] S.C. Chu and T.F. Li, *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao*, 1978, **10**, 199.

In an attempt to produce long-acting insulins the hormone was partially acylated with *N*-acetyl homocysteine thiolactone, and oxidized to a polymeric mixture with 20—700 insulins per molecule.[71]

Photochemical labelling of insulin was approached in two ways: Yip treated ^{125}I-insulin of high specific activity with 4-azidobenzoic acid *N*-hydroxysuccinimide ester and used the unfractionated product for affinity labelling.[72] The two derivatives described by Wisher *et al.*[73] contained photolabels specifically in positions B1 and B29, respectively.

The synthesis of human proinsulin by consecutive coupling of ten peptide azides to the *C*-terminal undecapeptide is described in a preliminary report.[79] Synthetic proinsulin exhibited 62 % of insulin immunoreactivity. Several C-peptides (human, dog, rat, and duck) and derivatives have also been synthesized.[79] The preparation of human proinsulin and C-peptide from human insulin[80] and a careful analysis of human blood with respect to proinsulin and intermediates have been described.[81]

In contrast to rigid proteins like lysozyme, the insulin hexamer is considered to be of intermediate rigidity.[82] Considerable flexibility even in the solid state is obvious from the fact that 2-zinc to 4-zinc transformations, and the reverse, can take place in rhombohedral crystals.[83] These involve significant rearrangements, particularly at the *N*-terminus, for monomer I.

The structure analysis of zinc-free cubic insulin[84] shows that the crystals are made of symmetrical dimers and are therefore very interesting in relation to hagfish insulin.[40,85] Differences in the primary structure of rat insulins I and II [Glu^{A4}→Asp, Asp^{B3}→Lys; Ser^{B9}→Pro(I); Lys^{B29}→Met (II)] are reflected both in crystallization behaviour and in c.d.-spectra in the absence and presence of zinc.[41]

The effect of replacement of Tyr^{A19} by Phe (22) on conformation has been studied by c.d.-spectroscopy in the absence and presence of zinc ions.[48] Further c.d.-spectral data for zinc insulin have been reported.[86] Analysis of the temperature dependence of derivative u.v.-spectra may be a useful technique for studying insulin analogues.[87]

Structure, Receptor Binding, and Activity. Further studies on the effect of modifications at the *N*-terminus of the A-chain support earlier findings (*cf.* vol. 10), that acylation generally reduces the activity markedly (compounds 2—5, 9, 10). A positive charge can be related to high activity (6,7), but its presence as such is not sufficient (13,14). D-Amino-acids in position A1 have little effect upon potency. The similarity of *in vitro* and *in vivo* potencies suggests that degradation is unaffected (11—19). While some inter-laboratory differences can be seen, there is, in general, good agreement of data, which is noteworthy

[79] N. Yanaihara, C. Yanaihara, M. Sakagami, N. Sakura, T. Hashimoto, and T. Nishida, *Diabetes*, 1978, **27**, 149.
[80] H. Kuzuya, R.E. Chance, D.F. Steiner, and A.H. Rubenstein, *Diabetes*, 1978, **27**, 161.
[81] C. De Haen, S.A. Little, J.M. May, and R.H. Williams, *J. Clin. Invest.*, 1978, **62**, 727.
[82] R.J.P. Williams, *Biochem. Soc. Trans.*, 1978, **6**, 1123.
[83] G. Bentley, G. Dodson, and A. Lewitova, *J. Mol. Biol.*, 1978, **126**, 871.
[84] E.J. Dodson, G.G. Dodson, A. Lewitova, and M. Sabesan, *J. Mol. Biol.*, 1978, **125**, 387.
[85] J.D. Peterson, C.L. Coulter, D.F. Steiner, S.O. Emdin, and S. Falkmer, *Nature*, 1974, **251**, 239.
[86] E.R. Arquilla, P. Thiene, T. Brugman, W. Ruess, and R. Sugiyama, *Biochem. J.*, 1978, **175**, 289.
[87] J. Hudecek, V. Kalous, and P. Anzenbacher, *Studia Biophysica*, 1978, **72**, 181.

when comparing semisynthetic and synthetic analogues, *e.g.* Ala^{A1} -insulins (11,12).

The carboxy-group in position 4 is not essential.[25] Removal of the aromatic OH-group in position A19 has a marked effect on activity, but not on conformation.[48] The finding that desamidoinsulin exhibits reduced potency[49] is important since deamidation is a frequent side-reaction.

Substitutions of the B1-amino-group have variable effects on bioactivity (compounds 24—27); partial inactivation by addition of amino-acids has been confirmed.[4] Positions B1 and B2 are rather insensitive to replacements by L-amino-acids (33,34,36,37) or des-amino-acids (29,30). The bulky di-iodo-(desamino)-tyrosine residue can be accommodated better in position B1 than B0. The Ala-Ala-sequence (analogue 39) is unrelated to the high biopotency of chicken insulin.[37] A decrease of activity is observed when B4-pyroglutamic acid is replaced by Lys (46).

The reduction of activity upon removal of Gln^4 and His^{B5} is not reflected in c.d.-spectra. Loss of secondary structure becomes apparent after cleavage of Leu^{B6} (52).[37] Replacement of His as well as Leu causes a marked inactivation (analogues 48,49,51) while Tyr^{B16} appears to be of minor importance.[53] It has been concluded that the *N*-terminus of the B-chain has a greater importance for full hormonal activity than hitherto assumed.[4, 37]

All attempts to interpret the results of modifications at the *C*-terminus of the B-chain are hampered by the fact that only *in vivo* data have been reported and that structural data are missing. Des-heptapeptide-insulin was reported to be equipotent to des-octapeptide-insulin[28, 58] (54). The elevated figures for 55*a* and *b* may be due to contamination. Further elongation of the chain brings about an increase in activity which is, in the first step, similar for Arg, Lys, and Ala analogues (58,59). In subsequent steps, the rise in potency is less pronounced when Arg is replaced by Lys or Ala (61, 64,66,67). It appears that Arg^{B22} is necessary for full activity, but it cannot play a unique role with respect to the stabilization of a biologically active conformation or in exhibiting biological effects directly. The recent work of Chu,[78] and also observations on Asn^{A21}, partner of the much discussed salt bridge B22—A21, support this view.[4]

Similar to the situation at B1, the effects of substituting the ε-amino group at B29-lysine are difficult to interpret. Potencies range between 100 %, even with very large substituents [*e.g.* (72)], and 30 % (70). The most exciting analogues are the disulphide isomers (87) and (88). While physiochemical properties (*e.g.* t.l.c., c.c.d.) suggest alterations of the three-dimensional structure, the suprisingly high biopotencies as well as immuno-reactivities indicate that essential regions are similar to those of insulin.[64] Rat insulins I and II show some reduction in affinity and potency.

An important finding with respect to receptor binding studies is that direct binding experiments with labelled analogue (89*b*) gave a ten-fold higher value for the affinity than that derived from normal displacement studies.[65]

Crosslinked Insulins. Comparison of new (96, 98,101,104,107,109) and already known derivatives shows that the length and chemical nature of the

crosslink are relatively unimportant. The cystine bridge (104) appears to be stable *in vivo*. Diaminosuberoyl-insulin (102) is more active than other A1-B1-linked insulins. A1-B29-crosslinked insulins are more stable than diacetyl-insulin (77) and insulin with respect to thiol-disulphide interchange[63] and enzymatic degradation by chymotrypsin,[63, 67] a cytosolic enzyme fraction.,[63] glutathione-insulin-transhydrogenase[42, 63, 66] and also *in vivo*. Removal of Asn^{A21} and cleavage at B22–B23 causes inactivation as with insulin, indicating that the crosslink cannot compensate for these destabilizing effects.[67]

Linking two insulin molecules between B1-amino groups has very little effect, between A1-groups a more pronounced effect, upon conformation and zinc binding.[67] Biological properties of insulin dimers deviate from those of modified monomers. The hypoglycaemic potencies are mostly very low, in contrast to *in vitro* activities.[67, 68] Receptor binding was found to be dependent on the tissue,[68] and affinity for receptors was higher than the ability to evoke the biological response, as demonstrated with the symmetrical dimer (114) and the B1-B29′-dimer. Similar discrepancies have also been reported for hagfish insulin.[38] Covalent conjugates of insulin and soluble carriers as rhodamine–lactalbumin and polyvinylpyrrolidone, as well as the 1 : 1 complex of biotinyl–insulin and avidin (118) have low activity, but can produce maximal insulin effects *in vitro*.[60, 69, 59]

Insulin-like activity in rat muscle, but no potentiating effect of insulin, has been reported for the hexapeptide amide B(21—26) when used at 10^5-fold concentration.[88] Three small insulins, designed to the three-dimensional structure of insulin, were biologically inactive.[25]

Further evidence has been produced that antibodies to insulin receptors elicit insulin effects in fat cells[89] and in rat muscle.[90] The biological properties are retained in the divalent $F(ab')_2$ fragments, but not in the monovalent F(ab′) fragments, which behaved as competitive antagonists.[89] Addition of antibodies against $F(ab')_2$ to fat cells preincubated with F(ab′), and also of anti-insulin antibodies to cells preincubated with submaximal amounts of insulin, produced a restoration or elevation of insulin effects. The conclusion is that receptor aggregation or crosslinking is important in insulin action.[89] Both the lipogenic and antilipolytic effect of insulin appear to be mediated through the same receptor.[91] While modified insulins had previously only been evaluated with respect to glucose transport and utilization, the effect on adrenaline-induced antilipolysis has now been studied in fat cells.[39] As shown with compounds (19), (77), (100), and (106), relative potencies are identical to the lipogenic effects.

These findings support the view that changes in biopotency caused by structural modifications are a direct consequence of changes in the affinity for insulin receptors. It appears, therefore, that information available with modified insulins is limited to this very first step. But there are further

[88] T. Toyota, A. Kitahara, and Y. Goto, *Horm. Metab. Res.*, 1978, **10**, 17.
[89] C.R. Kahn, K.L. Baird, D.B. Jarrett, and J.S. Flier, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 4209.
[90] Y. Le Marchand-Brustel, P. Gorden, J.S. Flier, C.R. Kahn, and P. Freychet, *Diabetologia*, 1978, **14**, 311.
[91] S.H.L. Thomas, K.P. Willey, and M.H. Wisher, *Diabetologia*, 1978, **15**, 213.

outlooks. A careful analysis of combined insulin–analogue action indicates small potentiating (77) or antagonistic (106) effects, both evidence against independent receptor sites in fat cells.[39] Detailed studies with dimers promise further insights.

A large number of modified insulins show a perfect correlation between binding to receptors in cultured human lymphocytes and their potency to accelerate the dissociation of labelled insulin (negative co-operativity). This has also been demonstrated in hepatocytes (at 12 °C, but not 37 °C) and in adipocytes (at 24 °C).[54] DOP- and DAA-insulin (54, 89*a*) do not exhibit the effect. A discrete surface domain of insulin, part of the receptor binding region, is thought to be responsible.

Receptor Binding, Internalization, and Degradation. Using sensitive fluorescence and photo-bleaching recovery techniques,[92, 61] binding, lateral diffusion, and aggregation into patches of Rtc-lac-insulin (115) could be visualized on living cultured 3T3 fibroblasts. Within 30 minutes at 37 °C, much of the label was found in endocytotic vesicles which moved within the cell.[92] Quantitative EM autoradiography studies with ^{125}I-insulin show that initial binding is restricted to the cell membrane at 15 °C.[93—95] Increase of temperature and time leads to a progressive translocation to a limited area within the cell (*ca.* 15 % of cell radius). Internalization is much greater with hepatocytes[93, 95] than with lymphocytes.[94] Binding and degradation of insulin by hepatocytes[38] and adipocytes[96] has been studied in detail. With adipocytes, internalization is suggested by the finding that at 37 °C labelled insulin becomes rapidly non-dissociable.[97]

Although until recently it was not considered seriously, there is now increasing experimental evidence for internalization of insulin and other polypeptide hormones; this is 'now the hottest topic in cell biology' (*cf.* Kolata[16]). There is no clear answer yet to the question whether internalization is related to action or only to degradation of hormone and/or receptor.[14, 16, 17] As the receptor can be triggered by antireceptor antibodies it has been questioned that this phenomenon is important for all of insulins actions.[89] Reversibility of hormone binding has been one of the functional definitions of receptors. It seems to be clear now that binding is a much more complicated process.

Further work on the degradation of insulin *in vitro* and *in vivo* has been described[9, 10] and a number of modified insulins tested.[42, 66] Purified preparations of glutathione-insulin-transhydrogenase (GIT) degrade monosubstituted B1-, B29-, A1-and also A1,B29-disubstituted derivatives at rates similar to insulin. The lowest relative value (68 %) was found with (3).[42]

[92] J. Schlessinger, Y. Shechter, M.C. Willingham, and I. Pastan, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2659.
[93] P. Gorden, J.L. Carpentier, P. Freychet, A. Le Cam, and L. Orci, *Science*, 1978, **200**, 782.
[94] J.L. Carpentier, P. Gorden, M. Amherdt, E. Van Obberghen, C.R. Kahn, and L. Orci, *J. Clin. Invest.*, 1978, **61**, 1057.
[95] P. Gorden, J.L. Carpentier, P. Freychet, A. Le Cam, and L. Orci, *Diabetes*, 1978, **27**, 450.
[96] J. Gliemann and O. Sonne, *J. Biol. Chem.*, 1978, **253**, 7857.
[97] C.R. Kahn, and K. Baird, *J. Biol. Chem.*, 1978, **253**, 4900.

The effect of substitution on metabolic clearance rates (m.c.r.) increased in the order B1 (24,25,32; like insulin) — B29 (68,73) — A1 (small to intermediate effect; acetyl < carbamoyl < succinyl).[66] Proinsulin and A1-B29-cross-linked insulins (94,100,106) were degraded much more slowly *in vitro*[42] and *in vivo*.[66] However, the data indicate that the determinants of m.c.r. are not identical to those defining cleavage by GIT.

First reports on successful affinity labelling have appeared. Glutaraldehyde-crosslinking of receptor-bound insulin gave a specifically labelled membrane constituent with a Stokes radius of 72 Å.[98] Photoinduced linking of (120) to adipocyte membranes led to labelling of a number of proteins, of which one, corresponding to a molecular weight of 130 000, was specifically labelled.[72] Wisher *et al.*[73] observed a specific binding of both B1- and B29-photolabelled insulins (121,122) to rat-liver plasma membranes. These findings compare well with earlier results on the size of the insulin receptor and its subunits.

Immunological Studies. A macrophage-mediated selection of antigenic sites is involved in the genetic control of the immune response to insulin.[99] Heterologous insulins (b,p,o) were immunogenic in animals of eight different species.[50] The response was independent of the state of purity and also of the removal of B1-phenylalanine (38). In cattle, sheep, and goats, homologous insulins are also immunogenic.[50] Measurements of specific IgE have been used to determine the immunogencity of various insulins.[100] Haptenic antibodies have been obtained from guinea-pigs immunized with Pht_3-insulin (83*a*), which cross-reacted readily with the partial hapten-bearing derivatives (8) and (28), to a smaller extent with tri-maleyl- and tri-succinyl-insulin, but not with tri-carbamoyl-insulin. Zinc insulin was found to be less reactive than zinc-free in the radioimmunoassay, but more reactive in the immune haemolysis inhibition assay. This indicates a conformational perturbance of determinants operative in the latter assay upon removal of zinc.[86] Treatment of patients with immunological insulin resistance with sulphated insulin resulted in an impressive decrease of both hormone requirement (550 units per day to 25), maximal ^{125}I-insulin binding by serum (191 mU ml^{-1} to 9) and half-life of labelled insulin.[55]

Glucagon.—A review covering many aspects of glucagon has been published.[101] The *X*-ray structure and its relation to the pancreatic storage form has been discussed.[102] The shape of certain A-cell granular inclusions is very similar to the rhombic dodecahedral crystal form, suggesting that aggregation of glucagon may play a role similar to that of insulin. Thermodynamic studies suggest that hydrophobic groups similar to those involved in aggregation may constitute the binding region of glucagon. The structured (α-helical) *C*-terminal sequence is

[98] N. Sahyoun, R.A. Hock, and M.D. Hollenberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 1675.
[99] A.S. Rosenthal, L.J. Rosenwasser, B.L. Baskin, J. Schroer, J.W. Thomas, and J.T. Blake, *Adv. Exp. Med. Biol.*, 1978, **98**, 447.
[100] H.G. Velcovsky, G. Schmidt, E.M. Jonatha, and K. Federlin, *Verh. Dtsch. Ges. Inn. Med.*, 1977, **83**, 1429.
[101] R.H. Unger, 'Yearbook Metabolism', 1977, p. 101.
[102] T.L. Blundell, S. Dockerill, J.E. Pitts, S.P. Wood, and I.J. Tickle, ref. 18, p. 249.

therefore important for binding, although the *N*-terminus seems more important for biological activity.

A reappraisal[103] of the biological activity of the glucagon fragment (1—21) shows that a potency of 0.1 % is retained in a binding assay and an adenylate cyclase stimulation assay, despite the absence of the *C*-terminal region. The fragment was produced by carboxypeptidase-A digestion of native glucagon and the authors feel that their stringent controls exclude the possibility of residual glucagon being responsible for the very low activity.

In a high-resolution ^{1}H-n.m.r. study,[104] very little ordered structure was observed in solutions of glucagon. Resonances from Val[23] were shifted upfield, but the remaining spectrum contained little difference to that of the randomized peptide. The shift was ascribed to the shielding effect of Trp-25. This effect requires that a structure other than an α-helix would have to exist at the *C*-terminus of glucagon. Such a structure is proposed, and is similar to that proposed for an almost identical partial sequence in PTH.[105]

S-Methyl glucagon[106] has now been investigated by high-resolution ^{13}C-n.m.r.[107] The pK_a of the amino and iminazole groups of the *N*-terminal histidine residue were determined as 7.23 and 5.32 respectively, and are almost identical to the values for histidinamide. The pK_c of formation of the carbamino adduct between CO_2 and glucagon was 4.66, meaning that the mole fraction of hormone present as adduct in physiological conditions is 0.35. In pathological states this fraction may vary from 0.1 to 0.55, raising the interesting question of whether the adduct has an altered biological potency as is known for other N^{α}-glucagon derivatives (see last year's Report, p. 445).

A new synthesis of N^{α}-trinitrophenyl-glucagon (TNP-glucagon) has been reported,[108] and its biological activities investigated. This derivative had been previously shown[109] to possess a binding potency (7 %) far in excess of its biological activity (0.1 % in the adenylate cyclase assay). Now it has been demonstrated that the derivative is a glucagon agonist in the cyclase assay, with a behaviour compatible with a binding potency of 7 % and negligible cyclase stimulatory activity. This suggested that a similar agonist activity would be found in other glucagon assays. However in a glycogenolysis assay, although a reduction in glucagon-stimulated cAMP production was observed, a stimulation of glycogenolysis occurred. The derivative alone had an activity of 5 % in this assay, but no increase in cAMP was observed as with glucagon. This must mean that TNP-glucagon stimulates glycogenolysis by a cAMP-independent mechanism, and not by the well-known cAMP-dependant mechanism of native glucagon. It will be of great interest to know whether the same cAMP-independent mechanism is available to glucagon.

Very little other work has appeared. A study of the aggregation of glucagon at different pHs[110] used the quenching of non-covalently bound Rhodamine G

[103] D.E. Wright, V.J. Hruby, M. Rodbell, *J. Biol. Chem.*, 1978, **253**, 6338.
[104] C. Boesch, A. Bundi, M. Oppliger, K. Wüthrich, *European J. Biochem.*, 1978, **91**, 209.
[105] A. Bundi, R. Andreatta, K. Wüthrich, *European J. Biochem.*, 1978, **91**, 201.
[106] T.M. Rothgeb, B.N. Jones, D.F. Hayes, and R.S. Gurd, *Biochemistry*, 1977, **16**, 5813.
[107] T.M. Rothgeb, R.D. England, B.N. Jones, R.S. Gurd, *Biochemistry*, 1978, **17**, 4564.
[108] T.E. Cote and R.M. Epand, *Biochim. Biophys. Acta*, 1979, **582**, 295.
[109] R.M. Epand and G.E. Wheeler, *Biochim. Biophys. Acta*, 1975, **393**, 236.
[110] M.L. Johnson, S. Formisano, H. Edelhoch, *J. Biol. Chem.*, 1978, **253**, 1353.

to give association constants close to previously reported values, but with large mean errors. A series of papers from Japanese groups on the total synthesis of mammalian glucagon,[111, 112] turkey glucagon,[113] and duck glucagon[114, 115] has appeared. An interesting new bioassay[116] is based on the ability of glucagon to relax rabbit renal artery strips previously primed with low doses of adrenaline.

Somatostatin.—The Proceedings of a Somatostatin Symposium have been published,[117] and includes articles on all aspects of somatostatin research. Pancreatic aspects of somatostatin are covered in a good review by Unger.[118] A review of hypothalamic hormones, including somatostatin, has appeared,[119] and various physiologic aspects have been discussed.[120, 121] The possible dual role of somatostatin as hormone and as neurotransmitter was the theme of the 28th Banting Memorial Lecture.[122]

Bigger and better methods for the solid-phase synthesis of somatostatin have been reported. Gramme quantities were synthesized on a convential resin and subsequently purified by preparative h.p.l.c. in yields of 13—20 %.[123] The details of the new Fmoc-strategy have been reported for the synthesis of fully active dehydrosomatostatin[124] in a yield, after purification, of 53 %.

Of the year's additions to the large list of synthetic somatostatin analogues, perhaps those of greatest interest are the truncated species, as short as octapeptides, which retain high, possibly even specific, suppressive activities against insulin, glucagon, or growth hormone. Thus des-(1,2,4,5,12,13), [D-Trp^8]-somatostatin is highly active in suppressing insulin secretion, whereas changing [L-Cys^{13}] to [D-Cys^{13}] in this sequence changes the specificity to suppression of glucagon.[125] The reduced active core correlates well with a model proposed for the biologically active conformation.[126]

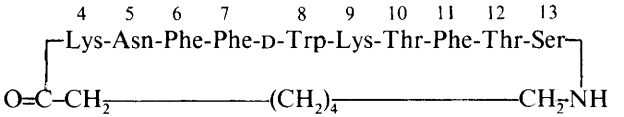
A new long-acting analogue, [des-Ala^{1}Gly^2]His^{4,5}D-Trp^8-somatostatin, appears to suppress the secretion of growth hormone and glucagon much better than that of insulin.[127] [D-Ala^5, D-Trp^8]somatostatin, seems to suppress specifically insulin and growth hormone secretions, but does not affect

[111] M. Fijino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, 1978, **26**, 539.
[112] T. Abiko, M. Kumikawa, S. Dazai, C. Uno, M. Ishizaki, H. Takahąshi, and H. Sekino, *Chem. Pharm. Bull.*, 1978, **26**, 3546.
[113] M. Wakimasu and M. Fujino, *Chem. Pharm. Bull*, 1978, **26**, 1522.
[114] H. Ogawa and H. Yajima, *Chem. Pharm. Bull.*, 1978, **26**, 1540.
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[122] R. Luft, S. Efendić, and T. Hökfelt, *Diabetologia*, 1978, **14**, 1.
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[127] D. Sarantakis, J. Teichman, R. Fenichel, E. Lien, *F.E.B.S. Letters*, 1978, **92**, 153.

glucagon.[128] The replacement of Trp[8] by L-6-F-Trp, L-5-Br-Trp results in analogues with activities of 118 %, 476 %, and 548 % in the isolated pituicyte assay.[129] The corresponding D-isomers were each about six times more active, which is comparable to the activity ratio between [L-Trp[8]] and [D-Trp[8]]somatostatins. The halogenated somatostatins were synthesized from the appropriate racemic amino-acids and the diasteromers separated by partition chromatography.[130] Replacement of Trp[8] by either 3-(1-naphthyl)-L-Ala, 3-(2-naphthyl)-L-Ala, or 1,2,3,4-tetrahydro-β-carboline gave derivatives with very low biological activities.[131] Several analogues seem promising for controlling functioning metastatic endocrine tumours[132] and in lowering the insulin requirements of certain diabetics.[133]

A note of caution comes from the Ciba-Geigy group[134] which claims that, contrary to previous reports,[135, 136] [D-Cys[14]] and [D-Trp[8], D-Cys[14]]somatostatins are *not* selective in suppressing glucagon. It is to be hoped that this may stimulate workers in this field to publish their work in proper detail so that it may be reasonably evaluated.

An interesting pair of publications seeks to promote a new model for the structure of somatostatin based on arguments from the activities of analogues[126] and the results of a ^{1}H-n.m.r. study.[137] The analogues are modifications of the fully active carbacyclic derivative:



This has been reported to have prolonged suppressive activity on pituitary and pancreatic hormone release, possibly because of its resistance to degradation.[138] The synthesis of the [L-Trp[8]]-diastereomer has been published in detail.[139] This core peptide was modified by the pair-wise substitution of Cys(S-Acm) residues with subsequent formulation of a disulphide bridge between the substituents to make bicyclic analogues. The [$Cys_2^{5—12}$] and [$Cys_2^{6—11}$] derivatives retained full activity while the [$Cys_2^{5—10}$] derivative had a very low activity. The authors interpret this as meaning positions 5 and 12 and positions

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6 and 11 are close together, allowing an unstrained formation of a disulphide bridge without loss of biological activity. This can be explained if the active configuration of somatostatin contains a Type II[1] β-turn at Trp[8], bringing Phe[6]-Phe[7] into a short anti-parallel β-sheet structure with Thr[10]-Phe[11]. The loss of activity on substituting Thr[10] by Pro is interpreted as being due to destabilization of the structure through loss of the hydrogen-bond $NH^{10} \ldots CO^{7}$. The enhanced activity of [D-Trp[8]] analogues is ascribed to the necessary introduction of a turn at bond 8—9. The high yields of monomeric products on cyclization are taken as evidence for the dominance of the proposed structural conformer in solution.

Support for the model comes from the identification of an upfield shift in the γ-CH_2 proton resonances of Lys-9 in analogues.[137] This probably arises from the proximity of the Trp-8 indole ring to the side-chain of Lys-9, which requires the constraint of a structure such as proposed. The degree of shift does not parallel the biological activity, but inactive derivatives have very low shifts. As somatostatin exhibits no shift, it seems that only a very minor population exists in the active configuration. This predicted from the requirements of the model which requires an unfavoured β-turn at L-Trp[8].

N^{α}-Tyrosyl-somatostatin has been radiolabelled with ^{125}I to a specific activity of 200—250 μCi μg^{-1}, and used in the radioimmunoassay of various tissues.[140] Like other similar derivatives, the tracer deteriorates quite rapidly. Iodinated [Tyr[1]]somatostatin was prepared at a specific activity of 1360 μCi μg^{-1} to characterize receptors on rat-pituitary tumour cells.[141] A tritiated derivative had a specific activity of 10 μCi μg^{-1} after the catalytic exchange of iodine for ^{3}H in [4-iodophenylalanine]^{6}-somatostatin.[142]

Insulin-related Growth Factors.—The excitement caused by the sequencing and theoretical model-building of relaxin was reviewed in last year's report. Insulin-related growth factors – insulin, insulin-like growth factor (IGF), nerve growth factor (NGF), and relaxin – have now been reviewed briefly.[143] This year IGF makes the running. The complete sequences of both IGF2[144] and IGF1[145] have been published. The sequence of IGF1 was accomplished with 5 μmoles of material isolated from three tons of dried plasma extract.

Four structural regions have been defined.[145] The A-region is strongly homologous to the insulin A-chain, and the B-region to the insulin B-chain. The C-region corresponds to the C-peptide of proinsulin, but consists of only 12 residues, many of which are serine or arginine. A D-region is attached as an 8-residue *C*-terminal extension of the A-region. Of the 19 invariant residues of the insulin sequence, 17 are totally conserved in the IGF1 sequence, and the other two are conservative alterations ($Gln^{A5}n \rightarrow Glu^{IGF}$; $Asn^{A21} \rightarrow Ala^{IGF}$). As the residues involved in the structure-determining hydrophobic core of insulin

[140] N. Yanaihara, H. Sato, N. Sakura, C. Yanaihara, *Endocrinol., Japan*, 1978, **25**, 95.
[141] A. Schonbrunn and A.H. Tashjian jun., *J. Biol. Chem.*, 1978, **253**, 6473.
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[144] E. Rinderknecht and R.E. Humbel, *J. Biol. Chem.*, 1978, **253**, 2769.
[145] E. Rinderknecht and R.E. Humbel, *F.E.B.S. Letters*, 1978, **89**, 283.

are thereby also maintained, the proposal is that a structural homology might exist.

The same arguments apply for IGF2[144] which is 62 % identical to IGF1. If one restricts the sequence comparison to the A- and B-regions, a figure of 76 % is obtained. The C-region contains only eight residues and the D-region only six residues. It should be noted that in the gene sequence of Ullrich *et al.*[146] there is a short redundant sequence after the proinsulin nucleotide sequence which may be the remnant of a similar D-region in proinsulin.

The theme of structural homology has been further developed.[148] A model has been built by fitting the sequence of IGF1 to the basic structure of insulin, and refining by computer. Sequence changes were accommodated without distortion, and lay mainly on the surface. The dimerization surface of insulin was shown to have an analogy in the IGF structure, implying that weak dimerization may be possible. The lack of cross-reactivity of IGF with anti-insulin antibodies was seen to be due to an absence of the necessary antigenic determinants. Similarly, the weak interaction of IGF with cell receptors for insulin is probably due to a partially preserved receptor binding region.

4 Gastrointestinal Hormones

Contributed by D. Gillessen and R.O. Studer

The proceedings[1] of a special symposium on gut hormones present a comprehensive view of present knowledge on gastrointestinal hormones. They include, besides articles on general techniques and on the individual hormones, some overviews on gut hormones,[2] hormone storage,[3] hormone isolation,[4] and chemical synthesis.[5] In addition some general reviews on digestive hormones have appeared.[6—8] The evolution of gastrointestinal hormones has been studied by different groups.[9—13]

Since some studies apply to or deal with several gastrointestinal hormones they will be reported here. A new technique for the identification of gastro-

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[148] T.L. Blundell, S. Bedekar, E. Rinderknecht, and R.E. Humbel, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 180.

[1] 'Gut Hormones', ed. S.R. Bloom, Churchill Livingstone, Edinburgh, 1978.
[2] S.R. Bloom and J.M. Polak, ref. 1, p. 3.
[3] M.G. Bryant, S.R. Bloom, P.E. Jones, and T.J. Peters, ref. 1, p. 82.
[4] V. Mutt, ref. 1, p. 21.
[5] E. Wünsch, ref. 1, p. 28.
[6] J.A. Chayvialle and R. Lambert, *Lyon Méd.*, 1977, **238**, 389.
[7] E. Zamrazilová and I. Skala, *Česk. Gastroenterol. Vyz.*, 1978, **32**, 306.
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[9] N.S. Track, *Int. Congr. Ser. Excerpta Med.*, 1977, **403** (Endocrinology), Vol 2, p. 87 (*Chem. Abs.*, 1978, **89**, 72 199).
[10] H. Gottfried, G. Mamikunian, S. Falkmer, S.O. Emdin, E. Landaw, and B. Dadourian, *Acta Paediatr. Scand., Suppl.*, 1977, **270** (Insulin Juv. Diabetes), 26 (*Chem. Abs.*, 1978, **89**, 192 682).
[11] J. Dlugosz, *Endokrynol. Pol.*, 1978, **29**, 99.
[12] G.J. Dockray, ref. 1, p. 64.
[13] L.I. Larsson and J.F. Rehfeld, ref. 1, p. 68.

intestinal hormones ending with a *C*-terminal amide group like gastrin, cholecystokinin, secretin, and vasoactive intestinal peptide in crude extracts or column eluates has been proposed. This procedure consists of enzymatic degradation of the hormones, dansylation of the resulting mixtures, and identification of the respective *C*-terminal dipeptide and amino-acid amides by thin layer chromatography.[14] The hepatotrophic effect of gastrointestinal hormones,[15] their effects on gall bladder function,[16] and on basic electric rhythm of the stomach and duodenum[17] as well as their co-ordination with the autonomic nerve system[18] were subjects of recent investigations.

Gastrins.—(Figure 1). Several reviews on gastrins have been published,[19—23] dealing with their role in health and disease,[19] their release,[20] their role in the regulation of the lower oesophageal sphincter,[21] or serving as general overviews.[22,23]

The synthesis of human [Leu[11]]minigastrin I,* having full gastrin activity, has been reported[24, 25] including the structure elucidation of a synthetic side-product.[25]

A great number of analogues of pentagastrin containing a free or protected α-amino-oxy acid at the *N*-terminus and leucyl, norleucyl, norvalyl, L- and D-2-aminodecanoyl residues instead of the methionyl residue,[26] or D-2-(amino-oxy)-3-phenylpropionic acid, L-(4-chlorophenyl)glycine, L- and D-phenylglycine or L- and D-cyclohexylglycine instead of the *C*-terminal phenylalanine[27] have been described. Their biological activities and structure–activity relationships with respect to stimulation of gastric flow when given i.v., intrajejunally, and intrarectally to rats have been reported.[28] The 4,5-bis(*p*-methoxyphenyl)-4-oxazolin-2-one derivative of pentagastrin showed a potent secretagogue action in chickens with an acute gastric fistula[29] and choline esters of peptides related to tetragastrin were reported to have smooth muscle

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[23] G.J. Dockray, ref. 1, p. 129.
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[28] I. Schön, L. Kisfaludy, J. Náfrádi, L. Varga, and V. Varró, *Z. physiol. Chem.*, 1978, **359**, 917.
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*Some initial confusion regarding the structures of human minigastrin I has now been resolved (R.A. Gregory, *Gastroenterology*, 1976, **71**, 545). There is an additional *N*-terminal tryptophan residue and the synthetic compound obtained is therefore des-Trp[1]-[Leu[12]]-human minigastrin I.

contracting activity.[30] *N*-(3-carboxypropionyl)-Sar-Tyr-Leu-Asp-Phe-NH_2[31] and Trp-Met-Asp-Phe-NH_2, where the *N*-terminal amino-group is blocked by either the t-butyloxycarbonyl-, the benzyloxycarbonyl-group or by a t-butyloxycarbonyl-*β*-alanine residue[32] have been reported to have gastrin activity. Gly-Lys-G-17 has been synthesized by conventional methods and its gastrin activity determined.[33]

A synthesis of tetragastrin in which the methyl group of methionine is enriched with [^{13}C] without loss of biological potency has been reported[34] as well as a [^{14}C]glycine-labelled *C*-terminal pentapeptide.[35]

2-nitrophenylsulphenyl-derivatives of pentagastrin and gastrin I have been found to be antagonistic to the action of gastrin.[36]

Further radioimmunoassays have been described[37,38] as well as the preparation and use of ^{125}I-labelled synthetic human [Leu^{15}]-G-17 for the determination of conventional human gastrins.[39] The clinical usefulness of radioimmunological determinations of gastrin in diseases has been reviewed.[40] Increased amounts of gastrin were measured in hypergastrinemia.[41] Using sensitive sequence-specific radioimmunoassays the molecular heterogeneity in pig pituitary[42] and human cerebrospinal fluid[43] was found very much alike and probably identical to antral gastrins. Further evidence was presented that the gastrin-like peptidic material extracted from human cerebral cortical grey matter is closely related to the *C*-terminal octapeptide of CCK in its complete sulphated biologically active form.[44] The heterogeneity of gastrins was reviewed[45] as well as the question of gastrin precursors.[46] The higher values found in sera of Zollinger–Ellison patients by bioassay than by immunoassays was attributed to non-gastrin substances with intrinsic or potentiating activity.[47] The simultaneous occurrence of both the *N*-terminal fragment of G-34 and G-17 in approximately equimolar amounts in the same cells of

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[33] F. Shimizu and N. Yanaihara, *Japan Kokai*, 78 84 966 (*Chem. Abs.*, 1979, **90**, 23 710).
[34] S. Yamada, M. Sakagami, N. Yanaihara, H. Nishikawa, and H. Watari, *Experientia*, 1977, **33**, 1566.
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[36] S.J.E. Bonfils, J.M. Dubrasquet, P. Fromageot, J.P. Girma, M. Lewin, and J.L. Morgat, *Belg. P.*, 858 507 (*Chem. Abs.*, 1978, **89**, 24 816).
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[47] S. Bonfils, M. Dubrasquet, J.P. Accary, J. Girodet, and M. Mignon, ref. 1, p. 158.

antral mucosa supports the idea that G-34 is cleaved intracellularly by a trypsin-like enzyme to produce G-17.[48, 49]

Evidence for the occurrence of G-17, *N*-terminal tridecapeptide as well as precursors in the trunk and branches of the vagal nerve[50] and their release by electrical stimulation[51] has been presented. The role of neural influences[52—55] and of amines[56—59] on gastrin secretion as well as a review on gastrin release[20] have appeared. The existence of a gastrin-releasing peptide in porcine nonantral gastric tissue[60] and an antral inhibitor of gastrin release[61] have been proposed.

Further contributions to the metabolism of gastrins were presented in a study of the fate of ^{123}I-gastrin in the rat by external detection with a gamma camera indicating the importance of the kidneys[62] and in a study on the hepatic inactivation of gastrins indicating increasing resistance to inactivation with increasing chain length.[63]

The role of endogeneous gastrin in the control of the lower oesophageal sphincter is still the subject of controversy.[21, 64] Effects of pentagastrin, G-17 and G-34 on the electrical and mechanical activities of canine antral smooth-muscle indicate the presence of receptors on the smooth-muscle cell.[65] Interactions of cholecystokinin and gastrin on the motor activity of isolated guinea-pig antrum and fundus[66] as well as a quantitative comparison of the effects of cholecystokinin, secretin, and pentagastrin on the gastrointestinal myoelectric activity in the conscious fasted dog[67] were presented. Pentagastrin, like caerulein and glucagon, was able to increase intraluminal release of enzymes in the rat small bowel.[68] Based on the increase of labelling and mitotic indexes as well as the thickness of the muscle layer after injection of pentagastrin into fasted rats it was suggested that gastrin is a trophic factor.[69]

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[69] A. Bosshard, D. Pansu, and R. Lambert, *Gastroenterol. Clin. Biol.*, 1978, **2**, 165.

The physiological action of gastrin as a trophic hormone for human gastric mucosa however seems to be limited.[70]

Based on the suppression of LH, TSH, and prolactin after injection of [Leu[15]]human gastrin into the third ventricle of ovariectomized rats, the possibility was discussed that gastrin may function as a modulator of neuronal activity controlling pituitary hormone release.[71]

Cholecystokinin-Pancreozymin (CCK).—(Figure 1). Several reviews on CCK have appeared.[72—76] They deal with the physiology,[72,73] the pathophysiology[73, 74] of CCK, its role as short-term satiety hormone[75] and the occurrence of CCK-like immunoreactivity in the brain.[76]

A new route for the preparation of *C*-terminal peptides of CCK has been described[77] in which Boc-Tyr-Met-Gly-OEt was first sulphated then saponified and coupled *via* the mixed anhydride procedure with the free *C*-terminal tetrapeptide. Side reactions which take place during the sulphation reaction at the tryptophan residue were avoided in this way and the *N*-protected heptapeptide was obtained in good yield. The protected *C*-terminal CCK-octapeptide (CCK-8) bearing a formyl group on its indole nitrogen was sulphated in high yield by the use of the *N*-methylmorpholine–SO_3 complex in dioxane and dimethylformamide as solvents.[78]

An *N*-t-butyloxycarbonyl-protected analogue of the *C*-terminal heptapeptide of CCK (CCK-7) with ε-hydroxynorleucine sulphate replacing tyrosine sulphate was synthesized in stepwise fashion and showed about 10 % of the biological potency of the parent compound with regard to amylase release from pancreatic acinar cells and about 40 % with regard to gall bladder contraction. Since these activities were about 100-fold higher than those of the corresponding serine analogue the conclusion was drawn that the distance of the sulphate ester group from the peptide backbone plays a major role for the biological activity of CCK.[79] The biological potency of *N*-(3-carboxypropionyl)-CCK-7 with regard to gall bladder contraction was of the order of magnitude of caerulein, while those of the corresponding *N*-[(*p*-hydroxyphenyl)acetyl]- and *N*-[3-(ethoxycarbonyl)propionyl] derivatives were only about 1 % as active. These analogues were prepared by treating the free sulphated heptapeptide with succinic anhydride or with the respective trichorophenyl ester.[77]

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G-34	Pyr-Leu-Gly-Pro-Gln-Gly-His-Pro-Ser-Leu-Val-Ala-Asp-Pro-Ser-Lys-
CCK-33	Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-
CCK-39	Tyr-Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-
G-14	Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
G-17	Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
G-34	-Lys-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
CCK-33	-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2
CCK-39	-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2
Caerulein	Pyr-Gln-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH_2

Figure 1 *Sequences of human gastrins,*[a,b] *porcine cholecystokinin-pancreocymins (CCK),*[c] *and caerulein*[a,c]
[a] Pyr = pyroglutamyl, [b] Tyr is present as sulphate ester in gastrin II-s, [c] Tyr is present as sulphate ester.

The intramolecular distance between tyrosine and tryptophan in the unsulphated, *N*-protected *C*-terminal heptapeptide of CCK was determined to be 10 Å by quantitative evaluation of the transfer of excitation energy from the phenol ring to the indole moiety.[80] The stability of small samples of CCK-8 lyophilized in vials in the presence of sodium chloride was good provided the relative humidity in the headspace did not exceed 30 %.[81]

The isolation of CCK and secretin has been improved by heating the intestine in salt solution instead of in acetic acid or water.[82] Two peptides with CCK-like immunoreactivity were isolated from sheep brain. Although the amino-acid sequence of both compounds corresponded to CCK-8 one compound migrated differently upon thin layer chromatography and electrophoresis and proved to be slightly less acidic than CCK-8 while the other was indistinguishable from CCK-8. The difference between the two forms has to be established.[83]

A sensitive radioimmunoassay for CCK crossreacting only slightly with gastrin was used to determine basal serum levels in dogs and man and their increase after stimulation with amino-acids or after eating.[84] Three types of sequence-specific antisera were raised and used for the development of radioimmunoassays.[85] The heterogeneity of intestinal CCK and the presence of CCK-8 was established by chromatography of gut extracts on Sephadex and monitoring of the effluents by radioimmunoassays.[86] The presence of at least six different components with CCK-like immunoreactivity was detected by a similar procedure using sequence-specific radioimmunoassays.[87, 88] These peptides ranged in size from a molecular form larger than CCK-39 to the *C*-terminal tetrapeptide (CCK-4), CCK-8 being the predominant form. Three components with CCK-like immunoreactivity corresponding to CCK-33, CCK-8, and CCK-4 were identified in brain extracts and it was calculated that the central nervous system contains more CCK than the intestine on a molar basis.[88] The heterogeneity of CCK in cerebrospinal fluid corresponded to that in brain.[43] It was demonstrated by bioassays and competitive binding assays that most of the peptidic material from human cerebral cortex showing CCK- or gastrin-like immunoreactivity corresponded to CCK-8.[44] Species differences in the *N*-terminal part of the sequence of CCK were found with the use of *N*- and *C*-terminal-specific antisera.[89] The evolution of CCK-like hormones was studied by immunohistochemical techniques using sequence-specific antibodies.[90] The A cell (glucagon-cell) of the endocrine pancreas was found to

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[90] L.I. Larsson and J.F. Rehfeld, ref. 1, p. 68.

contain CCK-like peptides by the same methodology[91] and electron microscopic immunohistochemical evidence was provided for the intestinal I cell as source of CCK.[92]

A comparison of different molecular forms of CCK showed that CCK-39 and CCK-33 were equipotent but 10—30 times less potent than CCK-8 with regard to stimulating calcium and cGMP outflux and release of amylase from dispersed acinar cells but that the efficacies were the same for all three compounds.[93] However, with regard to inducing contractions of antral circular muscle preparations, CCK-39, and CCK-8 possessed about equal potency and were more potent than CCK-33.[94] CCK-33 stimulated *in vitro* the release of trypsinogen from rabbit pancreas four times more strongly than that of chymotrypsinogen whereas CCK-8 affected the secretion of both proenzymes to almost the same degree.[95] The importance of external potassium concentration for CCK-induced pancreatic juice flow and amylase output from acinar cells was demonstrated and a model for the release mechanism was proposed.[96] The effect of CCK on adenylate cyclase[97] or guanylate cyclase,[98] the role of cGMP in the stimulus-secretion coupling in exocrine pancreas,[99] the interactions of CCK and gastrin,[66] CCK and secretin and CCK and glucagon[100] on motor activity of isolated antrum and fundus as well as the enhancement of electrically stimulated contractions of isolated gall bladder preparations by CCK-8 and pentagastrin in subthreshold concentrations[101] were studied.

The role of CCK and CCK-8 with regard to satiety was studied in rats[102—107] and rabbits[108] in many different experimental designs. Most authors concluded that CCK acts possibly as an endogenous satiety signal but one group[107] still questions this function. The effects of CCK on the biliary sphincter,[109] splanchnic circulation,[110] mesenteric blood flow and carotid artery pressure,[111]

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bile flow,[112] bile flow and biliary bile salt secretion[113] and enzyme secretion from the pancreas[114] were studied *in vivo*. It was shown that chronic administration of CCK increased the weight of the pancreas, its content of amylase and trypsin,[115] and its maximal output of bicarbonate and protein upon CCK-stimulation.[116] Daily infusions of CCK in combination with secretin prevented intestinal mucosal hypoplasia in dogs caused by total parenteral nutrition.[117] CCK and CCK-8 increased the intestinal lymph flow[118, 119] and produced an effect on the parotid gland similar to that on the pancreas.[120] The influence of the experimental design using either single dose or cumulative dose infusions of CCK on the response of the exocrine pancreas was studied.[121] Since intranasal administration of CCK-8 improved the condition of patients with chronic pancreatitis it was concluded that CCK is a trophic hormone of the exocrine cells of the pancreas.[122] On the other hand it was reported that CCK-induced pancreatic juice flow was independent of the secretion of enzymes.[123] Intraluminal administration of magnesium salt solutions,[124] perfusion of the intestinal loop with solutions of amino-acids other than threonine and cysteine[125] and infusion of bombesin[126] were found to release CCK in man and dogs. The effects of CCK or CCK-8 on myoelectric activity of the intestine,[67, 127—129] on gastric emptying[130] and on the diameter of the pylorus[131] were investigated.

Secretin.—(Figure 2). The pharmacology, physiology, and pathophysiology of secretin have been reviewed.[73, 132—134]

Substitution of the acidic residues at positions 9 and 15 of $S_{5—27}$ by

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[130] T. Yamagishi and H.T. Debas, *Amer. J. Physiol.*, 1978, **234**, E375.
[131] J.F. Munk, R.M. Gannaway, M. Hoare, and A.G. Johnson, ref. 127, p. 349 (*Chem. Abs.*, 1979, **90**, 955).
[132] W.H. Haecki, G.R. Greenberg, and S.R. Bloom, ref. 1, p. 182.
[133] W.Y. Chey, R.A. Rhodes, and H.H. Tai, ref. 1, p. 193.
[134] K.D. Buchanan and R.W. Henry, *Int. Congr. Ser. Excerpta Med.*, 1977, **413** (Diabetes), p. 83 (*Chem. Abs.*, 1978, **89**, 39 826).

glutamine and/or asparagine did not affect the significant stimulatory activity on pancreatic secretion of S_{5-27} in rats and guinea-pigs.[135] In contrast to S_{5-27}, however, no inhibitory effect of pentagastrin-stimulated acid secretion could be detected.[136] [Lys[15]]-secretin-(5—27), in which Asp[15] of secretin is replaced by Lys[15] of VIP, synthesized on an insoluble support, showed increased VIP-like activity on smooth muscle preparations under full retention of secretin-like activity on pancreatic juice secretion in the rat.[137] A number of secretin analogues (S_{1-13}), in which the *N*-terminal histidine is replaced by DNP-His and the *C*-terminus elongated by Asp, Asp-Ser, Asp-Ser-Ala, Asp-Ser-Ala-Arg, and Asp-Ser-Ala-Arg-Leu either in D- or L-form have been prepared by solid phase synthesis.[138] Iodination of the *N*-terminal histidine and subsequent catalytic exchange against tritium yielded labelled secretin. Both products had the same activity as secretin in stimulating pancreatic adenylate cyclase.[139]

A process for the manufacture of secretin and its subsequent purification by chromatography on LH-20[140] as well as a sequential synthesis of secretin by the repetitive excess mixed anhydride method, using reverse-phase h.p.l.c. for purification of intermediate peptides and protected end product have been published.[141] The preparation of a stable secretin salt has been described.[142] A comparison of the biological potency of a synthetic secretin with that of natural porcine secretin has appeared, assigning a specific activity of 5000 C.U. mg^{-1} to the synthetic preparation.[143]

The radioimmunological determination of secretin has been reviewed.[144, 145] In order to relate physiological effects and secretin release highly sensitive radioimmunoassays are essential.[146] Increased sensitivity has been claimed by chromatography of plasma on XAD-2 resin columns followed by extraction of secretin with acidic methanol[147] or by extraction of secretin from plasma by ethanol.[148] Based on this type of gradual improvement of assay methods there is increasing evidence that plasma secretin concentration is regulated by endogenous acid and that secretin under physiological conditions is secreted in minute short-lived spikes,[149—156] although under special conditions no change

[135] G.M. Makhlouf, M. Bodanszky, M.L. Fink, and M. Schebalin, *Gastroenterology*, 1978, **75**, 244.
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[145] W.H. Haecki, *Z. Gastroenterologie*, 1978, **16**, 294.
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[151] J. Fahrenkrug and O.B. Schaffalitzky de Muckadell, *European J. Clin. Invest.*, 1977, **7**, 201.

in secretin concentrations were found.[157, 158] Neural influences seem to be relatively unimportant for the release of secretin in man.[52] Radioimmunoassays have also been used to study the distribution of immunoreactive secretin in the gastrointestinal mucosa of several mammalian species.[159, 160]

Several commercially available secretins have been compared as to their effect on pancreatic stimulation.[161—163] The clinical value of secretin given by infusion or injection[164, 165] or used in the skin test[166] to discriminate between Zollinger–Ellison syndrome and duodenal ulcer was further evaluated. Secretin exhibited no effects on glucose resorption by the intestine,[167] on basal- and glucose-stimulated insulin secretion in man,[168, 169] on glucagon,[169, 170] on hemodynamics and function of the liver,[171] or on the splanchnic circulation.[110]

The kidneys and the liver account for the metabolic clearance rate for porcine secretin in the pig.[172] Based on the good plasma stability questions were raised against an earlier postulated secretinase. The mean half-life was determined to be 2.6 min, a value which corresponds well with the 3.8 min found in man.[173]

Indications that secretin interacts with gastrin in the physiological regulation of human gastroduodenal motility were presented.[174] Secretin suppressed the motilin- but not the CCK-induced duodenal myoelectric activity[129] but there is little evidence that it plays a physiological role in the modification of motor

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activity.[67] No evidence was found that secretin performs an appetite regulatory function by a neurohumoral feedback mechanism.[106]

Cell surface receptors for secretin as well as for VIP and glucagon have been reviewed.[175—177] The question whether secretin has a trophic, an atrophic, or no effect at all on the intestine is still open.[115—117, 178, 179] The action of secretin and VIP on the adenylate cyclase system of pancreatic acinar cells has been reviewed.[180]

Vasoactive Intestinal Peptide (VIP).—(Figure 2). Reviews on membrane receptors for VIP and secretin,[177] the expression of cell surface receptors for VIP and secretin in normal and transformed cells of the digestive tract[175] and on the action of VIP and secretin on the adenylate cyclase system of pancreatic acinar cells[180] have been published. In addition short summaries on VIP,[181] its systemic release and pharmacokinetics,[182] its pharmacokinetics and pharmacodynamics in man,[183] its nervous release[184] and its pathophysiology[185,186] have appeared.

Synthetic and natural porcine VIP produced identical effects on pancreatic and biliary secretion.[187] VIP inhibited pentagastrin-induced acid and pepsin secretion in dogs in a competitive fashion. However, the *C*-terminal fragments $VIP_{14—28}$, $VIP_{15—28}$, and $VIP_{18—28}$ at approximately eight times the molar concentration of the parent hormone had no detectable inhibitory effect under the same conditions.[136]

Results from different experiments in dogs and from patients in hepatic failure indicated that the liver influences the catabolism of VIP not only in the periphery but also within the central nervous system.[188]

A further highly specific radioimmunoassay for VIP with a sensitivity in the range of pmole l^{-1} was described.[189] The tissue distribution of VIP in the intestinal tract of the rat was studied by radioimmunoassay and a radioreceptor assay using rat-liver plasma membranes. VIP was detected throughout the digestive tract and the maximal quantity was observed in the jejuno-ileum. Material showing VIP-like immunoreactivity extracted from brain was also completely active in the receptor assay.[190] When VIP levels were quantified by

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[176] P. Robberecht, M. Deschodt-Lanckman, J.P. Dehaye, and J. Christophe, ref. 1, p. 97.
[177] J.D. Gardner, B.W. Long, E.R. Uhlemann, and S.R. Peikin, ref. 1, p. 92.
[178] L.R. Johnson and P.D. Guthrie, *Proc. Soc. Exp. Biol. Med.*, 1978, **158**, 521.
[179] A. Bosshard, D. Pansu, and R. Lambert, *Gastroenterol. Clin. Biol.*, 1978, **2**, 165.
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[181] S.I. Said, ref. 1, p. 465.
[182] I.M. Modlin, S.J. Mitchell, and S.R. Bloom, ref. 1, p. 470.
[183] S. Domschke, W. Domschke, S.R. Bloom, P. Mitznegg, S. Mitchell, G. Lux, U. Strunz, and L. Demling, ref. 1, p. 475.
[184] J. Fahrenkrug, O.B. Schaffalitzky de Muckadell, and J.J. Holst, ref. 1, p. 488.
[185] A.M. Ebeid, J. Escourrou, P. Murray, and J.E. Fischer, ref. 1, p. 479.
[186] T.M. O'Dorisio, ref. 1, p. 484.
[187] G.M. Makhlouf, W.M. Yau, A.M. Zfass, S.I. Said, and M. Bodanszky, *Scand. J. Gastroenterol.*, 1978, **13**, 759.
[188] A.M. Ebeid, J. Escourrou, P.B. Soeters, P. Murray, and J.E. Fischer, *Ann. Surg.*, 1978, **188**, 28.
[189] P.G. Burhol, I. Lygren, and H.L. Waldum, *Scand. J. Gastroenterol.*, 1978, **13**, 807.
[190] J. Besson, M. Laburthe, D. Bataille, C. Dupont, and G. Rosselin, *Acta Endocrinol.*, 1978, **87**, 799.

radioimmunoassay in three layers of hamster bowel wall it was found that VIP was mainly concentrated near or within the neural elements of the gut.[191] Nerves displaying VIP-like immunoreactivity were demonstrated in the oesophagus of rats, cats, pigs, and human foetus.[192] Four components with VIP-like immunoreactivity were identified in extracts from human colon mucosa after ion and gel chromatography by the radioimmunoassay technique. One component moved exactly like porcine VIP while two components were slightly less positively charged and the fourth had a smaller molecular size. Since, however, VIP-like immunoreactivity extracted from human colon muscle was almost homogeneous and identical with porcine VIP under similar conditions the question of different forms of VIP-like immunoreactivity occurring in nerves and endocrine cells was raised.[193] The possible role of VIP as a neurotransmitter or neurohormone was stressed by its localization in the vesicular fraction of hypothalamic tissue and its calcium-dependent release evoked by potassium.[194]

VIP stimulated the release of insulin and glucagon from the isolated perfused porcine pancreas in a dose-dependent manner. Depending on the glucose concentration of the perfusate either insulin or glucagon was secreted.[195] No such glucose-dependent separation of the effect on glucagon or insulin release was seen in studies using the perfused newborn rat pancreas.[196] VIP was also found to release somatostatin-like immunoreactivity from the isolated canine pancreas[197] and to cause a dose-related increase in plasma prolactin levels in the rat *in vivo*.[198] It was demonstrated that VIP binds to receptors present in certain human gut carcinoma cell lines in culture and that it is a potent stimulator of cAMP accumulation in this system[199, 200] as well as in slices of specific brain regions of the rat[201] and in guinea-pig trachea ring preparations.[202] VIP also stimulated adenylate cyclase activity in homogenates of human gastric and duodenal mucosa[203, 204] and in rat adipose tissue preparations.[205] Two classes of binding sites for VIP were located in brain

[191] T.S. Gaginella, H.S. Mekhjian, and T.M. O'Dorisio, *Gastroenterology*, 1978, **74**, 718.
[192] R. Uddman, J. Alumets, L. Edvinsson, R. Hakanson, and F. Sundler, *Gastroenterology*, 1978, **75**, 5.
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[197] E. Ipp, R.E. Dobbs, and R.H. Unger, *F.E.B.S. Letters*, 1978, **90**, 76.
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[199] M. Laburthe, M. Rousset, C. Boissard, G. Chevalier, A. Zweibaum, and G. Rosselin, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 2772.
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membranes from the guinea-pig.[206] *In vitro* studies showed that VIP exerts an inhibitory effect on spontaneous, acetylcholine- or pentagastrin-induced mechanical and electrical activity of canine antral smooth muscle[207] and on basal and motilin-induced contractions of rabbit pyloric antral but not of duodenal muscle preparations[208] and that it reduces sodium movement from lumen to interstitium and transepithelial potential difference in the salivary duct of the rabbit.[209] The inhibitory effects of VIP on lower oesophageal sphincter pressure were studied in the opossum[210] and in healthy volunteers.[211] Other studies dealt with the effects of VIP on excitation of central neurons,[212] on gall bladder function,[213] on intestinal absorption and blood flow,[214] on intestinal water and electrolyte transport and transmucosal potential difference,[215] and on small bowel propulsion[216] as well as on VIP release by electrical stimulation of both vagi[217] or by ischaemic bowel.[218] The role of VIP as a possible factor in the causation of the watery diarrhoea syndrome was extensively discussed.[219—225]

	1	5	10
Secretin	His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg		
VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-		
Glucagon	His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-		
GIP	Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-		

	15	20	25
Secretin	-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2		
VIP	-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2		
Glucagon	-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr		
GIP	-Asp-Lys-Ile-Arg-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln		

	30	35	40
GIP	-Gln-Lys-Gly-Lys-Lys-Ser-Asp-Trp-Lys-His-Asn-Ile-Thr-Gln		

Figure 2 *The secretin family (porcine sequences)*

[206] P. Robberecht, P. de Neef, M. Lammens, M. Deschodt-Lanckman, and J.P. Christophe, *European J. Biochem.*, 1978, **90**, 147.
[207] K.G. Morgan, P.F. Schmalz, and J.H. Szurszewski, *J. Physiol.*, 1978, **282**, 437.
[208] U. Strunz, P. Mitznegg, S. Domschke, W. Domschke, E. Wünsch, and L. Demling, in ref. 127, p. 125 (*Chem. Abs.*, 1978, **89**, 209 409).
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[214] D. Mailman, *J. Physiol.*, 1978, **279**, 121.
[215] G.J. Krejs, R.M. Barkley, N.W. Read, and J.S. Fordtran, *J. Clin. Invest.*, 1978, **61**, 1337.
[216] S. Gustavsson, H. Johansson, G. Lundqvist, and F. Nilsson, *Scand. J. Gastroenterol.*, 1977, **12**, 993.
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[218] I.M. Modlin, S.R. Bloom, and S.J. Mitchell, *Experientia*, 1978, **34**, 535.
[219] I.M. Modlin, S.R. Bloom, and S.J. Mitchell, *Gastroenterology*, 1978, **75**, 1051.
[220] J.D. Gardner and D.M. McCarthy, ref. 1, p. 570.
[221] A.M. Ebeid and J.E. Fischer, ref. 1, p. 574.
[222] J. Fahrenkrug and O.B. Schaffalitzky de Muckadell, ref. 1, p. 576.
[223] S.I. Said, ref. 1, p. 578.
[224] T.M. O'Dorisio, ref. 1, p. 581.
[225] S.R. Bloom, ref. 1, p. 583.

Gastric Inhibitory Peptide (GIP).—(Figure 2). The properties and actions of GIP[226] as well as its physiology,[227] its physiology in man,[228] its pathophysiology,[229] and its effects on the isolated pancreas[230] have been reviewed.

A new synthesis of GIP has been reported[231] in which this tritetracontapeptide was constructed exclusively by azide fragment condensation in solution with the exception of the tryptophan residue in position 25 which was introduced as active ester. The tosyl group was employed for protection of the ε-amino groups of the lysine residues and the benzyloxycarbonyl group for protection of the α-amino-group of the *N*-terminal fragment and those fragments which were coupled before the methionine residue in position 14 was incorporated. The t-butyloxycarbonyl group served the same purpose in the remaining two fragments. The acid side-functions of the various fragments were temporarily protected by t-butyl groups and the guanido group was protonated. Final deprotection was achieved by treatment with sodium in liquid ammonia. The final product, after extensive purification by gel and ion exchange chromatography, showed about 50 % of the immunoreactivity of the natural hormone, and a partially purified fraction possessed about 30 % of the insulinotropic activity.

A radioimmunoassay for the measurement of GIP in unextracted plasma showing negligible crossreactivity with other gastrointestinal and pancreatic hormones has been developed[232] and the K-cell has been identified as GIP-cell by electroimmunocytochemical evidence.[233] GIP, like VIP, reduced the sodium transport from the lumen to the interstitium but in contrast to VIP had no effect on the transepithelial potential differences in the salivary duct of the rabbit.[209] GIP was found to release insulin and glucagon from the perifused newborn rat pancreas and to be more effective on a molar basis than VIP at high glucose concentrations.[196] The effects of GIP and its interactions with arginine and glucose were studied with regard to insulin and glucagon release from the isolated pancreas.[234] Physiological amounts of GIP stimulated the secretion of insulin and glucagon in monolayers of neonatal pancreatic cells and from immunohistochemical evidence a direct action of GIP on glucagon release from the A cell, but an indirect action on insulin release from the B cell, was postulated.[235] Intraduodenal infusions of amino-acid mixtures which had very little effect on the release of CCK were found to stimulate the secretion of

[226] J.C. Brown, J.R. Dryburgh, J.L. Frost, S.C. Otte, and R.A. Pederson, ref. 1, p. 277.
[227] J.C. Brown and R.A. Pederson, *Int. Congr. Ser. Excerpta Med.* 1977, **413** (Diabetes), 56 (*Chem. Abs.*, 1978, **89**, 53 583).
[228] S. Cataland, ref. 1, p. 288.
[229] R. Ebert and W. Creutzfeldt, ref. 1, p. 294.
[230] R.A. Pederson, J.R. Dryburgh, J.C. Brown, and A.H. Dupree, ref. 1, p. 283.
[231] N. Yanaihara, T. Mochizuki, C. Yanaihara, N. Sakura, T. Hashimoto, M. Sakagami, T. Kaneko, A. Kanetoh, and J.C. Brown, ref. 1, p. 271.
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GIP.[236] The effects and interactions of insulin and glucose on plasma GIP levels were investigated in volunteers[237, 238] and dogs.[239] The release of GIP by intraduodenal glucose infusion and its modulation by other polypeptides[240] or by the sympathetic neuroendocrine system[241] as well as its inhibition by atropine[242] were studied. Atropine was also shown to reduce the meal-stimulated GIP release in dogs.[243] GIP was found to induce a dose-dependent increase of mesenteric blood flow in the rat by reducing vascular resistance,[244] to reduce the pressure of the lower oesophageal sphincter in cats,[245] and to suppress motilin- but not CCK-8-stimulated spike potentials in the antrum and duodenum of dogs.[129] The response of GIP to various stimuli obtained in healthy volunteers was compared with that determined in diabetics,[246—248] obese subjects,[248, 249] and patients with duodenal ulcers.[250, 251]

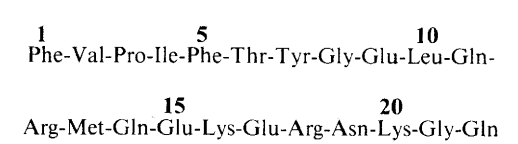


Figure 3 *The sequence of porcine motilin*

Motilin.—(Figure 3). The isolation,[252] the cellular origin,[253] the physiology,[254, 255] the pharmacology[256] and pharmacokinetics[257] of motilin as well as

[236] F.B. Thomas, D. Sinar, E.L. Mazzaferri, S. Cataland, H.S. Mekhjian, J.H. Caldwell, and J.J. Fromkes, *Gastroenterology*, 1978, **74**, 1261.
[237] D.K. Andersen, D. Elahi, J.C. Brown, J.D. Tobin, and R. Andres, *J. Clin. Invest.*, 1978, **62**, 152.
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[248] W. Creutzfeldt and R. Ebert, *Int. Congr. Ser. Excerpta Med.*, 1977, **413** (Diabetes), p. 63 (*Chem. Abs.*, 1978, **89**, 40 346).
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[250] K.B. Lauritsen and A.J. Moody, *Diabetologia*, 1978, **14**, 149.
[251] R. Arnold, W. Creutzfeldt, R. Ebert, H.D. Becker, H.W. Boerger, and A. Schafmayer, *Scand. J. Gastroenterol.*, 1978, **13**, 41.
[252] J.C. Brown and J.R. Dryburgh, ref. 1, p. 327.
[253] P. Heitz, J.M. Polak, and A.G.E. Pearse, ref. 1, p. 332.
[254] P. Mitznegg, W. Domschke, S.R. Bloom, E. Schubert, S. Domschke, U. Strunz, R. König, E. Wünsch, and L. Demling, ref. 1, p. 339.
[255] N.D. Christofides, S.R. Bloom, and H.S. Besterman, ref. 1, p. 343.
[256] W. Domschke, U. Strunz, P. Mitznegg, S. Domschke, W. Sprügel, H. Ruppin, E. Wünsch, and L. Demling, ref. 1, p. 335.
[257] P. Mitznegg, in 'Klin. Pharmacol. Exp. Ther.', Kolloq. 5th, ed. W. Vogt and H.E. Bock, Editio Cantor, Aulendorf, Germany, 1977, p. 77.

its effects on the pressure in the lower oesophageal sphincter[258] have been reviewed.

Motilin, [D-Phe[1]]-, [Met(O)[13]]-, and [D-Phe[1],Met(O)[13]]motilin were synthesized by the classical fragment condensation procedure.[259] The protecting groups of the side functions were designed in such a way that final deprotection was achieved by treatment with methanesulphonic acid-anisole. Motilin and its D-Phe[1]-analogue were obtained in good yield by treatment of the corresponding methionine sulphoxide derivatives with thioglycolic acid. [D-Phe[1]]- and [Met(O)[13]]motilin showed 20 and 80 % respectively of the biological potency of the parent hormone. The authors conclude from this result that the *N*-terminal region is important for the exhibition of biological activity while the methionine residue is not essential as was already demonstrated by the finding that [Leu[13],Glu[14]]- and [Nle[13],Glu[14]]motilin possessed full biological potency.[260] [Ala[8]]-, [D-Ala[8]]-, [Ala[8],Met(O)[13]]- and [DAla[8],Met(O)[13]]motilin were synthesized in a way similar to that utilized for the former analogues and it was found in an *in vitro* assay that the replacement of glycine in position 8 with L-alanine lowers the biological potency to about 30 % of that of the natural hormone while the replacement with the D-isomer increases it slightly.[261]

Further highly specific and sensitive radioimmunoassays for motilin using either labelled motilin[262] or [Nle[13],Glu[14]]motilin[263] as tracers have been described but as yet no correlation between plasma concentration of motilin and certain pathological states has been observed.

Under *in vitro* conditions acid strongly stimulated the release of motilin from duodenal mucosa while alkali abolished basal secretion.[264] When acid was infused into the duodenum of pigs it was observed that the increase of plasma motilin levels was associated with a marked reduction of immunostainable motilin in the upper intestine and that the release of motilin seems to be an entirely local phenomenon.[265] Contrary to previous reports no rise of plasma motilin concentration could be detected in man after infusion of acid into the duodenum although duodenal motor activity was increased.[266] The effect of oral and intravenous nutriments on release of motilin was studied and it was seen that fat increases plasma concentration and glucose lowers it while proteins have no effect. These results explained why normal meals caused only very slight changes.[267] The close correlation between blood glucose and motilin

[258] H.M. Jennewein, ref. 1, p. 359.
[259] M. Fujino, S. Shinagawa, M. Wakimasu, C. Kitada, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1978, **26**, 101.
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[266] N.S. Track, S. Collins, T. Lewis, and E.E. Daniel, ref. 1, p. 351.
[267] N.D. Christofides, S.R. Bloom, H.S. Besterman, T.E. Adrian, and M.A. Ghatei, *J. Endocrinol.*, 1978, **77**, 42P.

levels during insulin-induced hypoglycemia prompted the suggestion that motilin may play a role in carbohydrate metabolism.[268]

Studies of the relationship between plasma motilin levels and interdigestive myoelectric activity of the duodenum[269, 270] as well as gastrointestinal contractile activity[271] and motor activity of the lower oesophageal sphincter[272] revealed that motilin plays an important role on these activities during the interdigestive period but not during digestion. Likewise exogenous motilin caused simultaneous contractions of the stomach and of the lower oesophageal sphincter[273] or the sphincter[272] only in the interdigestive period. The effects of motilin and gastrin on gastric motility[274] were compared as well as those of motilin and CCK-octapeptide on antral and duodenal myoelectric activity in the interdigestive state and during inhibition by secretin and GIP.[129] The role of the cholinergic nervous system in the gastrointestinal response to motilin *in vivo* was studied[275] and one group reported that motilin promotes gastric emptying in rats.[276]

Urogastrone.—(Figure 4). A review dealing with urogastrone and epidermal growth factor has been published.[277] Human urogastrone which is structurally closely related to mouse epidermal growth factor has been found to be localized

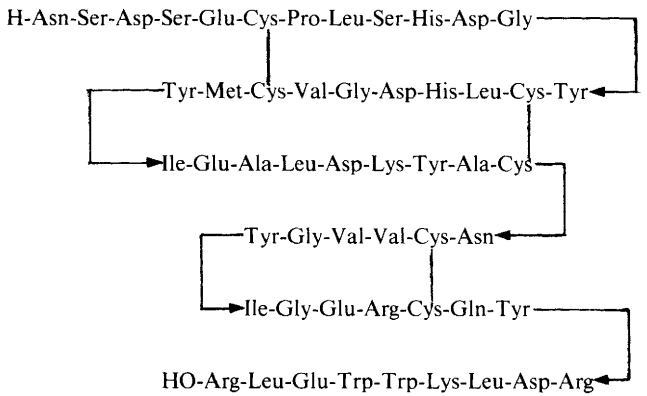


Figure 4 *The amino-acid sequence of β-urogastrone*

[268] N.D. Christofides and S.R. Bloom, *Experientia.* 1978, **34,** 809.
[269] K.Y. Lee, W.Y. Chey, H.H. Tai, H. Yajima, and D. Wagner, *Amer. J. Dig. Dis. Nutr.,* 1978, **23,** 789.
[270] W.Y. Chey, K.Y. Lee, and H.H. Tai, ref. 1, p. 355.
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[272] I. Aizawa, K. Hiwatashi, I. Takahashi, and Z. Itoh, ref. 127, p. 101 (*Chem. Abs.,* 1979, **90,** 16 749).
[273] Z. Itoh, I. Aizawa, R. Honda, K. Hiwatashi, and E.F. Couch, *Amer. J. Dig. Dis. Nutr.,* 1978, **23,** 341.
[274] M. Tani, K. Sugawara, M. Katoh, M. Manabe, Y. Akasaka, and K. Kawai, *Nippon Heikatsukin Gakkai Zasshi,* 1978, **14,** 55 (*Chem. Abs.,* 1979, **90,** 48 861).
[275] H.S. Ormsbee III and S.S. Mir, ref. 127, p. 113 (*Chem. Abs.,* 1979, **90,** 16 750).
[276] S. Nakazawa, K. Segawa, H. Hidano, Y. Tsukamoto, and T. Ichikawa, *Gastroenterol. (Japan),* 1978, **13,** 50 (*Chem. Abs.,* 1978, **89,** 100 637).
[277] H. Gregory, J.M. Bower, and I.R. Willshire, *Proc. F.E.B.S. Meet.,* 1978, **48,** 75 (*Chem. Abs.,* 1978, **89,** 209 293).

in specialized cells of the human submandibular salivary gland and also in cells of the glands of Brunner in the duodenum by immunohistochemical techniques.[278, 279] A homologous radioimmunoassay for human epidermal growth factor–urogastrone has been developed and was used for measurement of the excretion of urogastrone by normal adults and patients suffering from peptic ulcers, Cushing's syndrome or psoriasis, or recovering from major burns. No correlation between the amount of urogastrone excreted and the pathological state has yet been established.[280] Radiolabelled human epidermal growth factor–urogastrone was covalently linked to specific rat-liver membrane binding-sites and yielded, after solubilization of the membranes, one major and two minor ligand-specific soluble affinity-labelled components. Further investigations of these fractions suggested that the major specific binding-component is a glycoprotein subunit with an approximate molecular weight of 100 000.[281]

5 Vasoactive Peptides

Contributed by P.D. Roy

Bradykinin.—The kallikrein–kinin system has been reviewed in relation to kidney function,[1] activation,[2–4] kallikrein antagonists and inhibitors,[5] urinary kallikrein,[6] and urticaria.[7] A brief survey of the history of inflammation has been presented by Rochae Silva (discoverer of bradykinin).[8] The concept of using retro-sequences of peptides including bradykinin has been reviewed.[9]

Table 1 lists new bradykinin analogues with available biological data which have appeared in publications during 1978 and 1979. In general structure–activity relationship studies have examined the importance of phenylalanine residues in receptor interaction and of the proline residues in determining bradykinin conformation. In addition, interesting new analogues (30—33) have been reported which antagonize bradykinin activity.

The above antagonist analogues form part of a large structure–activity study carried out on fifty-five bradykinin analogues or fragments prepared by the solid phase method. Several of these compounds have previously been reported and only the new peptides are listed in Table 1 [analogues (1), (4—7), (14—16), (21), (22), (24), (26—33), (41—49), (52—55)].[10]

[278] J.B. Elder, G. Williams, E. Lacey, and H. Gregory, *Nature*, 1978, **271**, 466.
[279] P.U. Heitz, M. Kasper, S. Van Noorden, J.M. Polak, H. Gregory, and A.G.E. Pearse, *Gut*, 1978, **19**, 408.
[280] G.E. Dailey, J.W. Kraus, and D.N. Orth, *J. Clin. Endocrinol. Metab.*, 1978, **46**, 929.
[281] N. Sahyoun, R.A. Hock, and M.D. Hollenberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 1675.

[1] H.S. Margolius, *J. Lab. Clin. Med.*, 1978, **91**, 717.
[2] D. Ogston and B. Bennett, *Brit. Med. Bull.*, 1978, **34**, 107.
[3] A.P. Kaplan, *Prog. Hemost. Thromb.*, 1978, **4**, 127.
[4] I.H. Mills, L.F. Obika, and P.A. Newport, *Contrib. Nephrol.*, 1978, **12**, 132.
[5] J.D. Geratz and R.R. Tidwell, *Haemostasis*, 1978, **7**, 170.
[6] L.F. Obika, *Life Sci.*, 1978, **23**, 765.
[7] A. Taaffe, *Postgrad. Med. J.*, 1977, **53**, 732.
[8] M. Rocha e Silva, *Agents and Actions*, 1978, **8**, 45.
[9] M. Goodman and M. Chorev, *Accounts Chem. Res.*, 1979, **12**, 1.
[10] W.K. Park, S.A. St.-Pierre, J. Barabé, and D. Regoli, *Canad. J. Biochem.*, 1978, **56**, 92.

Table 1 *Analogues of bradykinin*

		1 5 9 Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg Bradykinin		
Compound number	*Structure*	*Biological activity*[b]	*Test*[c]	*Ref.*
1	[Glu[1,9]]	+++	B	10
		—	A,C	10
2	[3,4-dehydro-Pro[2]]	++++	E	17
		+++	A,F	17
3	[3,4-dehydro-Pro[3]]	+++	A,E,F	17
4	[Leu[5]]-(1–8)	+++	B	10
		—	A,C	10
5	[Tyr[5]]-(1–8)	+++	B	10
		—	A,C	10
6	[Tyr(Me)[5]]	+++	A,B,C,D	10
7	[Cha[5]]	+++	A,B,C,D	10
8	[Phe(4-NO_2)[5]]	< 5 %	A,E	14
9	[Phe(4-I)[5]]	< 5 %	A,E	14
10	[Phe(4-^{3}H)[5]]			15
11	[Phe(α-Me)[5]]	++	A,E,G,	16
12	[Car[5]]			12
13	[Car[5]]-(1–8)			12
14	[Cha[5,8]]	++++	B	10
		+++	A,D	10
		++	C	10
15	[Leu[5,8]]	+++	D	10
		++	B	10
		+	A	10
		—	C	10
16	[Ala[5,8]]	—	A,C	10
		++	B	10
17	[Car[5,8]]-(1–8)			12
18	[Car[5],LeuNH$_2$[8]]-(1–8)			12
19	[Phe(4-I)[5,8]]	rel. inactive	A,E	14
20	[Phe(4-NO_2)[5,8]]	rel. inactive	A,E	14
21	[Abu[6]]	+++	C	10
		+	A	10
		—	D	10
22	[D-Ala[6]]	+	A,C	10
		—	D	10
23	[20 %-1,2-$^{13}C_2$-Gly[6]]			20
24	[Acpc[7]]	+++	A,D	10
		++	C	10
25	[3,4-dehydro-Pro[7]]	+++	A,E,F	17
26	[Trp[8]]	++++	A,C	10
		+++	D	10
		—	B	10
27	[Nle[8]]	+++	D	10
		++	B	10
		+	A,C	10
28	[Pro[8]]	+	C	10
		—	A,D	10
29	[Tyr(Me)[8]]	++++	A,C	10
		+++	D	10
		++	B	10

Table 1. (*Cont.*)

1 5 9
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Bradykinin

Compound number	*Structure*	*Biological activity*[b]	*Test*[c]	*Ref.*
30	[Ala^8]-(1–8)	*	B	10
		—	A,C	10
31	[Leu^8]-(1–8)	**	B	10
		—	A,C	10
32	[Nle^8]-(1–8)	**	B	10
		—	A,C	10
33	[Leu(OMe)^8]-(1–8)	**	B	10
		—	A,C	10
34	[Car^8]-(1–8)			12
35	[Car^8]			12
36	[Phe(α-Me)^8]	+++	E	16
		++	A,G	16
37	[Tyr^8]-(1–8)	αE 1.0	B	21
		pD_2 4.39	B	21
38	[Phe(OMe)^8]-(1–8)	αE 0.8	B	21
		pD_2 6.81	B	21
39	[Phe(4-I)^8]	+++	A,E	14
40	[Phe(4-NO_2)^8]	+++	A,E	14
41	[Ala^{8,9}]	—	A,B,C	10
42	[Arg^8,Phe^9]	+++	D	10
		+	A,B	10
		—	C	10
43	[Ser^9]	+++	B	10
		+	C	10
		—	A,D	10
44	[Ile^9]	+++	B	10
		+	C	10
		—	A	10
45	[Glu^9]	+++	B	10
		—	A,C	10
46	[Nle^9]	+++	B	10
		—	A,C	10
47	[Arg(OMe)^9]	+++	B,C,D	10
		++	A	10
48	Sarcosyl-bradykinin	++++	A,C	10
		+++	D	10
49	D-Alanyl-bradykinin	++++	A	10
		+++	C,D	10
50	Lysyl{[Car^5,LeuNH$_2$^{8}]-(1–8)}			12
51	Lysyl{[Car^5,Leu^8]-(1–8)}			12
52	Bradykininyl[Ala^{10}]	++++	B	10
		+++	D	10
		++	C	10
		+	A	10
53	Bradykininyl[D-Ala^{10,11}]	+++	D	10
		++	A,C	10
54+	*endo*-Ala^{6a}	+++	D	10
		++	C	10
		+	A	10

Table 1. (*Cont.*)

		1 5 9 Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg Bradykinin		
Compound number	*Structure*	*Biological activity*[b]	*Test*[c]	*Ref.*
55+	*endo*-Ala[8][a]	++++	B	10
		+++	D	10
		+	C	10
		—	A	10

[a]Cha = cyclohexylalanine, Car = L-*o*-carboranylalanine, Abu = γ-amino-butyric acid, Acpc = amino-cyclopentane carboxylic acid. +Analogues with insertion of alanine in the bradykinin sequence after position 6 or 8. [b]Symbols for potency: 0—0.99 % +, 1–9.99 % ++, 10—99.9 % +++; 100 % or greater ++++, * and ** indicate inhibition (of increasing potency). [c]A, Rat blood-pressure (bradykinin = ++++); B, rabbit-aorta strip; C, cat-ileum strip; D, pulmonary inactivation in rat lung [bradykinin = +++ (86 %)]; E, rat uterus; F, guinea-pig ileum; G, cat jejunum. αE and pD_2 are intrinsic activity and affinity values, respectively, taken from Ref. 21.

The analogues (31) and (32) derived by replacement of phenylalanine in position 8 of des-[Arg[9]]bradykinin by leucine or norleucine, respectively, act as competitive and specific antagonists of the myotropic effects of bradykinin in rabbit-aorta strip. Methyl esterification at the *C*-terminus (33) does not markedly alter activity though substitution at position 8 with alanine gives an analogue (30) with weaker antagonist activity, presumably due to reduced side-chain hydrophobicity. In contrast, analogues which retain high agonist activity are obtained by replacement of phenylalanine in position 8 of bradykinin by tryptophan (26) or *O*-methyltyrosine (29) (125 and 270 %, respectively, on rat blood pressure). Bradykinin analogues which totally resist pulmonary inactivation in the rat-lung assay were obtained by substitution either at position 6 by γ-aminobutyric acid (21) or D-alanine (22), or at position 8 by proline (28), or at position 9 by serine (43). It is suggested that the latter positions are therefore important for substrate binding to kininase II.[10]

The synthesis of a series of novel bradykinin analogues in which phenylalanine in position 5 and/or 8 has been replaced by the boron containing new amino-acid L-*o*-carboranylalanine (Car)[11] has been reported. The resulting analogues (12), (13), (17), (18), (34), (35), (50), and (51) await biological evaluation. The 1,2-carborane cage in the side-chain of carboranyl-alanine has comparable dimensions to the benzene ring of phenylalanine and gives rise to close structural analogues. The thereapeutic potential of peptides containing carboranylalanine is discussed.[12]

Studies previously reviewed[13] on the effect of *p*-halogeno-substitution of the phenyl rings of bradykinin, have continued with the report of six analogues (8), (9), (19), (20), (39), and (40) in which the phenylalanine residue in position 5

[11] O. Leukart, M. Caviezel, A. Eberle, E. Escher, A. Tun-Kyi, and R. Schwyzer, *Helv. Chim. Acta*, 1976, **59**, 2184.
[12] O. Leukart, E. Escher, and D. Regoli, *Helv. Chim. Acta*, 1979, **62**, 546.
[13] P.D. Roy, in 'Amino-acids, Peptides, and Proteins', Vol. 10, A Specialist Periodical Report, ed. R.C. Sheppard, The Chemical Society, London, 1979, p. 463.

and/or 8 has been replaced by either 4-iodo-phenylalanine or 4-nitro-phenylalanine. Substitution of position 5 or both positions 5 and 8 yields relatively inactive analogues (8), (9), (19), and (20). The activity of the corresponding analogues (39) and (40) substituted at position 8 of bradykinin was 67 and 52 %, respectively, in the rat-uterus and blood-pressure assays, confirmining the importance of position 5 for hormone-receptor interaction.[14] In a separate study bradykinin specifically labelled at position 5 (10) was obtained on catalytic dehalogenation of the corresponding [4-Br-Phe^5]bradykinin analogue with 10 % Pd–C catalyst and ^{3}H tritium gas.[15]

Biological activity of the conformationally restricted α-methylphenylalanine bradykinin analogues (11) and (36) has been compared with bradykinin in a variety of *in vivo* and *in vitro* preparations. The two analogues retained 1.5—3.0 % hypotensive activity in rats *in vivo* and in *in vitro* preparations of rat-stomach strips. In the isolated rat uterus however the [Phe(α-Me)^8]bradykinin analogue (36) was ten times more potent than [Phe(α-Me)^5]bradykinin (11) and possessed 31 % of the potency of bradykinin. Such variation in the order of potency in different preparations indicates the heterogeneity of bradykinin receptors. Degradation profiles of these analogues in a number of systems are also described.[16]

The influence on activity of the proline residues in bradykinin has been examined through evaluation of the three analogues (2), (3), and (25) containing L-3,4-dehydroproline substituted at position 2,3, or 7, respectively. The 2- and 3-substituted analogues were equipotent to bradykinin in *in vitro* and *in vivo* assays used, and were found to be more resistant to enzyme degradation than bradykinin itself. By comparison the 7-substituted analogue (25) was less potent suggesting that the ring and/or bound angles of proline in this position is important for receptor interactions. The latter interpretation is supported by c.d. spectral data on the three analogues. These compounds are also of interest as precursors for the preparation of labelled bradykinins for metabolic studies. For example, catalytic reduction of analogue (3) in the presence of deuterium or tritium gas gives the corresponding [^{2}H-Pro^3]- or[^{3}H-Pro^3]bradykinin.[17]

The complete ^{13}C n.m.r. chemical shift assignments of bradykinin have been made on the basis of pH studies and the examination of constituents peptide fragments. Chemical shifts of proline carbons indicate that the *trans* configuration about the three X-Pro bonds predominates.[18] The *cis–trans* isomerism in X-Pro-Pro tripeptides has been characterized quantitatively[19] and the role of serine in reducing structural heterogeneity about the sixth peptide bond of bradykinin has been studied using analogue-(23).[20] Evidence

[14] A. Chung, J.W. Ryan, P. Berryer, and A. Day, Abs. Amer. Chem. Soc. Meeting, 1978 (176), BIOL. p. 54.
[15] A. Chung, J.W. Ryan, and P. Berryer, Abs. Amer. Chem. Soc. Meeting, 1978 (176), BIOL. p. 24.
[16] L.E. Leduc, G.R. Marshall, and P. Needleman, *Mol. Pharmacol.*, 1978, **14**, 413.
[17] G.H. Fisher, D.I. Marlborough, J.W. Ryan, and A.M. Felix, *Arch. Biochem. Biophys.* 1978, **189**, 81.
[18] R.E. London, J.M. Stewart, J.R. Cann, and N.A. Matwiyoff, *Biochemistry*, 1978, **17**, 2270.
[19] R.E. London, N.A. Matwiyoff, J.M. Stewart, and J.R. Cann, *Biochemistry*, 1978, **17**, 2277.
[20] R.E. London, J.M. Stewart, R. Williams, J.R. Cann, and N.A. Matwiyoff, *J. Amer. Chem. Soc.*, 1979, **101**, 2455.

has not been obtained to substantiate previously reported intramolecular salt-bridge formation between the guanidine group of arginine in position 1 and the carboxy-group of arginine in position 9. ^{13}C Spin-lattice relaxation data indicate that the side-chains of most residues are free to rotate internally relative to the peptide backbone supporting the view that bradykinin exists in solution primarily in a disordered conformation.[18]

Angiotensin.—Reviews of a general nature have covered the renin–angiotensin system[22] and its significance in hypertension[23] the clinical pharmacology and therapeutic use of anti-hypertensive drugs,[24] the effect of angiotensin* on behaviour[25] and its actions in the brain,[26,27] the physiology of hypertension,[28] and the relationship between converting enzyme and angiotensin in hypertensive disease.[29]

Table 2 lists a number of new angiotensin analogues with available biological activities reported during the review period. The emphasis and interest in clinically useful inhibitors of the renin–angiotensin system has moved from classical angiotensin antagonists (structural analogues) to simpler orally active compounds (proline derivatives) which block angiotensin converting enzyme and thereby the formation of angiotensin (see following section). This may account for the relatively few new analogues reported.

Attempts have been made to raise the pressor potency of angiotensin by increasing the hydrophobicity of the peptide. Derivatives bearing acetyl (56) propionyl (57), butyryl (58), pentanoyl (59), or hexanoyl (60) substituents at the *N*-terminus have been synthesized and evaluated both *in vivo* (rat blood-pressure) and *in vitro* (rat uterus; guinea-pig ileum). Results obtained show no correlation between myotropic activity *in vitro* and partition coefficients of the acylated peptides, though a significant negative correlation was found between increase in rat blood-pressure and hydrophobicity. As it is known that bulky substituents at the *N*-terminus of angiotensin do not affect activity, these findings indicate that hydrophobicity for this series is above an optimum value. The partition between aqueous medium and the non-polar receptor environment is not a limiting factor for angiotensin activity, whilst further data presented show that a free amino-group is important for the duration of angiotensin effects.[30]

The solid-phase synthesis of analogues of [Sar^1, Val^5]angiotensin in which the boron-containing amino-acid L-*o*-carboranylalanine (Car) replaces either

[21] D. Regoli, J. Barabe, and J. St.-Louis, in 'Peptides', Proceedings of the 5th American Peptide Symposium, ed. M. Goodman and J. Meienhofer, John Wiley and Sons, New York, 1977, p. 157.
[22] I.A. Reid, B.J. Morris, and W.F. Ganong, *Ann. Rev. Physiol.*, 1978, **40**, 377.
[23] J.J. Brown, R. Fraser, B. Leckie, A.F. Lever, J.J. Morton, P.L. Padfield, P.F. Semple, and J.I. Robertson, *Cardiovasc. Clin.*, 1978, **9**, 55.
[24] G.L. Wollam, R.W. Gifford jun., and R.C. Tarazi, *Drugs*, 1977, **14**, 420.
[25] C.B. Nemeroff and A.J. Prange jun., *Arch. Gen. Psychiatry*, 1978, **35**, 999.
[26] M.I. Phillips, *Neuroendocrinology*, 1978, **25**, 354.
[27] J.B. Simpson, M.L. Mangiapane, and H.D. Dellmann, *Fed. Proc.*, 1978, **37**, 2676.
[28] A.W. Cowley jun., *Cardiovasc. Clin.*, 1978, **9**, 1.
[29] R.L. Soffer and D.B. Case, *Amer. J. Med.*, 1978, **64**, 147.
[30] V.L.A. Nouailhetas, C.R. Nakaie, L. Juliano, and A.C.M. Paiva, *Biochem. J.*, 1977, **165**, 547.

*The term angiotensin is used to refer specifically to [Asp^1,Ile^5]angiotensin II; other derivatives are named with reference to this.

Table 2 *Analogues of angiotensin*

		1 5 8 Asp-Arg-Val-Tyr-Ile-His-Pro-Phe Angiotensin II *Biological activity*[b]		
Compound Number	*Structure*	*(Myotropic–Pressor)*	*Test*[c]	*Ref.*
56	[Ac-Asp[1]]	71	A	30
		25	B	30
		101	C	30
57	[Pr-Asp[1]]	67	A	30
		27	B	30
		89	C	30
58	[Bu-Asp[1]]	52	A	30
		22	B	30
		93	C	30
59	[Pen-Asp[1]]	53	A	30
		20	B	30
		49	C	30
60	[Hex-Asp[1]]	46	A	30
		17	B	30
		47	C	30
61	[Sar[1],Car[4],Val[5]]			12
62	[Sar[1],Val[5],Car[8]]			12
63	[Sar[1],dehydro-Ala[8]]	*		31

[a] Ac = acetyl, Pr = propionyl, Bu = butyryl, Pen = Pentanoyl, Hex = hexanoyl. [b] Values shown are percentages relative to angiotensin (100 %); * = antagonist. [c] A, rat uterus; B, guinea-pig ileum; C, rat blood pressure.

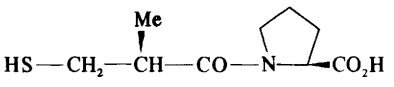
tyrosine in position 4 (61) or phenylalanine in position 8 (62) has been described. The increased hydrophobicity of the Car-peptides reflects the higher lipophilic character of the carborane side-chain with respect to those of tyrosine and phenylalanine. The biological activities of analogues (61) and (62) will appear in forthcoming publications.[12] Substitution of phenylalanine in position 8 of [Sar[1]]angiotensin by dehydroalanine provides an antagonist (63) claimed to be devoid of agonist activity.[31]

Studies on the ^{13}C n.m.r. properties of angiotensin in aqueous solution at pH 4.3 have been reported, and from the data provided correlation times for both overall motion of the molecule and for the internal motion of each peptide residue have been calculated to specify the nature of the molecular association of angiotensin in solution.[32]

Potentiating Peptides and Enzyme Inhibitors.—Several reviews have covered the important development of potent and specific inhibitors of angiotensin converting enzyme based on a hypothetical model of the active site of the enzyme.[33,34,35] Initial interest in peptidic inhibition of converting enzyme by

[31] Ger. Offen. 2 846 200 G.D. Searle and Co., 26th April, 1979.
[32] J.A. Ferretti and G.R. Marshall, *Biophys. J.* 1978, **21**, 79.
[33] D.W. Cushman, H.S. Cheung, E.F. Sabo, and M.A. Ondetti, *Progress in Cardiovascular Diseases*, 1978, **21**, 176.
[34] L. Favre, J.W. Hollifield, and D.N. McKinstry, *J. Tenn. Med. Assoc.*, 1978, **71**, 201.
[35] M.A. Ondetti, E.F. Sabo, K.A. Losee, H.S. Cheung, D.W. Cushman, and B. Rubin, Ref. 21, p. 576.

the nonapeptide, Glp-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro (Squibb 20 881) and several related peptides isolated from snake venom, has progressed to a series of orally active proline derivatives of greater potency. The most active compound 2-D-methyl-3-mercaptopropanoyl-L-proline (64) (Squibb 14 225) appears to have great potential for the treatment of hypertensive disease.[36,37] Experimental studies have further demonstrated the effectiveness of (64) as an antihypertensive agent in rat,[38,39] dog,[40,41,42,43] cat,[44] and rabbit[45] models. The mechanism of action of inhibitors of converting enzyme is probably a function of a combination of factors including reduction in levels of angiotensin (reduced vasoconstriction) and angiotensin III (reduced aldosterone secretion), and prolongation of the hypotensive effect of bradykinin (by inhibition of degradation).



(64)

Angiotensin converting enzyme purified from human plasma is an acidic glycoprotein consisting of a single polypeptide chain of molecular weight 140 000. The purified preparation cleaves bradykinin and angiotensin and is inhibited by SQ 14225.[46] Chemical modification studies carried out on the enzyme obtained from rabbit lung indicate the presence of a functional tyrosyl residue at the active site of angiotensin converting enzyme.[47] Other studies on the enzyme have demonstrated it is excreted in human urine.[48]

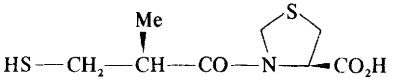
The synthesis of a dehydro (Δ) amino-acid containing tetrapeptide, Z-Pro-ΔPhe-His-Leu, which inhibits angiotensin converting enzyme has been described. The compound was prepared by two separate routes to authenticate the structure. The implications of the finding that replacement of phenylalanine by dehydro-phenylalanine at the enzyme cleavage point converts known substrate into an inhibitor are particularly interesting.[49]

[36] H.R. Brunner, J.P. Wauters, D. McKinstry, B. Waeber, G. Turini, and H. Gavras, *Lancet*, 1978, ii, 704.
[37] H. Gavras, H.R. Brunner, G.A. Turini, G.R. Kershaw, C.P. Tifft, S. Cuttelod, I. Gavras, R.A. Vukovich, and D.N. McKinstry, *N. Engl. J. Med.*, 1978, **298**, 991.
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[41] D.N. Harris, C.L. Heran, H.J. Goldenberg, J.P. High, R.J. Laffan, B. Rubin, M.J. Antonaccio, and M.E. Goldberg, *European J. Pharmacol.*, 1978, **51**, 345.
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[43] V.S. Murthy, T.L. Waldron, and M.E. Goldberg, *Proc. Soc. Exp. Biol. Med.*, 1978, **157**, 121.
[44] G.J. Trachte and A.M. Lefer, *Circulation Research*, 1978, **43**, 576.
[45] V.S. Murthy, T.L. Waldron, M.E. Goldberg, and R.R. Vollmer, *European J. Pharmacol.*, 1977, **46**, 207.
[46] J.J. Lanzillo and B.L. Fanburg, *Biochemistry*, 1977, **16**, 5491.
[47] P. Bünning, B. Holmquist, and J.F. Riordan, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 1442.
[48] T. Kokubu, I. Kato, K. Nishimura, K. Hiwada, and E. Ueda, *Clin. Chim. Acta*, 1978, **89**, 375.
[49] S. Konno and C.H. Stammer, *Internat. J. Peptide Protein Res.*, 1978, **12**, 222.

The use of dehydroamino-acids has also been reported in studies on analogues of the nonapeptide converting enzyme inhibitor, SQ 20 881. Substitution of proline in the parent structure at position 3 or 5 by L-3,4-dehydroproline gave analogues with approximately twenty times greater inhibitory potency. Such dehydropeptide analogues subjected to catalytic tritiation would yield ^{3}H-labelled SQ 20 881.[50]

Structure–activity studies on another potentiating peptide, Val-Glu-Ser-Ser-Lys (A-VI-5) have demonstrated that the side-chain functional groups of serine, glutamic acid, and lysine, are not essential for activity and similarly the *N*-terminal amino-group is not a requirement. Chain length (five-residues) and lipophilicity of the *N*-terminal residue are of greater importance whilst substitution at position 3 of the peptide chain with aromatic amino-acids results in highly active bradykinin potentiating peptides.[51]

The search for more potent inhibitors of angiotensin converting enzyme based on proline derivatives continues,[52] and recently new sulphydryl analogues with high antihypertensive activities have been reported. Replacement of the proline ring in SQ 14 225 by the thiazole ring provides an equipotent inhibitor (65) of angiotensin converting enzyme.[53]



(65)

Tachykinins.—The peptide groups in this section [tachykinins, bombesins (including litorin) and caeruleins] are potent hypotensive and myotropic agents derived mainly from the amphibian skin.[54] Inclusion of substance P in this category is due to its close chemical resemblance to the tachykinin group (*e.g.* physalaemin, uperolein, and phyllomedusin). Their biological significance as a whole is not established but is thought to involve control of secretions and regulation of the local vasculative in amphibian skin.[55]

A review has appeared on substance P.[56] Table 3 lists analogues of substance P and other tachykinins with available biological data. Results of studies on a series of substance P analogues and fragments (66—73) containing D-amino-acid substituents indicate that the phenylalanine in position 7 (but not position 8) is important for activity. Replacement of glycine in position 9 by D-leucine (68) reduced agonist activity in guinea-pig ileum to 0.5 %, whilst [D-Phe^7]substance P (66) exhibited weak antagonist activity. The heptapeptide

[50] G.H. Fisher, J.W. Ryan, and L.C. Martin, *Abs. Amer. Chem. Soc. Meeting*, 1978 (176), MEDI 26.
[51] J.G.R. Ufkes, B.J. Visser, G.A. Heuver, and C. Van Der Meer, *European J. Pharmacol.*, 1978, **50**, 119.
[52] E.W. Petrillo jun., M.A. Ondetti, D.W. Cushman, E.R. Weaver, and J.E. Heikes, *Abs. Amer. Chem. Soc. Meeting*, 1978 (176), MEDI 27.
[53] I. Mita, J.I. Iwao, M. Olya, T. Chiba, and T. Iso, *Chem. Pharm. Bull.*, 1978, **26**, 1333.
[54] V. Erspamer, P. Melchiorri, C.F. Erspamer, and L. Negri, *Adv. Exp. Med. Biol.*, 1978, **106**, 51.
[55] 'Fogarty International Center Proceedings, No. 27', ed. J.J. Pisons and K.F. Austen, U.S. Government Printing Office, Washington, 1976, p. 287.
[56] E.A. Mroz and S.E. Leeman, *Vitam. Horm.*, 1977, **35**, 209.

Table 3 *Analogues of substance P and other tachykinins*

Compound Number	*Structure*	*Biological Activity g.p.i.*[a]		*Ref.*
	1 5 10 Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 Substance P			
66	[D-Phe^7]substance P	2 %;*		57
67	[D-Phe^{7,8}]substance P	0.3 %		57
68	[D-Leu^9]substance P	0.5 %		57
69	[Glp^5,D-Phe^7[hepta (5–11)substance P	0.25 %;-*		57
70	[Glp^5,D-Phe^{7,8}]hepta (5–11)substance P	0.11 %;-*		57
71	[Lys^5,D-Leu^8,D-Phe^9]hepta (5–11)substance P	<0.06 %;-*		57
72	[Lys^5,Ile^8,D-Phe^9]hepta (5–11)substance P	<0.07 %;-*		57
73	[Glp^5,D-Leu^9]hepta (5–11)substance P	<0.02 %;-*		57
74	[Car^7]octa (4–11)substance P			12
75	[Car^8]octa (4–11)substance P			12
76	Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (substance P heptapeptide)	3.2		58
77	Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2 (eledoisin heptapeptide)	55.3		58
78	Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2 (phyllomedusin heptapeptide)	9.6		58
79	Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 (physalaemin heptapeptide)	20.3	*b*	58
80	Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH_2 (uperolein heptapeptide)	38.3		58
81	Asp-Glu-Phe-Val-Gly-Leu-Met-NH_2 (kassinin heptapeptide)	10.8		58
82	Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (bombesin heptapeptide)	0.007		58
83	Trp-Ala-Val-Gly-His-Phe-Met-NH_2 (litorin heptapeptide)	0.012		58

[a] Guinea-pig ileum *in vitro* (substance P = 100 %; *antagonist; -* devoid of antagonist activity).
[b] Relative potency (on Mol. basis) to substance P = 1.0.

analogues (71), (72), and (73), with a D-amino-acid in position 9 had the lowest agonist activity but failed to antagonise substance P activity.[57] The contractile activities of another *C*-terminal heptapeptide series of tachykinins and bombesins were in the order eledoisin (77) > uperolein (80) > physalaemin (79) > kassinin (81) = phyllomedusin (78) ≫ litorin (83) and bombesin (82).[58]

Substitution of phenylalanine in position 7 or 8 of the octapeptide fragment (4—11) of substance P by the boron-containing amino-acid, L-*o*-carboranyl alanine (Car) provides novel analogues (74) and (75) which await biological evaluation.[12]

A large number of peptides related to bombesin have been examined for effects on thermoregulation in rat.[59] The action of tachykinins on uptake of calcium by pancreatic acinar cells has been reported.[60]

[57] I. Yamaguchi, G. Rackur, J.J. Leban, U. Björkroth, S. Rosell, and K. Folkers, *Acta Chemica Scandinavica B*, 1979, **33**, 63.
[58] K. Kitagawa, K. Ujita, Y. Kiso, T. Akita, Y. Nakata, N. Nakamoto, T. Segawa, and H. Yajima, *Chem. Pharm. Bull.*, 1979, **27**, 48.
[59] J.E. Rivier and M.R. Brown, *Biochemistry*, 1978, **17**, 1766.
[60] R.J. May, T.P. Conlon, V. Erspamer, and J.D. Gardner, *Amer. J. Physiol.* 1978, **235**, E112.

6 Enkephalins and Endorphins

Contributed by P.W. Schiller

In 1978 research on opioid peptides continued at a rapid pace and resulted in more than 300 publications dealing with the chemistry and biology of enkephalins and endorphins. While the majority of the contributions aimed at a clarification of the physiological role of opioid peptides, the present review will be limited to a presentation of structure–activity relationships observed with enkephalin and endorphin analogues in various assay systems. Activities will be interpreted at the receptor level and conformational aspect of the enkephalin–opiate receptor interaction will be focused upon. The initial source of references has been 'Current Contents, Life Sciences' (Institute for Scientific Information, Philadelphia, U.S.A.).

The reader with an interest in the physiology and pharmacology of opioid peptides is referred to a number of excellent review articles which appeared in the past year. Comprehensive reviews on enkephalins and endorphins, opiate receptors, analgesia, and other behavioural and biochemical effects of opioid peptides include those by Uhl *et al.*,[1] Snyder,[2] Terenius,[3] Guillemin,[4] Kosterlitz,[5] Goldstein,[6] Taube,[7] and Chau-Pham.[8] A general article by Miller and Cuatrecasas[9] includes structure–activity relationships obtained with analogues of enkephalin and the role of the latter peptide in neurotransmission was reviewed by Snyder.[10] Progress in the localization, isolation, and characterization of opiate receptors is described in a detailed article by Simon and Hiller.[11] Several papers on brain peptides and opiate actions are contained in a recent monograph[12] and the now available volume 5 of the series 'Hormonal Proteins and Peptides'[13] is dealing with lipotropin and related peptides.

A fair amount of research related to the actions of opioid peptides was presented at several meetings which took place in 1978. While the proceedings of the Montreal meeting[14] have appeared, those of the Waterloo conference[15]

[1] G.R. Uhl, S.R. Childers, and S.H. Snyder in 'Frontiers in Neuroendocrinology', Vol. 5, ed. W.F. Ganong and L. Martini, Raven Press, New York, 1978, p. 289.
[2] S.H. Snyder, *Amer. J. Psychiatry*, 1978, **135**, 645.
[3] L. Terenius, *Ann. Rev. Pharmacol. Toxicol.*, 1978, **18**, 189.
[4] R. Guillemin in 'The Hypothalamus', ed. S. Reichlin, R.J. Baldessarini, and J.B. Martin, Raven Press, New York, 1978, p. 155.
[5] H.W. Kosterlitz and J. Hughes in 'Bases of Addiction', Life Sciences Report, Vol. 8, ed. J. Fishman, Dahlem Konferenzen, Berlin, 1978, p. 411.
[6] A. Goldstein in 'Recent Developments in Chemotherapy of Narcotic Addiction', Ann. New York Acad. Sci., Vol. 311, ed. B. Kissin, J.H. Lowinson, and R.B. Millman, New York Academy of Sciences, New York, 1978, p. 49.
[7] H.-D. Taube, *Anaesthesist*, 1978, **27**, 2.
[8] T.T. Chau-Pham, *Drug Metab. Rev.*, 1978, **7**, 255.
[9] R.J. Miller and P. Cuatrecasas, *Naturwiss*, 1978, **65**, 507.
[10] S.H. Snyder, ref. 4, p. 233.
[11] E.J. Simon and J.M. Hiller, *Ann. Rev. Pharmacol. Toxicol.*, 1978, **18**, 371.
[12] 'Factors Affecting the Action of Narcotics', ed. M.W. Adler, L. Manara, and R. Samanin, Raven Press, New York, 1978.
[13] 'Hormonal Proteins and Peptides', Vol. 5, 'Lipotropin and Related Peptides', ed. C.H. Li, Academic Press, New York, 1978.
[14] 'Central Nervous System Effects of Hypothalamic Hormones and Other Peptides', ed. R. Collu, J.R. Ducharme, A. Barbeau, and J.G. Rochefort, Raven Press, New York, 1979.
[15] Proceedings of the 'International Symposium on Brain Peptides: The New Endocrinology', held in Waterloo, October 29—31, 1978, in press.

are still in the press. Work on enkephalins and endorphins disclosed at the 15th European Peptide Symposium[16] will soon be published.

Opiate Activity in Various Assay Systems.—Like the classical opiates, the enkephalins and endorphins display a broad spectrum of biological activities. Among the *in vitro* bioassays, those based on inhibition of electrically stimulated contractions of the mouse vas deferens (MVD) or guinea-pig ileum (GPI) are the most common. Progress in the synthesis of radiolabelled opiate agonists and antagonists during the past few years has led to the development of highly specific binding assays which are now widely used. A plethora of procedures for measuring *in vivo* analgesic activities and other behavioural effects has been worked out. Other activities permitting studies of structure–activity relationships include stimulation of growth hormone and prolactin secretion, electrophysiological effects, and inhibition of adenylate cyclase activity.

New developments and structure–activity relationships in the various assay systems will be presented in individual sections. While novel analogues only are being considered, old ones are occasionally listed again for comparative purposes. Activities are expressed as molar potencies relative to standard compounds, whereby data were transformed, if necessary. For enkephalin analogues standard compounds in the *in vitro* assays included [Met^5] enkephalin, [Leu^5]enkephalin and the carboxamidated D-Ala^2-analogues of the latter peptides, while *in vivo* analgesic potencies were expressed relative to morphine. In the series of β-endorphin analogues and β-LPH fragments [Met^5]enkephalin and β-endorphin were employed as standards both in the *in vitro* and in the *in vivo* assays. Such standardization facilitates comparison of results obtained in different laboratories and of structure–activity relationships observed in different assays.

Isolated Tissue Assays.—Aside from studies with the well established MVD and GPI assays, a new assay based on the inhibition of electrically evoked twitches of the medial smooth muscle of the cat nictitating membrane[17] was used for the measurement of opiate activities.[20] The motor transmitter in the cat nictitating membrane is noradrenaline, as it is the case with the vas deferens.

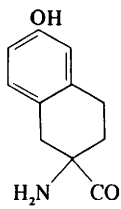
The activities of enkephalin and endorphin analogues on isolated tissue preparations are listed in Table 1. Protection against enzymatic degradation without loss of potency remained one of the major aims in the design of enkephalin analogues during the period covered by this review. Substitution of D-amino-acids in position 2 and modifications of the *C*-terminal continued to be the best strategy for protection against hydrolysis by aminopeptidases and carboxypeptidases. Thus, Day *et al.*[19] synthesized [D-Ala^2, des-carboxy-Nle^5]enkephalin (5) which is four times more potent than [Met^5]enkephalin in the GPI-assay. Following the initial lead by Bajusz *et al.*[39] further analogues

[16] Proceedings of the '15th European Peptide Symposium', held in Gdansk, ed. G. Kupryszewski, in the press.

[17] G. Henderson, J. Hughes, and H.W. Kosterlitz, *Brit. J. Pharmacol.*, 1975, **53**, 505.

Table 1 *Activity of analogues in isolated tissue assays*

(a) Enkephalins

Compound number	*Structure**	*Test* *MVD potency*		*Ref.*	*GPI potency*		*Ref.*
1	D-Ala^2, Leu^5	834	*a*	18	183	*a*	18
2	I-Tyr^1,D-Ala^2,Leu^5	63	*a*	18	55	*a*	18
3	I_2-Tyr^1,D-Ala^2,Leu^5	<2.5	*a*	18	<27	*a*	18
4	*N*-MeTyr^1, Nle(descarboxy)^5				120	*a*	19
5	D-Ala^2,Nle(descarboxy)^5				393	*a*	19
6	DesMet^{5}NH-n-butyl				77	*b*	20
7	DesMet^{5}NH-cyclohexyl				120	*b*	20
8	*β*-Ala^2,DesGly^3,Met^5-OMe				<8	*b*	20
9	*β*-Ala^2,Met^5-OMe				<8	*b*	20
10	D-Ala^2,Pro^5-NH_2				4286	*b*	20
11	D-Met^2,Pro^5-NHEt				17 143	*b*	20
12	D-Ala^2,Met^5-NH_2	381	*a*	21	775	*a*	21
13	D-Ser^2,Met^5-OMe	2950	*a*	21	1430	*a*	21
14	D-Met^2,Leu^5-OMe	299	*a*	21	6300	*a*	21
15	D-Met^2,Leu^5-NH_2	368	*a*	21	275	*a*	21
16	D-Ala^2,Pro^5-NH_2	49	*a*	21	549	*a*	21
17	D-Ser^2,Pro^5-NH_2	19	*a*	21	220	*a*	21
18	D-Met^2,Pro^5-NH_2	97	*a*	21	2920	*a*	21
19	D-Ala^2,Pro^5-NHEt	53	*a*	21	272	*a*	21
20	D-Ser^2,Pro^5-NHEt	31	*a*	21	211	*a*	21
21	*N*-MeTyr^1,Leu^5-NH_2	85	*a*	21	485	*a*	21
22	*N*-MeTyr^1,D-Ala^2,Leu^5-NH_2	640	*a*	21	1078	*a*	21
23	*N*-MeTyr^1,D-Ser^2,Pro^5-NHEt	7	*a*	21	218	*a*	21
24		114	*a*	21	767	*a*	21
25	Met^5-NH$(CH_2)_2$NH-dansyl				86	*b*	22
26	D-Ala^2,Met^5-NH$(CH_2)_2$NH-dansyl				147	*b*	22
27	Met^5-NH$(CH_2)_5$NH-dansyl				13	*b*	22
28	*N*-Dansyl-Tyr^1				4	*b*	22
29	D-Ala^2,F_5Phe^4,Met^5-NH_2	50	*c*	23			
30	D-Ala^2,Lys^6(N^ε-D-Ala^2-enkephalyl)-NH_2	61	*c*	23			
31	D-Ala^2,Δ^ZPhe^4,Met^5-NH_2				515	*c*	24

(b) β-LPH fragments and endorphin analogues

I	β_S-LPH-(60—65)	90	*a*	25
II	β_S-LPH-(59—65)	7	*a*	25
III	β_S-LPH-(58—65)	4.4	*a*	25
IV	β_S-LPH-(57—65)	0.3	*a*	25
V	β_S-LPH-(56—65)	0.11	*a*	25
VI	β_S-LPH-(41—91)	<9	*d*	26
VII	β_C-EP-(6—31)	4	*d*	27

Table 1 (*Cont.*)

Compound number	*Structure**	*Test* *MVD potency*	*Ref.*	*GPI potency*	*Ref.*
VIII	β_h-EP-(1—5)-(16—31)			135 *d*	27
IX	β_c-EP-(1—5)-(28—31)			35 *d*	27
X	β_h-EP-(1—5)			25 *d*	28
XI	β_h-EP-(1—15)			17 *d*	28
XII	β_h-EP-(1—21)			46 *d*	28
XIII	β_h-EP-(1—26)			48 *d*	28
XIV	β_h-EP-(1—28)			89 *d*	28
XV	β_h-EP-(1—29)			104 *d*	28
XVI	Phe^{27},Gly^{31}β_h-EP			128 *d*	29
XVII	Ala^{6,7},Phe^{27},Gly^{31}β_h-EP			118 *d*	29
XVIII	Nle^5,Phe^{27},Gly^{31}β_h-EP			118 *d*	29
XIX	D-Thr^2,Phe^{27},Gly^{31}β_h-EP			136 *d*	29
XX	D-Ala^2,β_s-EP	158 *d*	12	93 *d*	30
XXI	D-Ala^2,MePhe^4β_s-EP	260 *d*	12	10 *d*	30
XXII	D-Ala^2,MePhe^4,Met(O)^5β_s-EP	250 *d*	12	10 *d*	30

[a]Potency relative to Met^5-enkephalin = 100. [b] Potency relative to Leu^5-enkephalin = 100. [c]Potency relative to D-Ala^2,Met^5-enkephalinamide = 100. [d]Potency relative to β_h-endorphin = 100 (β_h-EP and β_c-EP (= β_s-EP) are equipotent in the GPI assay).

*The numbers indicating the position of amino-acids refer to the sequence of [Met^5]enkephalin (H-Tyr^1-Gly^2-Gly^3-Phe^4-Met^5-OH), unless a β-LPH or β-endorphin (β-EP) sequence is specified. The subscripts h, c, and s depict human, camel, or sheep β-endorphin or β-LPH, respectively.

with proline-amide in position 5 were synthesized and tested.[20, 21] Among these [D-Met^2, Pro^5]enkephalin-ethylamide (11) was found[20] to be 170 times more potent than [Leu^5]enkephalin in the GPI assay which is in accordance with the previously observed high *in vivo* analgesic potency of this compound.[40] In a novel approach English and Stammer[24] introduced 2,3-dehydrophenylalanine in Z-configuration (Δ^zPhe) in position 4 in order to achieve protection against enzymolysis. The resulting analogue (31) was resistant toward chymotryptic digestion and was five times more potent than [D-Ala^2,Met^5]enkephalinamide in the GPI assay. Obviously, the introduction of a double bond reduces the conformational flexibility of the aromatic side-chain. A similar conformational restriction was introduced at the tyrosyl residue in position 1 in an analogue (24) synthesized by the ICI-group and tested by Shaw and Turnbull.[21] It is interesting to note that enhanced rigidity in the important side-chains in positions 1 and 4 does not decrease potency in the isolated tissue assays. On the basis of their experience with highly potent, branched LH-RH analogues Coy *et al.*[23] synthesized a dimer of [D-Ala^2]enkephalin (30) which showed 60 % of the potency of the monomer in the MVD assay.

[18] R.J. Miller, K.-J. Chang, J. Leighton, and P. Cuatrecasas, *Life Sci.*, 1978, **22**, 379.

[19] A.R. Day, J.M. Carney, J.A. Rosecrans, W.L. Dewey, and R.J. Freer, *Res. Comm. Chem., Pathol. Pharmacol.*, 1978, **20**, 59.

[20] J. Knoll, P. Illés, and K. Medzinradszky, *J. Pharm. Pharmacol.*, 1978, **30**, 394.

[21] J.S. Shaw and M.J. Turnbull, *European. J. Pharmacol.*, 1978, **49**, 313.

[22] M.C. Fournie-Zaluski, G. Gacel, B.P. Roques, B. Senault, J.M. Lecomte, B. Malfroy, J.P. Swerts, and J.C. Schwartz, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 300.

[23] D.H. Coy, A.J. Kastin, M.J. Walker, R.F. McGivern, and C.A. Sandman, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 977.

Miller *et al.*[18] investigated the influence of iodine substitution in the phenol ring of tyrosine on opiate activity. While the monoiodinated analogue (2) retained 8—30 % activity in the MVD- and GPI-assay, the potency of the di-iodinated compound was very low. These findings are of interest for the use of radioiodinated enkephalin analogues in receptor binding studies. A series of dansylated enkephalin analogues was prepared by Fournie-Zaluski *et al.*[22] Analogues carrying a 1-dimethylaminonaphthalene-5-sulphonate (dansyl) group at the *C*-terminal (25, 26) showed undiminished potency in the GPI-assay. The low potency of the analogue dansylated at the *N*-terminal (28) emphasizes once again the importance of an intact α-amino-group for opiate activity. Dansylated enkephalin analogues are of potential use for opiate receptor studies by fluorescence techniques. An analogue with pentafluorophenylalanine substituted in position 4 (29) was prepared by Coy *et al.*[23] Since it is still half as potent as the corresponding Phe[4]-analogue, it should be useful for ^{19}F n.m.r. spectroscopic studies.

Several authors focused on the comparison of structure–activity relationships in different tissues. Knoll *et al.*[20] found a good correlation between potencies observed in the GPI- and cat nictitating membrane assay in a series of enkephalin analogues with the exception of [Leu[5]]enkephalin. The agonist potencies of 13 enkephalin analogues (12—24) in the MVD- and GPI-assay were compared by Shaw and Turnbull.[21] There was little correlation between the agonist potencies observed with the MVD-assay and those determined with the GPI-assay. In confirmation of the report by Waterfield *et al.*,[41] The K_e-values for naloxone against the analogues were much lower on the vas deferens than on the ileum. Among the enzyme-resistant analogues of this series the ileum/vas potency ratios varied inversely with the K_e-values on the vas deferens. The results can be explained with the existence of a single, naloxone-sensitive receptor (μ- receptor) on the ileum and of two types of receptors (μ-and δ-receptors) on the vas. According to this hypothesis the enkephalin analogues would display high affinity for both types of receptors, while naloxone would interact poorly with δ-receptors.

Extension of [Met[5]]enkephalin at the *N*-terminal with amino-acid residues according to the sequence of β-LPH was performed by Ling *et al.*[25] In confirmation of the findings by Law *et al.*[42] β_S-LPH-(60—65) (I) showed only slightly reduced potency relative to [Met[5]]enkephalin in the GPI-assay, while further extension reduced potency drastically. It has often been assumed that the intact α-amino-group of tyrosine in enkephalin is important for high activity and that it may correspond to the tertiary nitrogen of morphine. The retained activity of β-LPH-(60—65) raises new questions regarding the role of the tyrosine amino-group. An extension analogue of β-endorphin, β_S-LPH-(41—91) (VI) synthesized by Lemaire *et al.*[26] showed weak potency in the GPI-assay. Li *et al.*[27] synthesized three analogues of β-endorphin which were

[24] M.L. English and C.H. Stammer, *Biochem. Biophys. Res. Comm.*, 1978, **85**, 780.
[25] N. Ling, S. Minick, and R. Guillemin, *Biochem. Biophys. Res. Comm.*, 1978, **83**, 565.
[26] S. Lemaire, D. Yamashiro, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 179.
[27] C.H. Li, D. Yamashiro, L.-F. Tseng, and H.H. Loh, *Internat. J. Peptide Protein Res.*, 1978, **11**, 154.

designed to clarify the role of the *C*-terminal segment for opiate activity. β_h-Endorphin-(1—5)-(16—31) (VIII) was more potent in the GPI-assay than the native peptide in contrast to β_c-endorphin-(6—31) (VII) and β_c-endorphin-(1—5)—(28—31) (IX) which were significantly less active. As reported previously,[43] the action of VII is not naloxone-reversible. Li's group also prepared a series of enkephalin analogues with increasing extension at the *C*-terminal according to the sequence of β_h-endorphin[28] (X—XV). A progressive decline in potency up to the 17-residue length was observed in the GPI-assay. Further extension to the 21-peptide increased potency again and it became evident that nearly the entire sequence of β_h-endorphin is required for full activity. Several analogues of β_h-endorphin with substitutions in the enkephalin part and the *C*-terminal segment were synthesized by Blake *et al.*[29] (XVI—XIX). Relative to the native peptide they all showed slightly enhanced potency in the GPI-assay. β_h-Endorphin-analogues with substitutions analogous to those which had previously been performed with enkephalin[44] were prepared by Lemaire *et al.*[30] Poor correlation between potencies in the MVD-, GPI-, and receptor binding assay was observed and interpreted in terms of multiple opiate receptors.

On the basis of structural and conformational similarities[45] between the active analogue [Trp^4, Met^5]enkephalin[46] and the unsulphated *C*-terminal 7-peptide of cholecystokinin (CCK-7), Schiller *et al.*[47] determined the activity of the latter peptide in the GPI-assay. The observed reduction in potency by at least two orders of magnitude relative to [Met^5]enkephalin was parallelled by a similar drop in affinity. The *in vitro* opiate activity of CCK-7 is thus comparable to that of ACTH-peptides.[48]

Opiate Receptor Binding.—Binding assays with opiates performed in different laboratories often differ from one another in the selection and preparation of tissue, the choice of radiolabelled opiate, the composition and temperature of the incubation medium and the duration of the incubation. A new, highly sensitive receptor assay based on displacement of [^{3}H-Tyr]Leu-enkephalin from binding sites on a neuroblastoma × glioma hybrid cell line (NG 108–15) was developed by Diekmann-Gerber *et al.*[35] This radio-receptor assay has pmole sensitivity and should be useful for the isolation of small amounts of opioid peptides.

A detailed analysis of the binding of [^{3}H-Tyr]Met-enkephalin, [^{3}H]dihydromorphine and [^{3}H]naloxone to opiate receptors in rat-brain membranes was performed by Simantov *et al.*[49] A Scatchard plot revealed two classes of binding sites for [^{3}H-Tyr]Met-enkephalin with respective dissociation constants of 1.8 and 5.8 nM. Kinetic experiments showed that enkephalin associates to and dissociates from opiate receptors more slowly than alkaloid opiates. The results of competitive binding experiments suggested that part of the

[28] H.-W. Yeung, D. Yamashiro, W.-C. Chang, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 42.
[29] J. Blake, L.-F. Tseng, W.-C. Chang, and C.H. Li, *Internat. J. Peptide Protein Res.*, 1978, **11**, 323.
[30] S. Lemaire, A. Bérubé, G. Derome, I. Lemaire, J. Magnan, D. Regoli, and S. St.-Pierre, *J. Med. Chem.*, 1978, **21**, 1232.

enkephalin binding may involve different sites than does the binding of alkaloid opiates. Finally, the authors observed that alkaloid and peptide opiate binding have comparable organ and subcellular distributions and ion specificity.

Relative affinities of enkephalin- and endorphin analogues are listed in Table 2. Substitution of chlorine in the *para*-position of the phenyl ring in position 4 (36,40) does not produce a loss in affinity, while introduction of a methoxy-substituent at the same site (42) lowers affinity significantly.[31] Further enzyme-resistant analogues with D-configuration in positions 2 and 5 and with modified α-amino- and carboxy-groups were tested in binding assays. Some new analogues of this type include those prepared by the Burroughs-Wellcome group and tested by Meltzer *et al.*[31] as well as those synthesized by Belluzzi *et al.*[32] Several of these compounds (35,39,40,41,44) showed higher affinity than [Met^5]enkephalin under conditions where enzymatic degradation is eliminated. McGregor *et al.*[34] demonstrated that the 4-peptide H-Tyr-D-Ala-Gly-Phe-NH_2 (63) has 84 % affinity relative to [D-Ala^2,Met^5]enkephalinamide. Comparison with the affinities of other 4-peptide analogues (65,68,69) revealed that aside from preventing enzymatic degradation the *C*-terminal carboxamide function may directly enhance the affinity for the receptor.

Table 2 *Opiate receptor affinities*

(a) Enkephalins

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
2	I-Tyr^1,D-Ala^2,Leu^5	54,39.5	*a,b*	18,18
3	I_2-Tyr^1,D-Ala^2,Leu^5	<0.46,<0.36	*a,b*	18,18
25	Met^5-NH$(CH_2)_2$NH-dansyl	50	*c*	22
26	DAla^2,Met^5-NH$(CH_2)_2$NH-dansyl	100	*c*	22
27	Met^5-NH$(CH_2)_5$NH-dansyl	24	*c*	22
28	*N*-Dansyl Tyr^1	10	*c*	22
32	D-Ala^2,D-Leu^5-NHEt	20	*d*	31
33	D-Ala^2,DesMet^5	29	*d*	31
34	D-*N*-MeAla^2,D-Leu^5-OMe	22	*d*	31
35	D-Ala^2, D-Leu^5	769	*d,e*	31,36
36	D-Ala^2, *p*-ClPhe^4,D-Leu^5-NHEt	25	*d*	31
37	*N*-MeTyr^1,D-Ala^2,D-Met^5-NH_2	6.7	*d*	31
38	*N*-MeTyr^1,D-Ala^2,D-Leu^5-NH_2	100	*d*	31
39	D-Ala^2,Thr^6	357	*d*	31
40	D-Ala^2,*p*-ClPhe^4,D-Leu^5-OMe	1000	*d*	31
41	D-Trp^2,Leu^5	222	*d*	31
42	Tyr(OMe)^4,Leu^5-OMe	0.04	*d*	31
43	D-Trp^2,D-Leu^5	0.25	*d*	31
44	D-Ala^2,D-Met^5	200	*d*	31
45	D-Ala^3,Leu^5	0.25	*d*	31
46	D-Phe^4,Leu^5	<0.02	*d*	31
47	D-Ala^2,Leu^{4,5}	<0.02	*d*	31
48	D-Tyr^1,D-Ala^2,Leu^5	0.007	*d*	31
49	DesTyr^1,DesGly^2,DesPhe^4, Leu^5-OMe	<0.02	*d*	31

[31] H.Y. Meltzer, R.J. Miller, R.G. Fessler, M. Simonovic, and V.S. Fang, *Life Sci.*, 1978, **22**, 1931.
[32] J.D. Belluzzi, L. Stein, W. Dvonch, S. Dheer, M.I. Gluckman, and W.H. McGregor, *Life Sci.*, 1978, **23**, 99.

Table 1 (*Cont.*)

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
50	Phe[1],D-Ala[2],Leu[5]	20	*d*	31
51	D-Ala[2],D-Met[5]-NH_2	11	*e*	32
52	D-Ala[2],Leu[5]-NH_2	100	*e*	32
53	D-Ala[2],D-Leu[5]-NH_2	60	*e*	32,34
54	Trp[4]	129	*d*	33
55	Trp[4],Leu[5]	32	*d*	33
56	Trp[4],DesMet[5]	2	*d*	33
57	Trp[4],Thr[6]	59	*d*	33
58	D-Ala[2],Trp[4]	34	*d*	33
59	Ala[2],Trp[4]	2	*d*	33
60	D-Trp[4]	<0.1	*d*	33
61	D-Ala[2],DesGly[3],DesPhe[4],DesMet[5]-NH_2	0.09	*e*	34
62	D-Ala[2],DesPhe[4],DesMet[5]-NH_2	<0.001	*e*	34
63	D-Ala[2],DesMet[5]-NH_2	84	*e*	34
64	D-Ala[2]-*N*-MePhe[4],DesMet[5]-NH_2	135	*e*	34
65	D-Ala[2],DesMet[5]	6.5	*e*	34
66	D-Ala[2],Leu[4],DesMet[5]-NH_2	3.5	*e*	34
67	D-Ala[2],Trp[4],DesMet[5]-NH_2	38	*e*	34
68	D-Ala[2],DesPhe[4],DesMet[5]-$NH(CH_2)_2Ph$	26	*e*	34
69	D-Ala[2],DesPhe[4],DesMet[5]-$NHCH(Me)CH_2Ph$	23	*e*	34
(b) Endorphins				
XX	D-Ala[2]β_S-EP	84	*f*	30
XXI	D-Ala[2],MePhe[4]β_S-EP	143	*f*	30
XXII	D-Ala[2],MePhe[4]β_S-EP	143	*f*	30
XXIII	α-EP	37	*g*	35
XXIV	β_C-EP	60	*g*	35
XXV	γ-EP	32	*g*	35
XXVI	β_S-LPH	0.9	*g*	35

[a]Affinity relative to D-Ala[2],Leu[5]-enkephalin = 100. N4TG1 cells, ^{125}I-D-Ala[2],D-Leu[5]-enkephalin, 25 °C. [b]Affinity relative to D-Ala[2], Leu[5]-enkephalin = 100. Whole rat-brain pellet without Na^+, [^{3}H]naloxone, 25 °C. [c]Affinity relative to Leu[5]-enkephalin = 100. Mouse striatum, [^{3}H]Leu[5]-enkephalin, 30 °C. [d]Affinity relative to Met[5]-enkephalin = 100. Whole rat-brain pellet without Na^+,[^{3}H]naloxone, 4 °C. [e]Affinity relative to D-Ala[2],Met[5]-enkephalinamide = 100. Whole rat-brain pellet, 100 mM-NaCl, [^{3}H]naloxone, 0 °C. [f]Affinity relative to β_S-endorphin = 100. Whole rat-brain pellet without Na^+,[^{3}H]naloxone, 25 °C. [g]Affinity relative to Met[5]-enkephalin = 100. NG 108—15 cells, [^{3}H]Leu[5]-enkephalin, 37 °C.

Good correlation between affinities and activities on isolated tissue preparations (Table 1) is observed with the iodinated[18] and dansylated[22] analogues of enkephalin. Schiller *et al.*[33] prepared a series of enkephalin analogues with tryptophan substituted in position 4 (54—60). The relative affinities of the latter compounds paralleled those determined for the corresponding analogues with phenylalanine in the same position. The series proved useful for conformational studies by fluorescence techniques (see below).

Structure–activity relationships observed in the assay based on displacement of [^{3}H-Tyr]Leu-enkephalin from NG 108–15 cells[35] are in reasonable

[33] P.W. Schiller, C.F. Yam, and J. Prosmanne, *J. Med. Chem.*, 1978, **21**, 1110.
[34] W.H. McGregor, L. Stein, and J.D. Belluzzi, *Life Sci.*, 1978, **23**, 1371.
[35] L. Diekmann-Gerber, S. Stein, M. Rubinstein, J. Wideman, and S. Udenfriend, *Brain Research*, 1978, **151**, 117.

agreement with those determined by displacement of the same radio-ligand from receptor sites in brain homogenates.[50] In particular, β_c-endorphin showed similar affinities relative to [Met^5]enkephalin in the two assay systems, while α and γ-endorphin displayed ten times higher affinities in the hybrid cell assay than in the brain membrane assay.

***In vivo* analgesic activities.**—A variety of test procedures have been employed to determine the antinociceptive activity of enkephalin and endorphin analogues. The mouse or rat tail flick test and the mouse hot plate test were the most frequently used, whereby analogues were administered by the intracerebroventricular (i.c.v.), intravenous (i.v.), subcutaneous (s.c.), or oral route. Progress has been made in the standardization of the results insofar as most activities were expressed in terms of molar potencies relative to morphine or β-endorphin. Relative molar potencies of enkephalin and endorphin analogues are indicated in Table 3.

Omission of the *C*-terminal carboxy-group was used by Day *et al.*[19] in an attempt to prevent enzymatic degradation at the *C*-terminal. The resulting analogues (4,5), however, had only 16—30 % potency relative to morphine in the mouse tail flick test (i.c.v.) in contrast to their enhanced potencies in the *in vitro* GPI assay (Table 1). Similar potency was observed with the fluorescent analogue (26) prepared by Fournie-Zaluski *et al.*[35] in the mouse hot plate test after i.v. administration. Aside from stabilization against enzymolysis the lipophilic dansyl group at the *C*-terminal may facilitate crossing of the blood–brain barrier.

Belluzzi *et al.*[32] tested analogues of [D-Ala^2]enkephalin with methioninamide or leucinamide in position 5 either in L- or D-configuration. The D-isomers (51, 53) were significantly more potent than their corresponding 5-position L-isomers (12,52) in the rat tail flick test both i.v. and i.c.v. On the basis of stability studies it was concluded that the enhanced analgesic potency of the D-isomers is due to some other factor than increased stability toward enzymatic degradation. Surprisingly, the analgesic potency (i.c.v.) of [D-Ala^2, Met^5]-enkephalinamide (12) was 25 times lower than that of [D-Ala^2, Leu^5]-enkephalinamide (52), even though the two analogues are equipotent in the binding assay (Table 2). Correlations between potencies obtained after central and peripheral administration was good in contrast to the discrepancies observed between analgesic potencies and opiate receptor affinities. The results could be explained by multiple opiate receptors. Using the same assays the Wyeth-group, McGregor *et al.*,[34] also determined the analgesic potencies of a series of *N*-terminal fragments with D-alanine in position 2 and with carboxamide termination (61—64). Unexpectedly, even the dipeptide (61) retained long-lasting, analgesic activity at a concentration 200-times higher than that required with [D-Ala^2, Met^5]enkephalinamide. The tetrapeptide (63) induced profound, naloxone-reversible activity, whereby potency ratios of $\frac{1}{2}$ and 5 relative to [D-Ala^2, Met^5]enkephalinamide were observed for i.v. and i.c.v. administration, respectively. The correlation between antinociceptive potencies and receptor affinities (Table 2) was good in this series of compounds (61—69).

Table 3 In vivo *data*

(a) Enkephalins

Compound number	*Structure*	*Activity*	*Method*	*Ref.*
4	*N*-MeTyr^1,Nle(descarboxy)^5	16	*a*	19
5	D-Ala^2,Nle(descarboxy)^5	30	*a*	19
12	D-Ala^2,Met^5-NH_2	<5,10	*b,c*	32, 32
26	D-Ala^2,Met^5-$NH(CH_2)_2NH$-dansyl	35	*d*	22
35	D-Ala^2,D-Leu^5	<5,10	*b,c*	32, 32
51	D-Ala^2,D-Met^5-NH_2	20, 250	*b,c*	32, 32
52	D-Ala^2,Leu^5-NH_2	10,50	*b,c*	32, 32
53	D-Ala^2,D-Leu^5-NH_2	20,250	*b,c*	32, 32
61	D-Ala^2,DesGly^3,DesPhe^4,DesMet^5-NH_2	<10,0.5	*b,c*	34, 34
62	D-Ala^2,DesPhe^4,DesMet^5-NH_2	<10,<0.5	*b,c*	34, 34
63	D-Ala^2,DesMet^5-NH_2	50,250	*b,c*	34, 34
64	D-Ala^2,*N*-MePhe^4,DesMet^5-NH_2	50,250	*b,c*	34, 34
65	D-Ala^2,DesMet^5	<10,10	*b,c*	34, 34
66	D-Ala^2,Leu^4,DesMet^5-NH_2	10,50	*b,c*	34, 34
67	D-Ala^2,Trp^4,DesMet^5-NH_2	10,20	*b,c*	34, 34
68	D-Ala^2,DesPhe^4,DesMet^5-$NH(CH_2)_2$Ph	<10,5	*b,c*	34, 34
69	D-Ala^2,DesPhe^4,DesMet^5-NHCH(Me)CH_2Ph	<10,10	*b,c*	34, 34
70	D-Thr^2,Thz^5-NH_2	275,475 152,174	*a,e* *f,g*	36, 36 36, 36

(b) Endorphins

VII	β_c-EP-(6—31)	<0.1	*h*	27
VIII	β_h-EP-(1—5)-(16—31)	0.3	*h*	27
IX	β_c-EP-(1—5)-(28—31)	<0.1	*h*	27
XI	β_h-EP-(1—15)	<0.1	*i*	37
XII	β_h-EP-(1—21)	0.3	*i*	37
XIII	β_h-EP-(1—26)	8	*i*	37
XIV	β_h-EP-(1—28)	6	*i*	37
XV	β_h-EP-(1—29)	20	*i*	37
XVI	Phe^{27},Gly$^{31}\beta_h$-EP	119,148	*i,j*	29,29
XVII	Ala^{6,7},Phe^{27},Gly$^{31}\beta_h$-EP	23,49	*i,j*	29, 29
XVIII	Nle^5,Phe^{27},Gly$^{31}\beta_h$-EP	35,65	*i,j*	29, 29
XIX	D-Thr^2,Phe^{27},Gly$^{31}\beta_h$-EP	21,32	*i,j*	29, 29
XXIII	D-Sar$^2\beta_c$-EP	<0.3	*i*	38
XXIV	Ala$^2\beta_c$-EP	12	*i*	38
XXV	D-Leu$^2\beta_c$-EP	48	*i*	38
XXVI	D-Lys$^2\beta_c$EP	15	*i*	38
XXVII	Pro$^5\beta_h$-EP	0.6	*i*	38
XXVIII	Leu$^5\beta_h$-EP	17	*i*	38
XXIX	D-Leu$^5\beta_h$-EP	0.2	*i*	38
XXX	D-Ala^2,D-Leu$^5\beta_h$-EP	8	*i*	38
XXXI	β_h-EP-(1—30)	72	*i*	37

[a]Mouse tail flick, i.c.v., potency relative to morphine = 100. [b]Rat tail flick, i.v., potency relative to morphine = 100. [c]Rat tail flick, i.c.v., potency relative to morphine = 100. [d]Mouse hot plate, i.v., potency relative to morphine = 100. [e]Mouse tail flick, i.v., potency relative to morphine = 100. Mouse tail flick, s.c., potency relative to morphine = 100. [g]Mouse tail flick, oral, potency relative to *morphine = 100.* [h]Mouse tail flick, i.c.v., potency relative to β_c-endorphin = 100. [i]Mouse tail flick, i.c.v., potency relative to β_h-endorphin = 100. [j]Mouse tail flick, i.v., potency relative to β_h-endorphin = 100.

An extremely potent and long-lasting analogue, [D-Thr^2, Thz^5]enkephalinamide (70), was synthesized by Tseng *et al.*[36] In i.c.v. and i.v. administration it showed potency similar to that of β-endorphin, while after s.c. or oral delivery it was about 1.5—1.7 times as potent as morphine sulphate. The two analogues (29,30) synthesized by Coy *et al.*[23] induced long-lasting analgesia in the rat tail flick test (i.c.v.) at a dose of 25 μg; however, log-dose–response curves were not presented for these compounds.

A considerable number of β-endorphin analogues were synthesized and tested for their *in vivo* analgesic activity in the mouse tail flick test by Li's group. While β_c-endorphin-(6—31) (VII) and β_c-endorphin-(1—5)—(28—31) were virtually inactive, β_h-endorphin-(1—5)—(16—31) (VIII) showed low but significant analgesic potency (i.c.v.),[27] which, in agreement with the results of the GPI-assay, was not completely naloxone-reversible. This finding, together with the observed poor correlation between potencies obtained in the *in vitro* GPI-assay and the *in vivo* analgesic test, provides further evidence for the existence of multiple opiate receptors. Removal of one amino-acid at a time starting with the COOH-terminus of β_h-endorphin reduced analgesic potency (i.c.v.) in a stepwise fashion[37] and demonstrated that the complete sequence of 31 amino-acid residues in β_h-endorphin is required for full analgesic activity. Once again the analgesic potencies determined with this series of analogues correlated poorly with the potencies obtained in the GPI-assay.[28] The same poor correlation was observed[29] with analogues XVI—XIX. Among these [Phe^{27}, Gly^{31}]-β_h-endorphin (XVI) is the first described analogue with higher *in vivo* analgesic potency (i.c.v. and i.v.) than native β_h-endorphin. Finally, Yamashiro *et al.*[38] synthesized a series of β-endorphin-analogues with substitutions in position 2 and/or 5 which all showed lower analgesic potency (i.c.v.) than the native peptide. [Leu^5]-β_h-endorphin (XXVII), whose natural existence has not been demonstrated so far, was about 1/6 as potent as [Met^5]-β_h-endorphin. This study also indicated that β-endorphin is less sensitive to alterations in position 2 than are the enkephalins to corresponding changes. On the other hand, [Pro^5]-β_h-endorphin (XXVII) and [D-Leu^5]-β-$_h$-endorphin (XXIX) displayed weak analgesic potencies in contrast to the high activities of [D-Ala^2]enkephalin analogues[40, 32] with corresponding substitutions in position 5. These findings indicate that extrapolation of structure–activity relationships established for enkephalins to endorphins is not always possible.

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Other Biological Activities.—DeWied *et al.*[51] found that β-endorphin, α-endorphin, and [Met⁵]-enkephalin delayed extinction of active avoidance behaviour in rats, whereby β-endorphin was less potent than α-endorphin or β-LPH-(61—69). Since these effects are not naltrexone-reversible, they are probably not mediated by opiate receptors. In contrast to these peptides, γ-endorphin and [des-Tyr¹]-γ-endorphin facilitated extinction of avoidance behaviour and attenuated passive avoidance, the latter peptide being the more potent of the two.[52] The authors speculated that [desTyr¹]-γ-endorphin may be an endogenous neuroleptic and its administration to schizophrenic patients diminished hallucinations and delusions.[53] However, binding studies indicate that [desTyr¹]-γ-endorphin cannot displace neuroleptic drugs from their binding sites in rat brain and that its site of action must be different from that of the currently used neuroleptics.[54] In tests based on fear habituation Olson *et al.*[55] demonstrated a profound behavioural effect of [D-Ala²]-β-endorphin on goldfish after both central and peripheral administration.

The stimulation of prolactin and growth hormone release by analogues of enkephalin was further investigated. An earlier study[56] had shown that lower concentrations of β-endorphin or of [Met⁵]enkephalin and some of its analogues were required for eliciting prolactin release than for inducing growth hormone secretion. In contrast to these results, [D-Ala², D-Leu⁵]enkephalinamide (53) stimulated growth hormone release in the rat at s.c. doses as low as 50 μg kg^{-1}, while significant increases in prolactin release were only observed at a dose of 1 mg kg^{-1}. Both effects were naloxone-reversible; however, in contrast to what is observed with morphine, growth hormone release induced by this analogue was not blocked by somatostatin. Meltzer *et al.*[31] measured circulating levels of prolactin in rats following i.c.v. injection of 19 enkephalin analogues (32—50). While in general structure–activity relationships for prolactin release correlated well with those observed in the conventional *in vitro* and *in vivo* assays, there were some interesting exceptions. Thus, in contrast to its high *in vivo* analgesic potency,[57] [D-Ala², D-Met⁵]enkephalin produced only a slight increase in the prolactin level. On the other hand, [D-Ala², *p*-Cl-Phe⁴, D-Leu⁵]enkephalinethylamide (36), the most effective

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[55] R.D. Olson, A.J. Kastin, G.F. Michell, G.A. Olson, D.H. Coy, and D.M. Montalbana, *Pharmacol. Biochem. Behav.*, 1978, **9**, 111.
[56] L. Cusan, A. Dupont, G.S. Kledzik, F. Labrie, D.H. Coy, and A.V. Schally, *Nature*, 1977, **268**, 544.
[57] R.J. Miller, K.J. Chang, P. Cuatrecasas, S. Wilkinson, L. Lowe, C. Bedell, and R. Follenfant in 'Centrally Acting Peptides', ed. J. Hughes, MacMillan Press, London, 1978, p.195.

analogue in eliciting prolactin release, shows relatively low affinity for opiate receptors in brain homogenates (Table 2). These discrepancies can again be explained by the existence of multiple classes of opiate receptors. I.m. injections of the Sandoz-analogue, [D-Ala^2, MePhe^4, Met(O)ol^5]enkephalin (FK 33–824) in man also stimulated prolactin and growth hormone secretion.[58] However, contrary to the results obtained with β-endorphin,[59] the effect of this analogue was not antagonized by nalorphine.

Palmer *et al.*[60] investigated the potencies of several enkephalin analogues on the depression of rat cerebral cortex neurons. The results were in agreement with structure–activity relationships observed in the *in vitro* opiate assays. Since this electrophysiological effect was also naloxone-reversible, the authors concluded that it may be mediated *via* opiate receptors.

Conformation of Enkephalins and Endorphins.—Soon after the discovery of the opioid peptides numerous conformational studies were performed and information is now available on the conformation of enkephalin in empty space, in the crystalline state, in solution, and in the complex with the opiate receptor. A comprehensive article by Gorin *et al.*[61] includes a critical review of earlier and more recent papers on this subject. An interesting aspect of these conformational investigations is the comparison of the topography of corresponding functions in enkephalin and in opiates. The results of structure–activity studies indicate a correspondence between the tyramine moïety in Tyr^1 of the peptide and the hydroxyphenethyl portion of morphine. According to an early suggestion by Bradbury *et al.*,[62] the phenyl ring of Phe^4 in enkephalin might correspond to the phenethyl substituent on carbon 19 of a potent derivative of the oripavine family, 7α-[1-(*R*)-hydroxy-1-methyl-3-phenylpropyl] -6,14-*endo*-ethenotetrahydro-oripavine (PEO). In model studies topographical similarities are observed between PEO and the various β-bend conformations proposed for enkephalin, mainly on the basis of theoretical studies and n.m.r.-evidence (see reviews of 1976 and 1977). In a different proposal Gorin and Marshall[63] consider carbons 5 and 6 of the C-ring of morphine as an additional receptor binding element which would correspond to the *meta* and *para* positions of the phenyl ring of enkephalin. The computer-generated, receptor-bound conformation of enkephalin proposed by these authors is characterized by a close proximity of the two aromatic rings in contrast to the phenol–phenyl distance of about 10Å which is observed in β-bend models and in PEO. Based on this model correct predictions of the activity were made for a number of analogues.[61] An argument against the latter conformation was put forward by Nagaraj and Balaram[64] on the basis of the high opiate activity of [Aib^2,Met^5]enkephalinamide which might assume the

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[60] M.R. Palmer, D.H. Morris, D.A. Taylor, J.M. Stewart, and B.J. Hoffer, *Life Sci.*, 1978, **23**, 851.
[61] F.A. Gorin, T.M. Balasubramanian, C.D. Barry, and G.R. Marshall, *J. Supramol. Struct.*, 1978, **9**, 27.
[62] A.F. Bradbury, D.G. Smyth, and C.R. Snell, *Nature*, 1976, **260**, 165.
[63] F.A. Gorin and G.R. Marshall, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 5179.
[64] R. Nagaraj and P. Balaram, *F.E.B.S. Letters* 1978, **96**, 273.

backbone angles of residue 2 proposed by Gorin and Marshall only at a high cost of energy. A third model of the receptor-bound conformation, proposed by Clarke *et al.*,[65] is characterized by a 5 ⟶2 hydrogen-bonded βII bend and the carbonyl of Tyr[1] is considered to be important for the interaction with the receptor on the basis of a comparison with the meperidines. However, the latter model is in disagreement with structure–activity data obtained with various enkephalin analogues.[61]

Further efforts, were made to describe the conformation of enkephalin by theoretical energy calculations. Balodis *et al.*[66] performed a semiempirical conformational analysis of [Met[5]]enkephalin, [D-Ala[2],Met[5]]enkephalin and [D-Ala[3],Met[5]]enkephalin, whereby they singled out a 'biologically active' conformation of [Met[5]]enkephalin which contains a 4 ⟶1 hydrogen-bonded β-I turn. The latter proposal, however, is incompatible with the high activity of enkephalin analogues containing *N*-methylphenylalanine substituted in position 4. Using both empirical and quantum mechanical methods, Loew and Burt[67] found that conformers with maximum resemblance to PEO had very high energy. However, these authors also described a relatively low energy conformer which showed overlap both with the tyramine portion (Tyr[1]) and the phenethyl substituent on carbon 19 (Phe[4]) of PEO. The latter model is similar to that originally proposed by Bradbury *et al.*[62] and it was again suggested that it might represent the receptor-bound conformation. Finally, Momany and Aubuchon[68] performed a conformational energy analysis of *retro-all*-D-methionine-enkephalin and found that low energy conformers of the *retro-all*-D-analogue show little resemblance to those obtained for the native peptide.

The determination of the crystal structure of [Leu[5]]enkephalin by *X*-ray diffraction was performed by Smith and Griffin.[69] The structure is characterized by a β-bend with two antiparallel hydrogen-bonds between Tyr[1] and Phe.[4] In addition, two different conformations for the tyrosine side-chain were found. The respective intramolecular phenol–phenyl distances[70] of 10.67 and 11.28Å are close to that observed in PEO. Nevertheless, the crystal structure is certainly dissimilar from the receptor-bound conformation because of the high biological activity of [Phe(NMe)[4]]enkephalin analogues mentioned above. Furthermore, as Gorin and Marshall[61] pointed out, there is no overlap between the tyramine portions of either one of the two crystal structures and the phenethyl moïety of morphine. The crystal structure of [Leu[5]]enkephalin is quite different from that of its *N*-terminal 4-peptide segment[71] both with regard to backbone angles and the side-chain angles of Tyr[1] and Phe[4]. No hydrogen

[65] F.H. Clarke, H. Jaggi, and R.A. Lovell, *J. Med. Chem.*, 1978, **21**, 600.
[66] Y.Y. Balodis, G.V. Nikiforovich, I.V. Grinsteine, R.E. Vegner, and G.I. Chipens, *F.E.B.S. Letters*, 1978, **86**, 239.
[67] G.H. Loew and S.K. Burt, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 7.
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[69] G.D. Smith and J.F. Griffin, *Science*, 1978, **199**, 1214.
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[71] M.C. Fournie-Zaluski, T. Prange, C. Pascard, and B.P. Roques, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 1199.

bonds were detected by Carson and Hackert[72] in the crystal structure of the *N*-terminal 3-peptide.

Numerous studies of the conformation of enkephalin in solution have been performed by n.m.r.-techniques in the concentration range from 10^{-1} to 10^{-3}M. A dependence of n.m.r.-, c.d.-, and u.v.-parameters on concentration in the range from 10^{-1} to 10^{-4}M was demonstrated by Khaled *et al.*[73] and taken as evidence for molecular aggregation at high concentrations. There is thus a possibility that most n.m.r.-studies may have dealt with the conformational properties of a dimer or oligomer. Nagaraj and Balaram[64] recorded the ^{1}H n.m.r. spectrum of [Aib^2, Met^5]enkephalin and detected non-equivalence of the methyl groups of Aib^2 and the $C^{\alpha}H_2$ protons of Gly^3. This observation provides evidence, even though no definite proof, for the existence of well defined conformations in solution. A ^{13}C n.m.r. study performed by Tancrède *et al.*[74] in ^2H_2O at enkephalin concentrations ranging from 0.16 M to 3×10^{-3}M showed that Gly^3 is conformationally more restricted than Gly^2. However, with increasing concentration the mobility of the Gly^2 residue is reduced relative to that of the Gly^3 residue. Whether or not this observation is due to molecular aggregation remains unclear.

Schiller *et al.*[33, 46, 75] performed conformational studies with enkephalin analogues at significantly lower concentrations (2×10^{-5}M) by means of fluorescence quantum yield and energy transfer measurements. A tyrosine fluorescence quantum yield determination showed that conformers of [Met^5]enkephalin with a hydrogen bond between the tyrosyl hydroxy-group and a carbonyl group of the backbone, as suggested by Isogai *et al.*[76] and by Khaled *et al.*,[73] are not predominant in aqueous solution or in butanol.[75] The latter finding was subsequently confirmed by Spirtes *et al.*[77] on the basis of absorption data. With the biologically active[46] analogue [Trp^4, Met^5]enkephalin Schiller *et al.*[33] found no change in tryptophan fluorescence quantum yield in the concentration range from 10^{-5}M to 5×10^{-7}M. This result provides evidence that aggregation does not occur at concentrations below 10^{-5}M and it is compatible with the data of Khaled *et al.*,[73] if self-aggregation with a dissociation constant of the order of 10^{-3}—10^{-4}M is assumed. The intramolecular Tyr^1–Trp^4 distance of about 10Å obtained from measurement of resonance energy transfer[46] indicates the existence of folded conformations of [Trp^4,Met^5]enkephalin in aqueous solution. However, as an alternative to hydrogen bonds, such folded structures may simply be stabilized by solvent interactions. With the same analogue no conformational change was observed upon titration of the *C*-terminal carboxy-group.[75] This indicates that a NH_3^+—CO_2^- interaction is not important for stabilizing the conformation at

[72] W.M. Carson and M.L. Hackert, *Acta Cryst.*, 1978, **B34**, 1275.
[73] M.A. Khaled, M.M. Long, W.D. Thompson, R.J. Bradley, G.W. Brown, and D.W. Urry, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 224.
[74] P. Tancrède, R. Deslauriers, W.H. McGregor, E. Ralston, D. Sarantakis, R.L. Somorjai, and I.C.P. Smith, *Biochemistry*, 1978, **17**, 1978.
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[77] M.A. Spirtes, R.W. Schwartz, W.L. Mattice and D.H. Coy, *Biochem. Biophys. Res. Comm.*, 1978, **81**, 602.

physiological pH, as it had been proposed by Garbay-Jaureguiberry *et al.*[78] and by Jones *et al.*[79] Finally, Schiller *et al.*[33] found no gross conformational differences in a series of [Trp^4]enkephalin analogues with widely differing opiate receptor affinity and demonstrated conformational analogy between [Trp^4, Met^5]enkephalin, [Trp^4, Leu^5]enkephalin, and the *N*-terminal 4-peptide comprised in the latter two analogues.

Little is known about the conformation of the endorphins. A ^{13}C-n.m.r.-study by Tancrède *et al.*[74] revealed that α-endorphin has a flexible structure with the enkephalin segment differing little in motional properties from the rest of the peptide. C.d.-experiments with β-endorphin performed by Hollósi *et al.*[80] indicated a small α-helix content in water which was considerably increased in trifluoroethanol solution. In c.d.- and sedimentation velocity experiments Yang *et al.*[81] found no evidence for a globular and compact structure of β-endorphin and observed the formation of helical segments in methanol and sodium dodecyl sulphate. The existence of a preferred conformation of β-endorphin has been suggested by Austen and Smyth[82] on the basis of enzymatic degradation experiments.

Concluding Remarks on Structure–Activity Relationships.—In the review period covered by this article stabilization against enzymatic degradation remained one of the major goals in the design of enkephalin analogues and several enzyme-resistant compounds with analgesic potency exceeding that of morphine are now known. Some of them permit i.v. or oral administration and induce long-lasting analgesia. No progress has been reported on the development of an analogue with antagonist properties. Tolerance and dependence remain problems which have not been overcome so far by chemical modification of enkephalin. Clinical trials of the Sandoz-analogue FK 33–824 in man have revealed undesirable side-effects.[58]

Numerous discrepancies between potencies of enkephalin and endorphin analogues in different *in vitro* and *in vivo* assays have been observed. Following original suggestions by Martin[83] and by Lord *et al.*[84] (see review of 1977), these discrepancies were almost unanimously attributed to the existence of multiple classes of opiate receptors with varying affinity for a given analogue. The observation that some alkaloid opiates cannot displace very well [^{3}H-Tyr]Met-enkephalin from its receptor sites and that enkephalin cannot compete very efficiently for [^{3}H]alkaloid opiate binding sites,[21, 49] has been explained by opiate receptor heterogeneity,[21] different modes of binding to the same receptor,[49] and ligand (enkephalin)-induced conformational changes of the receptor.[85]

[78] C. Garbay-Jaureguiberry, B.P. Roques, R. Oberlin, M. Anteunis, and A.K. Lala, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 558.
[79] C.R. Jones, V. Garsky, and W.A. Gibbons, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 619.
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[84] J.A.H. Lord, A.A. Waterfield, J. Hughes, and H.W. Kosterlitz, *Nature*, 1977, **267**, 495.
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The investigation of molecular details of enkephalin–opiate receptor interactions has been the focus of many contributions. To this end novel analogues for use in studies of opiate receptor localization and binding and for the application of n.m.r.- and fluorescence techniques in conformational investigations have been synthesized. Progress has been made in the elucidation of the solution conformation of enkephalin and models of its receptor-bound conformation have been proposed. The average intramolecular distance of about 10Å between the two aromatic rings in positions 1 and 4 determined in solution[46] is considerably larger than that observed in a proposed pharmacophoric conformation.[63] Even though there is some spectroscopic evidence against significant conformational heterogeneity in solution, an equilibrium situation with only a small fraction of enkephalin conformers being able to interact with the receptor cannot be ruled out at this time. Alternatively, enkephalin might undergo a more or less drastic conformational change during the binding process. The slow association and dissociation observed with enkephalin in receptor binding studies has been interpreted in favour of the latter hypothesis.[49] Further efforts towards a clarification of the relationship between solution conformation and receptor-bound conformation are needed.

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5

Metal Complexes of Amino-Acids, Peptides, and Proteins

BY R.W. HAY AND D.R. WILLIAMS

1 Introduction

This chapter describes work published during the years 1977 and 1978 in the general field indicated by the title. As in our previous review we have tried to satisfy the requirements of both organic and inorganic chemists who have interests in this broad area.

A considerable number of books and general review articles have appeared: 'Inorganic Aspects of Biological and Organic Chemistry',[1] an introductory textbook to the area of Bioinorganic Chemistry,[2] 'Structure and Function of Copper Proteins',[3] 'Copper and Iron Metalloproteins',[4] a new Journal, 'Inorganic Perspectives in Biology and Medicine',[5] 'Synthetic Approaches to the Active Sites of Iron–Sulphur Proteins',[6] 'Synthetic Models for the Oxygen-Binding Hemoproteins',[7] 'Aqueous Solution Chemistry of Methylmercury and its Complexes with Amino Acids',[8] 'The Binding of Heavy Metals to Proteins',[9] 'Electron Transfer Reactions of Copper Proteins',[10] 'Zinc and Copper in Clinical Medicine',[11] 'Iron and Copper Proteins',[12] 'Substitution—Inert Metal Ions as Probes of Biological Function',[13] and 'Metal Toxicity in Mammals'.[14]

Volume 7 of Sigel's 'Metal Ions in Biological Systems' has been published and deals with iron in model and natural compounds.[15] A second A.C.S. publication dealing with Bioinorganic Chemistry has appeared.[16] Further books and review articles are: 'Inorganic and Nutritional Aspects of Cancer',[17]

[1] R.P. Hanzlik, 'Inorganic Aspects of Biological and Organic Chemistry', Academic Press, New York, 1976.
[2] Ei-Ichiro Ochiai, 'Bioinorganic Chemistry; an Introduction', Allyn and Bacon, London, 1977.
[3] H. Beinert, *Co-ordination Chem. Rev.*, 1977, **23**, 119.
[4] E. Frieden, *Trends Biochem. Sci.*, 1976, **1**, 273 (*Chem. Abs.*, **86**, 51 617).
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[8] D.L. Rabenstein, *Accounts Chem. Res.*, 1978, **11**, 100.
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[16] 'Bioinorganic Chemistry II', Advances in Chemistry Series, No. 162, ed. K.N. Raymond, American Chemical Society, Washington, 1977.
[17] 'Inorganic and Nutritional Aspects of Cancer', ed. G.N. Schrauzer, Plenum, New York, 1978.

'Inorganic Biochemistry',[18] 'Metals and Metabolism',[19] Volume 2 of 'The Porphyrins'[20] dealing with structure and synthesis, 'The Principles of Bio-Inorganic Chemistry',[21] 'Trace Elements in Human and Animal Nutrition',[22] 'Asymmetric Transformation of α-Amino Acids Promoted by Optically Active Metal Complexes',[23] 'The Evolution of Metalloenzymes, Metalloproteins, and Related Materials',[24] 'Roles for Metal Ions in Biological and Organic Catalysts',[25] 'Tungsten, a Biologically Active Metal',[26] 'Biochemistry and Physiology of Magnesium',[27] and 'Recent Advances in the Chemistry of Nitrogen Fixation'.[28]

2 Amino-acids

Binding.—Over the years that we have been reviewing the field of metal complexes of amino-acids there has been a notable transition from a situation where there was a plethora of publications concerning simple binary complexes between amino-acid anions and the more conventional electron acceptor metal ions of the first transition series, to a situation where such binding characteristics had been sufficiently well documented in the literature to permit such studies to be utilized as cornerstones for further studies involving, for example, ternary mixed ligand or mixed metal complexes, mixed solvent studies, and also investigations of the heavier metal ions of the periodic table. This transition can now be said to be complete since this review contains but three references to simple amino-acid–first transition series metal ion systems: the histidine–copper(II) system,[29] glycine–zinc(II) binding characteristics investigated using ultrasonic absorption,[30] and edta–cobalt(III) and titanium(III).[31] Some of the earlier literature concerning binary systems has been combined into compilations of stability constants and a recent handbook on proton ionization heats.[32]

An equilibrium study of the ternary system formed from copper(II)–amino-acid complexes and 2,2′-bipyridyl, and from copper(II)–amino-acid

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complexes and thiodicarboxylate and pyridine dicarboxylate as secondary ligands has been reported.[33] Similarly, the formation constants for L-3,4-dihydroxyphenylalanine and a range of amino-acid–copper(II) and zinc(II) ternary complexes have been established.[34] Manganese(II) is an important biometal and yet its co-ordination compounds have often been overlooked when solution studies were being planned. Sigel has reported on an intensive study of the ternary complexes of manganese(II) based upon equilibrium constant data.[35] The increase or decrease in stability upon adding the second ligand is sensitive to the nature of the donor groups provided by the first ligand and the discrimination that the metal ion has for groups such as ATP^{4-} is sensitive to the donor groups of this first ligand. The participation of cobalt(II) and nickel(II) in biological systems, the evolution of co-ordination spheres, and ligand–ligand interactions in mixed ligand complexes are all discussed at length. In similar vein, there is a report concerning the stereoselectivity occurring in mixed ligand complexes of histidine and L-amino-acids.[36]

Certain amino-acids such as tryptophan and phenylalanine form amazingly stable mixed ligand complexes and it is further interesting to note that this stereoselective effect reaches a maximum at a pH value of 6 but disappears as protons ionize from the complex. The question of whether pyrrole hydrogens can ionize in mixed ligand complexes has been the subject of a study of cobalt(II), nickel(II), and zinc(II) complexes of histamine, and of histidine with glycinate, ethylenediamine, 4,5-dihydroxybenzene-1,3-disulphonate, and 2,2′-bipyridyl.[37] It appears that nickel and zinc ions do not affect the ionization of the pyrrole hydrogen whereas cobalt decreases the pK by 3 log units.

The preparation and properties of glycinato[β-aminoethanolato]cobalt(III),[38] histidinatocobalt(II),[39] and *fac*(*N*)-D-asparto-L-asparaginatocobalt(III)[40] complexes and adducts of arginine, histidine, lysine, and isoleucine with potassium hexacyanoferrate(III)[41] have been reported. Imidazole–, glycine–, and L-histidine–aquapentacyanoferrate(II) complexes have also been categorized.[42]

Gergely has studied the complex formation and redox reactions between copper(II) ion and D-pencillamine which is dependent upon the metal–ligand ratio and also the concentration of halide ions prevailing.[43] Mixed valence polymers having an intense red-violet colour are reported. Another polymeric complex, copper(II)–poly(L-ornithine), has been studied using potentiometric titration and absorption and circular dichroic spectra.[44] Charge transfer transitions are observed in this polymeric system. From a study of the formation constants of the mixed ligand complexes of copper(II)-1-(2-

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pyridylazo)-2-naphthol chelates with a variety of bidentate ligands which were known to accelerate its rate of colour change when the ligand is used as an indicator in copper–edta titrations, mechanisms have been proposed to explain the rate increase observed. This work was performed in dioxane–water mixed solvents.[45] However, changes in thermodynamic quantities accompanying protonation reactions occurring in solvents of different dielectric constant (from water through that of dioxane–water to methanol–water) cannot be entirely correlated with simple electrostatic theory, even for simple copper and nickel alanine complexes.[46]

It frequently happens that when a ligand has three or four potential donor groups complexing occurs through the most powerful electron donors leaving one or two donors uncomplexed, merely solvated. However, when the more powerful groups are involved in polymerization to form biopolymers, as occurs in peptide formation, the interactions between the remaining donor groups and metal ions become important. There are difficulties in studying peptide–metal-ion interactions and similarly there are difficulties in studying weak-donor metal-ion interactions in the presence of strong-donor metal-ion bonds. The recent introduction of oil continuous microemulsion techniques[47, 48] allows the study of specific interactions between amino-acid side-chains and metal ions. Both the metal and the ligand are microencapsulated as aqueous droplets in a dispersed phase. The ligand is introduced into the system as a surface-active derivative, an N^{α}-dodecanoyl amino alcohol, and so both the ligand side-chains and its metal complexes are located within the interfacial domain of the droplet. This system appears to have potential for the study of metal–ligand interactions such as those which occur in metalloproteins and other complexes having biological relevance. The copper(II) complexes of N^{α}-dodecanoyl-L-lysinol, N^{α}-dodecanoyl-L-glutaminol, and N^{α}-dodecanoyl-L-methioninol have been studied and it was found that this metal ion can be forced to interact with very weak ligand groups by making certain adjustments to the composition of the microemulsion. Studies involving copper and the side-chain of tryptophan have revealed salient features of the indole–copper interaction. This is a major chromophore contributing to the u.v. absorption spectra of proteins.[48]

Although formation constants indicate the stoicheiometry of a complex, a large constant does not mean that a complex will exist under competition conditions. The previous paragraph has shown that side-chain–metal-ion bonding becomes important under microemulsion conditions and recent computer simulation models of solutions occurring in very sophisticated biofluids have shown which complexes actually prevail. Provided the constants are carefully determined in terms of concentrations[49] rather than activities they can be used both for these speciation calculations and for determining stepwise

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heats of formation.[50] When many complexes co-exist in the same solution it is often impossible to analyse for each complex *in situ* and so models are composed based upon compendia of equilibrium constants listing each reaction possible in the complex mixture. The amino-acid–metal ion-complexes present in blood plasma have been studied in competition with other low molecular weight ligands, and with proteins, present by a large computer model based upon the ECCLES program.[51] One important feature of this approach is that the influence of administered exogenous ligands can be calculated. This model of competitive binding has been applied to bismuth therapy of ulcers,[52] antiviral complexes,[53] zinc absorption,[54] [55] the metal mobilizing characteristics of D-penicillamine and edta,[56] rheumatoid arthritis,[57–62] trace element liberating therapeuticals in general,[63–64] low molecular weight iron(III) complexes,[65] and the ability of ligands to mobilize the copper(II) complexed in ceruloplasmin.[66] A reinvestigation of the zinc(II)–citrate–cysteinate system has emphasized the necessity to determine carefully the constants used in these studies.[67]

With the movement away from first transition series–amino-acid binding studies to more sophisticated systems, it is becoming fashionable to add relevance to one's researches by turning to polluting heavy elements which are challenging the lighter transition series elements complexes *in vivo*. Stability constants for *mono* and *bis* lead–aminoacetate and gluconate complexes and their protonated forms have been reported,[68, 69] and additionally mixed ligand chelates formed between amino-acids, pyridoxamine, and cadmium ions. Ternary complexes between this vitamin B_6 component and glycine, alanine, valine, and β-phenylalanine have been reported from polarography.[70] Other polarographic studies involving cadmium and lead ions include those of the *N*-[tris(hydroxymethyl)methyl]glycine complexes and their mixed hydroxo

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derivatives[71] and a study of compounds formed between zinc and cadmium ions with cysteine.[72]

Newer metal-ions to be added to the amino-acid complexing field include a study of a range of amino-acids and carboxylic acids complexing with the uranyl ion. (This paper concludes that uranyl very much prefers oxygen donors to sulphur and nitrogen donors[73]), a spectral and pH titration study of lanthanides from terbium(III) to europium(III) complexing with a range of pyridinecarboxylic acids,[74] a range of rare-earth metal ions complexing with DL-α-aminobutyric acid anions,[75] the binding of triorganotin(IV) with amino-acids such as cysteine, and its ethyl ester,[76] the binding of cysteine to silver,[77] glycinate to hafnium,[78] DL-penicillamine to indium(III),[79] and *trans*-1,2-diaminocyclohexanetetra-acetate to americium(III).[80]

There have been some interesting studies concerning mixed ligand complexes of palladium and ethylenediamine[81, 82] in which palladium-(ethylenediamine)-asparagine, -glutamine, -glycine, -alanine, -sarcosine, -*N,N*-dimethylglycine, -L-leucine, -L-phenylalanine, -L-proline, -L-tryptophan, -L-methionine, and -*S*-methyl-L-cysteine were quantified in terms of formation constants. All form simple 1 : 1 :1 mixed ligand complexes but the methionine and *S*-methyl-L-cysteine systems also formed protonated ternary complexes.

Amino-acid phosphates have been complexed with copper(II), nickel(II), zinc(II), cobalt(II), manganese(II), magnesium(II), and calcium(II), seven membered chelate rings being formed.[83]

Synthetic and Spectroscopic Studies.—Preparative exercises are invariably associated with product analyses using a variety of spectral techniques and so these two subjects are considered under the same heading. Even an extremely well-studied ligand such as edta can still attract the interests of spectroscopists; there are reports of hydrogen and deuterium isotope substitution effects using Raman spectral and ^{1}H n.m.r. studies for the protonation of edta and cdta.[84] These solution structural studies were used as precursors for metal complex – edta solution studies using n.m.r. spectroscopy.[85] The main conclusions are that the stereochemistry in solution is similar to that determined by *X*-ray diffraction in the solid state, that the denticity of edta varies from one to six and that the co-ordination number about a range of metal ions varies from four to

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[85] A.A. McConnell, R.H. Nuttall, and D.M. Stalker, *Talanta*, 1978, **25**, 425.

nine. The nickel–dtpa system has been studied over a wide range of pH values using ^{1}H n.m.r. and i.r. spectra. Rate constants, enthalpies, and entropies for the proton–deuterium isotopic exchange reaction are reported and it is found that only some of the carboxylates co-ordinate to the metal ion.[86]

Whereas formation constant studies have now passed through the stage of studying simple binary complexes, there are still several reports of synthetic and spectroscopic studies on first transition series metal-ion–amino-acid ligand binary complexes. The isomers of bis(*S*-2,3-diaminopropionato)-cobalt(III) ion have been studied using ^{1}H n.m.r. and theoretical calculations.[87] Here again, the structures resolved are similar to those reported from solid state *X*-ray studies. The i.r. study of group IIB metals with the amino-acid derivatives *N*-acetyl-L-, α-*N*-benzoyl-DL-, and benzoyl-β-alanine have been reported[88] and the effect of thermal rearrangement on the copper(II)–L-cystine complex studied by i.r. and Raman spectroscopy.[89]

Structural and dynamical information concerning the nickel(II) complexes of histidine have been investigated using carbon-13 relaxation spectra.[90] The work concludes that in addition to the conventional bis(histidinato)nickel(II) complex present at room temperature, there is a minor species present in which a carboxylate group has become disengaged from the nickel ion. The three isomers of L-histidinato–iminodiacetatocobalt(III) complex have been prepared[91] and it is found that the mole fractions of the *cis*-N, *trans*-N and *fac* were in the ratio of 0.91 : 0.04 : 0.05 respectively at 80 °C.

Several amino-acids, and (*S*)-proline in particular, have been reported to mediate the asymmetric transformation of α-triethylenetetraminecobalt(III) moieties.[92] The isomers formed can be resolved using cation exchange chromatography. Synthetic, structural, and stereochemical researches of cysteine and penicillamine cobalt(III) complexes have been reported.[93]

Two copper(II) amino-acid spectroscopic studies have appeared. The poly-chromism involved in the monomer–dimer equilibrium for copper(II) complexes of glycine ethyl ester[94] and the charge transfer states and c.d. spectra of copper(II) amino-acid interactions studied using theoretical calculations have been correlated with visible c.d. spectra.[95] Spectroscopic studies of the benzoyl-β-alaninato nickel(II) complexes have been used to investigate the ligand field strength produced by aromatic heterocyclic amine adducts.[96]

Some interesting mixed ligand complexes of cobalt(III) containing 3-azapentane-1,5-diamine and amino-acids such as glycine or phenylalanine have been prepared and studied by spectroscopic and solution equilibrium

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[96] G. Marcotrigiano, L. Menabue, and G.C. Pellacini, *Bull. Chem. Soc. Japan*, **50** (3), 742.

investigations.[97] Similarly, complexes between copper, zinc, cobalt, and nickel ions with *N*-acetyl-DL-tryptophanate and pyridine adducts have been prepared and characterized.[98]

The bis(*N*-acetyl-L-alanine)copper(II) monohydrate has recently been prepared and magnetic, electronic, and i.r. spectra used to suggest copper acetate monohydrate type structures.[99] All eight geometric isomers of mixed cobalt(III) complexes containing glycinato and β-alaninato–metal chelate rings have been isolated by absorption chromatography using a cation exchanger in the protonated form and relying on the differences in the basicities of carbonyl groups hydrogen-bonded to the resin.[100] The wide range of isomers arises because the glycine anion forms five-membered rings whereas the β-alanine anion forms six-membered chelate rings. Radioactive labelled zinc-65 have been used to synthesize a range of zinc glycine complexes of biological relevance[101] and the synthesis and properties of histidine containing five-co-ordinate complexes of hemin have also been reported.[102]

There have been several reports of new complexes formed between the less familiar metal ions and amino-acids, *viz.* e.s.r. and solution structure studies on vanadyl glycine, histidine, cysteine, proline, valine, methionine, and aspartic acid.[103] N.m.r. studies of diethylenetriaminepentaacetic acid complexes of mercury having particular interests in the possibility of hydrogen bond formation,[104] gallium–edta, nta, and amine buffers studied using ^{1}H n.m.r. spectra[105] (the abilities of ligands to bind gallium in these studies are said to correlate well with the i.r. abilities of preventing the incorporation of gallium into L1210 leukemic cells), the gold(I) and gold(III) complexes of L-cysteine and D-penicillamine have been prepared in the presence of chloride and triphenylphosphine (the anion apparently influences the type of complexes formed) and studied using u.v.-visible spectra,[106] ^{15}N-chemical shifts for imidazole derivatives in aqueous cadmium solutions,[107] and a range of di-μ-oxo, μ-oxo-μ-sulphido, and di-μ-sulphido complexes of molybdenum(V) with edta, cysteine, and cysteine ester ligands have been prepared and characterized by their electrochemical and spectral properties.[108]

The dividing line between solid state chemistry and aqueous solution chemistry is indistinct. Gelatinous metal hydroxides of titanium(IV), zirconium(IV), iron(III), vanadium(III), and tin(II) have been reported and patented

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as gelatinous hydroxides which complex with enzymes.[109] Such complexes retain the enzyme in a specific position, often still give the same enzyme activity, and can sometimes be used to give a product the pH profile of which may differ from that of the soluble form of the enzyme. This suggests that enzymes which function best at physiological pHs can possibly be adapted to function equally as well at a pH more acceptable in industrial chemistry.

The clinical success of platinum anticancer drugs has led to a plethora of papers investigating platinum and palladium ion complexes involving biological ligands such as amino-acids. Proton n.m.r. studies of palladium(II) with *S*-methyl-L-cysteine and with the parent L-cysteine have been used to study the conformation of amino-acids in these complexes at different molar ratios,[110] and solid complexes of ligands related to L-cysteine such as methionine, *S*-methylcysteine, penicillamine, glutathione, and methylsulphoniummethionine have been prepared with nickel, palladium, and rhodium ions.[111] ^{13}C-n.m.r. spectra have been reported for platinum(II) complexes of both chelated [platinum(amino-acid)(Cl_2)] and *N*-co-ordinated[platinum(amino-acid)(NH_3)$_3$]$^+$ species involving proline and pipecolic acid.[112, 113] ^{13}C-shifts can be used to make assignments of isomer structure since *cis* fused rings exhibit shifts more upfield than their *trans* fused isomers. This work is related to a previous study of *N*- and *C*-methyl substituted methylglycine complexes of platinum(II).[114] The syntheses and spectroscopic properties of *cis*-dichloro and *cis*-dibromo *N*-methylimidazole platinum(II) have been published, the dichloro-complex crystallizing in four distinct crystal modifications which all contain the *cis*-Pt($C_4H_6N_2$)$_2Cl_2$ unit, whereas the dibromo complex has but two crystalline forms, these being isomorphous with two of the dichloro forms.[114, 115] Amino-acid complexes of the (+IV) oxidation state of platinum and trimethylplatinum with α-substituted amino-acids chosen from alanine, valine, phenylalanine, and α-aminoisobutyric acid have been isolated and studied using variable temperature n.m.r.[116] Optically active co-ordination compounds involving platinum(II) with L-proline and the L-enantiomers of alanine, serine, valine, and sarcosine, have been prepared and characterized by chemical analyses, spectroscopic studies (electronic, o.r.d., ^{1}H n.m.r., and i.r.) and the rates of inversion and of deuteriation of the asymmetric nitrogen centres in the sarcosine complexes have been compared.[117]

Diffraction Studies.—Several crystallographic investigations have been carried out. L-Cysteine complexes of Hg(II) are urinary excretory products of certain mercurial diuretics,[118] and have been implicated in the transport of Hg(II)

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across membranes[119] and the binding of Hg(II) to kidney proteins.[120] The crystal structure[121] of $HgCl_2[SCH_2CH(\overset{+}{N}H_3)CO_2H]$ shows a strong, almost symmetrical sulphur bridge between tetrahedrally co-ordinated mercury atoms. The Hg–S bond lengths to the bridging cysteine molecules (2.47 Å) are distinctly shorter than the Hg–Cl bonds (2.61 Å). The above complex is converted to $Hg[SCH_2CH(\overset{+}{N}H_3)COO][SCH_2CH(\overset{+}{N}H_3)COOH]Cl,0.5H_2O$ by heating in water. In this complex[121] the mercury ion is co-ordinated to the sulphur atoms of two cysteine molecules in an approximately linear array with anionic chloride. Crystallographic studies have also been carried out[122] on methyl-DL-penicillaminato-mercury(II) monohydrate $[HgMe(pen)],H_2O$ (pen = DL-penicillaminate) and μ-DL-penicillaminato-bis[methylmercury(II)], $[Hg_2Me_2(pen)]$ prepared from appropriate amounts of methyl mercury chloride (or hydroxide) and DL-penicillamine in aqueous ethanol. In $[HgMe(pen)],H_2O$, the amino-acid, present in the zwitterionic form $\bar{S}C(Me)_2CH(\overset{+}{N}H_3)COO^-$, is co-ordinated to methylmercury *via* a deprotonated sulphydryl group and in $[Hg_2Me_2(pen)]$, the methylmercury groups are bonded to the deprotonated sulphydryl and amino groups. The crystal structures of the 1 : 1 complexes formed by methylmercury(II) with L-tyrosine and L-2-amino-4-phenylbutanoic acid have also been determined by *X*-ray diffraction,[123] as has the structure of (DL-methioninato)methylmercury(II).[124] Bis(*S*-methyl-L-cysteinato)cadmium (II) and bis(*S*-methyl-L-cysteinato)zinc(II) have been prepared and their crystal structures determined.[125] The cadmium complex is a polymer, two ligand molecules co-ordinate to the cadmium atom through *amino*-N and *carboxylate*-O. The slightly distorted octahedral geometry at the metal atom is completed by two carboxylate oxygens of neighbouring ligands. The structure is very similar to that of bis(L-methionate)cadmium(II). The zinc complex is not isostructural with the cadmium derivative, the zinc complex consists of infinite chains with a trigonal-bipyramidal geometry on zinc with three Zn-O(*carboxylate*) bonds and two Zn-N(*amino*) bonds.

The crystal structure of $K[Co(D\text{-pen})(L\text{-pen})],2H_2O$ has also been reported,[126] the ligating atoms being, S, N, and O, the isomer is the all-*cis* isomer. The purple complex formed by the reaction of copper(II) and D-penicillamine at physiological pH has been shown to be[127] an anionic Cu(I),Cu(II) cluster-complex $[Cu(II)_6Cu(I)_8(D\text{-pen})_{12}Cl]^{5-}$. The synthesis and crystal structure of the chromium(III) complex $[Cr(L\text{-His})(D\text{-pen})],H_2O$ has also been reported.[128] Angelici and co-workers[129] have synthesized the potentially tetradentate

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ligand, *N*-(*O*-hydroxybenzyl)-L-histidine (L-OHBH) and its mixed cobalt(III) complexes [L-OHBH)(AA)Co^{III}], where AA = L-, D-, or DL-alaninate or α-aminoisobutyrate. The crystal structure of [(L-OHBH)(L-Ala)Co^{III}].$2H_2O$ has been determined[129] to investigate potential stereoselective effects in such ternary complexes of optically active amino-acids. The histidine fragment of the tetradentate ligand occupies one face of the co-ordination octahedron with the phenolate oxygen of the *o*-hydroxybenzyl substituent occupying the position *trans* to the histidinate carboxyl oxygen. The bidentate L-Ala ligand is attached with its amino nitrogen *trans* to the imidazole nitrogen and its carboxylate oxygen *trans* to the secondary amino nitrogen of the tetradentate ligand.

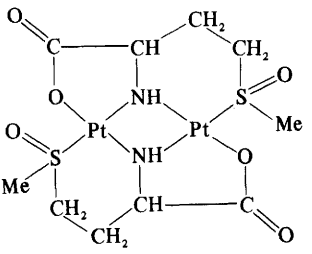
The crystal structure of the copper(II) complex of the Schiff base formed between (1*R*)-3-hydroxymethylene-camphor and (*S*)-phenylalanine has also been determined,[130] in an attempt to rationalize stereoselective effects.

Crystal structures have also been established for bis(DL-Val)calcium chloride dihydrate,[131] bis(DL-2-aminobutyric acid)calcium chloride dihydrate,[131] and bis(*N*-acetylglycinato)diaquacopper(II) dihydrate.[132] The structure of bis(β-alanine)zinc nitrate tetrahydrate has been determined and e.s.r. and optical studies of the copper(II) diluted complex carried out.[133] Structural and physico-chemical investigations of copper(II), nickel(II), cobalt(II), and cadmium(II) complexes of L-phenylalanine have also been reported.[134]

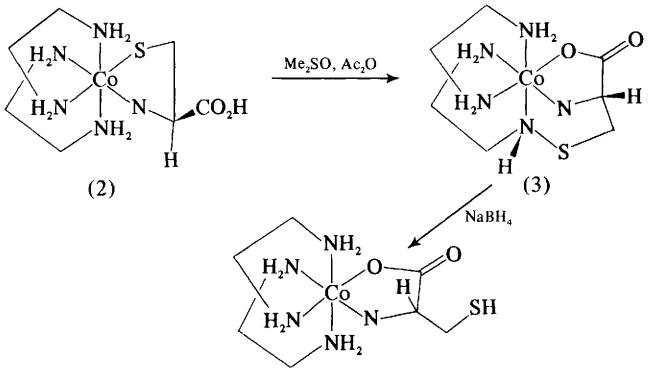
The preparation and characterization of four complexes containing equimolar proportions of platinum(II) and (*S*)-methionine *S*-oxide has been reported,[135] including the *X*-ray structure of di-μ-[2*S*, S*S*)-2-amino-4- methylsulphinylbutanoato (—2)-*S*,*O*μ-*N*]diplatinum(II). The dinuclear complex has two bridging imido nitrogens (ionized α-amino groups) from the amino-acid which link the platinum(II) centres in a four-membered ring. Each four-co-ordinate platinum atom is surrounded by nearly coplanar N, S, and O atoms from one methionine *S*-oxide ligand and the bridging imido nitrogen from the other. This complex (1) provides the first example of a double imido-bridge in a metal co-ordination compound.

N,*S*-Bound (*R*)-cysteine in Δ- and Λ-(*R*)-cysteinato-bis(ethylenediamine) cobalt(III) ions of known configuration (2) is oxidized in Me_2SO–Ac_2O to a sulphenamide (3).[136] A two electron reduction using $NaBH_4$ or $S_2O_4^{2-}$ regenerates the thiol sulphur (4) without reversing the N,O bonding arrangement. The *X*-ray structure of the cystine dimer of (4) has been published.[136]

[130] L. Casella, M. Gullotti, A. Pasini, G. Ciani, M. Manassero, and A. Sironi, *Inorg. Chim. Acta.*, 1978, **26**, L1.
[131] T. Glowiak and Z. Ciunik, *Bull. Acad. polon. Sci; Sér. Sci. chim.*, 1978, **26**, 43.
[132] M.R. Udupa and B. Krebs, *Inorg. Chim. Acta*, 1978, **31**, 251.
[133] I. Dejeht, R. Debuyst, B. Ledieu, J.P. Declercq, G. Germain, and M. Van Meerssche, *Inorg. Chim. Acta*, 1978, **30**, 197.
[134] A. Demaret, L. Abello, M. Fourati, and G. Lapluye, *J. Chem. Research(S)*, 1978, 354.
[135] W.A. Freeman, L.J. Nicholls, and C.F. Liu, *Inorg. Chem.*, 1978, **17**, 2989; W.A. Freeman, *J.C.S. Chem. Comm.*, 1977, 607.
[136] W.G. Jackson, A.M. Sargeson, and P.A. Tucker, *J.C.S. Chem. Comm.*, 1977, 199.



(1)



(2) (3) (4)

Studies are beginning to appear on iron(III) complexes of amino-acids. Thus the crystal structure of tri-μ_3-oxo-triaquohexakis(glycine)tri-iron(III) perchlorate $[Fe_3O(C_2H_5O_2N)_6,(H_2O)_3][ClO_4]_7$ has been determined.[137] The structure is similar to that of basic iron acetate $[Fe_3O(CH_3COO)_6,3H_2O]ClO_4$ with a trimeric unit having an oxygen atom at the centre. The remaining co-ordination sites of each iron are occupied by four carboxylate oxygens from bridging glycines and a single water molecule.

Several aspects of cadmium biochemistry suggest a greater similarity to Hg(II) than to Zn(II). Thus Cd(II) salts are, like their Hg(II) counterparts, exceeding toxic, with symptoms of Itai-Itai disease appearing in humans exposed to levels in excess of 250 μg per day. The mechanism of Cd^{II} poisoning may involve metal–sulphur (cysteine) bonding similar to that postulated to account for protein and enzyme inactivation by Hg^{II}. The reaction of cadmium bromide tetrahydrate with DL-penicillamine in aqueous ethanol gives aquo-(bromo)-DL-penicillaminato-cadmium(II) dihydrate. The crystal structure[138] consists of infinite chains of alternating cadmium atoms and bridging tridentate penicillamine molecules linked by asymmetric double bromide bridges. The ligand binding sites are a deprotonated sulphydryl group and two oxygens of

[137] R.V. Thundathil, E.M. Holt, S.L. Holt, and K.J. Watson, *J. Amer. Chem. Soc.*, 1977, **99**, 1818.
[138] A.J. Carty and N.J. Taylor, *Inorg. Chem.*, 1977, **16**, 177.

the carboxy-group; the amino group is protonated. A crystal structure of another Cd^{II} complex $[Cd(NH_2CH(COO)CMe_2S)].H_2O$ has also recently appeared.[139]

Square-pyramidal co-ordination is found in many copper(II)–peptide complexes. The attractive features of this geometry for copper(II)–protein–substrate interactions has been pointed out by Freeman.[155] The presence of an open axial position may allow the approach of a substrate, leading to tetragonally elongated octahedral co-ordination in the transition state, followed by the breaking of the former axial bond. The crystal structure[140] of (2,9-dimethyl-1,10-phenanthroline)(glycylglycinato)copper(II) pentahydrate establishes that the geometry about copper(II) is a five co-ordinate distorted square pyramid. The peptide ligand is tridentate *via* the amino nitrogen, ionized amide nitrogen, and the carboxylate oxygen. The distorted square about copper(II) is completed by one of the two dimethylphenanthroline nitrogens, while the other occupies the apical position of the distorted square pyramid. A similar structure has been established for the analogous phenanthroline complex.[141] An interesting feature of the dimethylphenanthroline complex is the high value of the extinction coefficient of the visible band ($\varepsilon = 313$ at 633 nm) which is considerably higher than the values ($\varepsilon \leqslant 100$) normally observed for copper(II) complexes of small peptides.

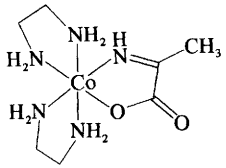
The predominant binary amino-acid complex in blood serum is Cu(II)–(L-His)$_2$, and the crystal structure of the complex Cu(L-His)(D-His).$4H_2O$ has now been reported.[142] The copper is octahedrally co-ordinated by amino and imidazole nitrogens in a square planar arrangement and by water molecules at the axial positions.

Stereochemistry and Stereoselectivity.—The rhodium(I) complex of the phosphine ligand (2*S*,3*S*)-bis(diphenylphosphino)butane acts as an efficient homogeneous hydrogenation catalyst at ambient temperature and pressure for α-*N*-acylaminoacrylic acids.[143] This catalyst gives leucine and phenylalanine in essentially complete optical purity; alanine, dopa, and tyrosine were obtained in 91, 83, and 92 % optical purity, respectively.

The stereospecific synthesis of the sulphenamide cobalt(III) complex (3) derived from (*R*)-cysteine has been described,[144] and the structure of the complex confirmed by *X*-ray crystallography. The same group[3] have also reported a stereoselective chelate and amino-acid synthesis by the reaction of CN^- with the complex (5) containing chelated 2-iminopropionate.[145]

A full account of the optical resolution of racemic α-amino-acids by formation of mixed ligand copper(II) complexes with electrostatic ligand–ligand interactions has been published.[146] The optical purities of the resolved

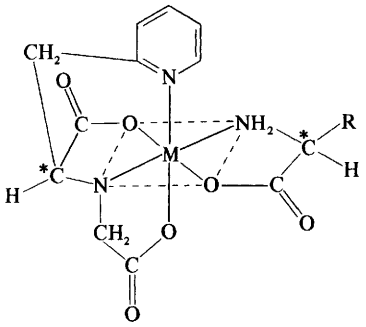
[139] See ref. 21 for reference No. 9.
[140] C.J. Simmons, M. Lundeen, and K. Seff, *Inorg. Chem.*, 1978, **17**, 1429.
[141] M.C. Lim, E. Sinn, and R.B. Martin, *Inorg. Chem.*, 1976, **15**, 807.
[142] N. Camerman, J.K. Fawcett, T.P.A. Kruck, B. Sarkar, and A. Camerman, *J. Amer. Chem. Soc.*, 1978, **100**, 2690.
[143] M.D. Fryzuk and B. Bosnich, *J. Amer. Chem. Soc.*, 1977, **99**, 6262.
[144] G.J. Gainsford, W.G. Jackson, and A.M. Sargeson, *J. Amer. Chem. Soc.*, 1977, **99**, 2383.
[145] J. Springborg, R.J. Geue, A.M. Sargeson, D. Taylor, and M.R. Snow, *J.C.S. Chem. Comm.*, 1978, 647.
[146] O. Yamauchi, T. Sakurai, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1977, **50**, 1776.



(5)

amino-acids were as high as 90 and 70 % for aspartic and glutamic acid respectively, and 40—50 % for the basic amino-acids.

The stereoselective binding of optically active amino-acids by nickel(II) and copper(II) complexes of *N*-carboxymethyl-*β*-(2-pyridyl)-L-*α*-alanine has been studied.[147] Steric crowding between the R side-chain of the amino-acid and the pyridyl group reduces the binding constants of the L-amino acidates as compared to their D-enantiomers (6). Spectrophotometric studies have provided evidence for zinc-mediated ternary complexes between ATP and aromatic amino-acids.[148] An interesting paper has appeared dealing with stereoselectivity in ion-pair formation between optically active (phenylalaninato)bis(ethylenediamine)cobalt(III) complexes and L-tartrate anion.[149] The differences in association constants are related to the elution order in column chromatography.



(6)

The synthesis of mixed-ligand cobalt(III) complexes with (*S*)-aspartic-*N*-monoacetic acid and different amino-acids has been described,[150] and a kinetic study of the interconversion of the *cis*-N and *trans*-N complexes reported.[151] The preparation of three isomers of the mixed ligand complexes of cobalt(III) with immodiacetate and L-histidinate has been described.[152] A number of chromium(III) complexes of L-*α*-amino-acids have been prepared,[153] all the tris complexes have a *fac*-structure.

[147] S.A. Bedell, P.R. Rechani, R.J. Angelici, and R. Nakon, *Inorg. Chem.*, 1977, **16**, 972.
[148] J.J. Toulme, *Bioinorg. Chem.*, 1978, **8**, 319.
[149] T. Taura, H. Tamada, and H. Yoneda, *Inorg. Chem.*, 1978, **17**, 3127.
[150] G. Colomb and K. Bernauer, *Helv. Chim. Acta*, 1977, **60**, 459.
[151] G. Colomb and K. Bernauer, *Helv. Chim. Acta*, 1977, **60**, 468.
[152] M. Watabe, M. Zamba, and S. Yoshikawa, *Bull. Chem. Soc. Japan*, 1978, **51**, 1354.
[153] H. Oki, *Bull. Chem. Soc. Japan*, 1977, **50**, 680.

Reactivity and Kinetics.—A number of interesting papers have appeared in this area. The catalytic dephosphorylation of *O*-phosphoserine by glyoxylate ion and copper(II) has been studied,[154] and the interaction of a number of amino-acid phosphate esters with a variety of metal ions investigated by potentiometry and by ^{31}P n.m.r.[83]

The activation of glycine methylene hydrogens by co-ordination to cobalt(III) has been known for some years.[156] Norman and Phipps[157] have observed different hydrogen–deuterium exchange rates for the three stereochemically different glycinato ligands of *mer*-[$Co(gly)_3$], and this behaviour has been rationalized.[157, 158] The comparative effects of charge and ligand structure on the activation of amino-acids co-ordinated to cobalt(III) has been discussed,[159] as has the origin of the stereoselectivity observed in the condensation of acetaldehyde with glycine co-ordinated to cobalt(III).[160] The hydroxide ion-catalysed epimerization of chelated α-amino-acids in cobalt(III) complexes has been reviewed.[161]

The hydrolysis of amino-acid esters in the co-ordination sphere of metal ions continues to be an active research field. The base hydrolysis of *cis*-[$CoCl(en)_2(NH_2(CH_2)_3CO_2Me)]^{2+}$ has been studied by pH-stat and stopped-flow spectrophotometry.[162] Ester hydrolysis is *ca.* 7-times faster in the complex compared with the unprotonated ester, and lactamization to 2-pyrrolidinone does not occur with the complexed ester. The second order rate constants, rate = k_{OH} [(CuL)$(GlyOMe)^{x+}$][OH^-] for the hydrolysis of a series of mixed complexes of methyl glycinate have been determined. Both the strength of the donor atoms of the auxiliary ligands and the charge on the metal complex are believed to be important in determining the catalytic activity of the various copper(II) complexes.[163] A correlation between the stability constant of CuL^{2+} and the base hydrolysis rates of [$CuL(glyOMe)^{x+}$] complexes has been noted.[164] The higher the value of the formation constant, the lower the base hydrolysis rate. Second order rate constants, rate = k_{OH}[$M(NTA)GlyOMe^-$][OH^-] have also been determined[165] for the base hydrolysis of several mixed complexes of methylglycinate with divalent metal nitrilotriacetates [$M(NTA)^-$]. The catalytic activity is due primarily to polarization of the carbonyl group of the ester by inductive withdrawal *via* the amino group. The effect of metal ions on the hydrolysis of L-asparagine by L-asparaginase has also been investigated.[166] A detailed study of the intramolecular hydration of nitriles in complexes of the

[154] M.S. Mohan and E.H. Abbott, *Inorg. Chem.*, 1978, **17**, 3083.
[155] H.C. Freeman, *Adv. Protein. Chem.*, 1967, **22**, 257.
[156] D.H. Williams and D.H. Busch, *J. Amer. Chem. Soc.*, 1965, **87**, 4644.
[157] P.R. Norman and D.A. Phipps, *Inorg. Chim. Acta*, 1977, **24**, L35.
[158] U. Sakaguchi, H. Yoneda, and Y. Monimoto, *Inorg. Chim. Acta*, 1978, **31**, L473.
[159] P.R. Norman and D.A. Phipps, *Inorg. Chim. Acta*, 1978, **28**, L161.
[160] D.A. Phipps, *Inorg. Chim. Acta.*, 1978, **27**, L103.
[161] S. Yoshikawa, M. Saburi, and M. Yamaguchi, *Pure Appl. Chem.*, 1978, **50**, 915.
[162] R.W. Hay, R. Bennett, and D.P. Piplani, *J.C.S. Dalton*, 1978, 1046.
[163] J.K. Walker and R. Nakon, *Inorg. Chem.*, 1978, **17**, 1151.
[164] R.D. Wood, R. Nakon, and R.J. Angelici, *Inorg. Chem.*, 1978, **17**, 1088.
[165] D.E. Newlin, M.A. Pellack, and R. Nakon, *J. Amer. Chem. Soc.*, 1977, **99**, 1078.
[166] A.J. Charlson, A.J. Coman, T.A. Karosssi, F.S. Stephens, R.S. Vagg, and E.C. Watton, *Inorg. Chim. Acta*, 1978, **28**, 217.

type *cis*-$[Co(en)_2(NH_2CH_2CN)X]^{2+}$ (X = Cl or Br) to give $[Co(en)_2(glyNH_2)]^{3+}$ and $[Co(en)_2(NH_2CH_2CONH)]^{2+}$ has been published.[167] The rate of inversion of sulphur in cobalt(III)-thioether complexes containing (*R*)-cysteine, cysteamine, and their *S*-methylated derivatives has been studied.[168] Sulphur inversion is slow on the n.m.r. time scale at 25 °C ($k_{inv} < 10\ s^{-1}$). A kinetic investigation of the oxidation of (cysteinato-*N,S*)bis(ethylenediamine)cobalt(III) and related thiolo complexes to sulphenatocobalt(III) complexes has been published.[169]

Metal ion catalysis of the reduction of the C=N bond has implications for the mechanism of action of glutamate dehydrogenase, and a number of α-amino-acid dehydrogenase models involving magnesium have been developed.[170]

There has been considerable interest in redox reactions of metal complexes containing amino-acids. The Cr^{II} reductions of the three complexes $[Co(en)_2(Met)]^{2+}$, $[Co(en)_2(Cys(Me))]^{2+}$, and $[Co(en)_2(Cys)]^{2+}$(Cys(Me) = *S*-methyl-L-cysteine) has been studied kinetically.[171] The *cis*-chloroaquo tetra-ammine cobalt(III) ion reacts with the α-amino-acids, Gly, Ala, Val, Ile, Lys, Arg, and His to give the amino-acid substituted tetrammine cobalt(III) complexes. Kinetic studies[172] have shown that the reduction by Cr^{II} proceeds by an inner sphere bridge mechanism in which the amino-acid is transferred from cobalt to chromium. The kinetics of the Cr^{II} reductions of complexes of the type $[Co(en)_2(AA)]^{2+}$ (AA = Gly.O, DL-Ala.O, DL-Phe.O, and DL-Leu.O) have also been investigated.[173] The mechanism of the Cr^{II}, V^{II}, and $Ru(NH_3)_6^{2+}$ reductions of *N*-bonded glycinato- and β-alaninatopenta-amminecobalt(III) complexes has been shown to involve precursor complex formation with outer-sphere electron transfer.[174]

Copper(II) ions have been found to catalyse the oxidation of lysine by alkaline chloramine-T.[175] The isomerization, exchange, and ring-closure reactions of glycinate complexes of *cis*-dimethylplatinum(IV) has been studied,[176] as has the kinetics of reaction of imidazole, glycine, and L-histidine with the aquapentacyanoferrate(II) ion.[177] The reaction of nickel(II)-*N,N'*-diglycylethylene diamine with ethylenediaminetetra-acetate has been studied kinetically.[178]

Schiff Bases.—A steady interest is being maintained in this area. Metal-catalysed racemization of amino-acids co-ordinated as Schiff bases has been well studied, the original work of Snell having stimulated effort in many analogous systems. These studies have generally utilized an aromatic aldehyde

[167] D.A. Buckingham, P.J. Morris, A.M. Sargeson, and A. Zanella, *Inorg. Chem.*, 1977, **16**, 1910.
[168] W.G. Jackson and A.M. Sargeson, *Inorg. Chem.*, 1978, **17**, 2165.
[169] D.L. Herting, C.P. Sloan, A.W. Cabral, and J.H. Krueger, *Inorg. Chem.*, 1978, **17**, 1649.
[170] U.K. Pandit, M.J. de Nie-Sarink, A.M. van der Berg, J.B. Steevens, and R.F.M. van Dokkum, *Rev. Trav. chim.*, 1978, **97**, 121.
[171] R.J. Balahura, N.A. Lewis, *Inorg. Chem.*, 1977, **16**, 2213.
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[173] R.D. Williams and D.E. Pennington, *J. Coord. Chem.*, 1978, **7**, 187.
[174] C.S. Glennon, J.D. Edwards, and A.G. Sykes, *Inorg. Chem.*, 1978, **17**, 1654.
[175] B.S. Yadav, D.R. Singh, and G. Chandra, *J. Inorg. Nuclear Chem.*, 1978, **40**, 161.
[176] N.H. Agnew, T.G. Appleton, and J.R. Hall, *Inorg. Chim. Acta*, 1978, **30**, L343.
[177] H.E. Toma, J.M. Martins, and E. Giesbrecht, *J.C.S. Dalton*, 1978, 1610.
[178] R. Pearson and G.K. Pagenkopf, *Inorg. Chem.*, 1978, **17**, 1799.

derived either from pyridoxal or salicylaldehyde. Gillard and O'Brien[179] have, however, shown that aqueous solutions containing copper(II), pyruvate, and L(+)-alanine in the molar ratio 1 : 2 : 4 lead to rapid racemization of alanine in the presence of base. Schiff bases of pyridoxal and amino-alkyl phosphonic acids and their complexes with metal ions have been studied spectrophotometrically.[180] A model for an intermediate in pyridoxal catalysed γ-elimination and γ-replacement reactions of amino-acids has been discussed,[181] and catalysis of the β-elimination of *O*-phosphoserine and β-chloroalanine by pyridoxal and zinc(II) studied.[182]

A reinvestigation[183] of the reaction between iron(II), glycine, and salicylaldehyde has shown that the product is hexa-aquoiron(II) bis(*N*-salicylideneglycinato)ferrate(III) trihydrate and not a binuclear iron(III) complex containing a bridging glycinato ligand.

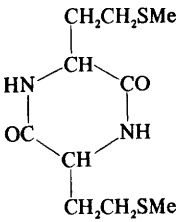
The crystal structure of *catena*-μ-(*N*-salicylidene-L-tyrosinato-*O*,*O'*)copper(II) has been reported.[184] The magnetic and spectroscopic properties of complexes of copper(II) and nickel(II) with *N*-salicyclidenearginine have been described,[185] as have the magnetic properties of (*N*-salicylideneglycinato) aquocopper(II) hemihydrate and (*N*-salicylidene-α-aminoisobutyrato) aquocopper(II).[186]

3 Peptides

Structural Aspects.—This continues to be an active research area. The complexes of a number of transition metals with *cyclo*-L-methionyl-L-methionine (7) have been prepared and their ^{1}H n.m.r. spectra investigated.[187] Spectral and magnetochemical studies of aquo-μ-bis [cyclo-(L-histidyl-L-histidyl)] dicopper(II) have been reported.[188] The complex contains a pseudo-tetrahedral $Cu^{II}N_4$ unit which is joined by two deprotonated amide bridges to a square-pyramidal $Cu^{II}N_2O_3$ unit. McLendon and Martell[189] have suggested that copper(II)-thioether bonding may be of importance in some blue copper-proteins. The first direct evidence for copper(II)-thioether bonding in complexes of methionine derivatives has been reported.[190] Such complexes are only stable up to pH 4 at low temperatures; at higher pH values thioether sulphur cannot compete with nitrogen donors.

Polarographic and spectroscopic studies of the copper(II)–glutathione system in basic solution have been reported[191] as have spectroscopic and n.m.r.

[179] R.D. Gillard and P. O'Brien, *J.C.S. Dalton*, 1978, 1444.
[180] M.F. Langohr and A.E. Martell, *J. Inorg. Nuclear Chem.*, 1978, **40**, 149.
[181] Y. Karube and Y. Matsushima, *J. Amer. Chem. Soc.*, 1977, **99**, 7356.
[182] K. Tatsumoto and A.E. Martell, *J. Amer. Chem. Soc.*, 1977, **99**, 6082.
[183] F.L. Bowden, R.P. Carpenter, R.V. Parish, and R.D. Pollock, *Inorg. Chim. Acta*, 1977, **23**, 35.
[184] R. Hämaläinen, M. Ahlgren, U. Turpeinen, and M. Rantala, *Acta Chem. Scand.*, 1978, **A32**, 235.
[185] S.-T. Chow, D.M. Johns, C.A. McAuliffe, and D.J. Machin, *Inorg. Chim. Acta.*, 1977, **22**, 1.
[186] W.E. Estes and W.E. Hatfield, *Inorg. Chem.*, 1978, **17**, 3226.
[187] R. Ettore, V. Guantieri, A.M. Tamburro, and L. de Nardo, *Inorg. Chim. Acta.*, 1979, **32**, L39.
[188] H.J. Schugar, T.G. Fawcett, D.N. Hendrickson, and T.R. Felthouse, *Inorg. Chem.*, 1978, **17**, 2707.
[189] G. McLendon and A.E. Martell, *J. Inorg. Nuclear Chem.*, 1977, **39**, 191.
[190] H. Kozlowski and T. Kowalik, *Inorg. Nuclear Chem. Letters*, 1978, **14**, 201.
[191] M. Ostern, G. Formicka-Kozlowska, B. Jezowska-Trzebiatowska, and H. Kozlowski, *Inorg. Nuclear Chem. Letters*, 1978, **14**, 351.



(7)

investigations on Ni^{II}, Cu^{II}, and Pd^{II} complexes of Gly-Leu-Tyr and Tyr-Gly-Gly.[192]

The new tripeptides *N*-mercaptoacetylglycyl-L-histidine (MAGH) and *N*-mercaptoacetyl-DL-histidyl-DL-histidine (MAHH) have been prepared. They form very stable complexes with copper(II).[193] The e.s.r. spectra of the 1 : 1 copper(II) complexes of MAGH and MAHH show the seven and nine lines of nitrogen superhyperfine splitting, indicating clearly the co-ordination of three and four nitrogen atoms toward copper(II), respectively. Square-planar and square-pyramidal stereochemistries are suggested for the MAGH and MAHH complexes. In addition ^1H n.m.r. of the 1 : 1 copper(I) complexes of the tripeptides establish that the sulphydryl and imidazole groups are involved in co-ordination.

Oxidation of nickel(II)-oligopeptides in aqueous solution gives paramagnetic nickel(III) complexes. E.s.r. spectra are consistent with nickel(III) in a tetragonal geometry with four donor-groups from the oligopeptide in the equatorial plane and two axially bound water molecules.[194] Nickel(III)-glycylglycylglycinamide forms two complexes with ammonia in which NH_3 replaces first one and then the second axially bound water molecules. Nickel(III) complexes with five or more nitrogen donors are slower to undergo self-decomposition reactions.

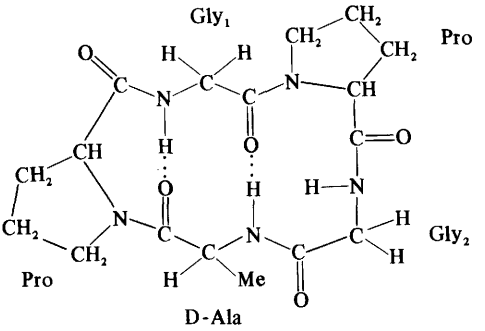
The 'blue' copper-proteins, which occur widely in nature have attracted considerable attention as a result of their interesting properties. The distinctive features of 'blue' copper centres are unusually high extinction coefficients near 600 nm ($\varepsilon = 1000$—5000), anomalously small copper hyperfine coupling constants and markedly positive copper redox potentials. Sugiura and Hirayama[195] have prepared a number of copper(II) and nickel(II) complexes of the peptides *N*-mercaptoacetyl-L-histidine (MAH) and 2-mercaptopropionyl-L-cysteine. The 1 : 1 MAH–Cu(II) complex has λ_{max} 600 nm ($\varepsilon = 830$) and a small copper hyperfine coupling constant. The model studies suggest that the 'blue' copper sites involve cysteine sulphydryl and histidine imidazole donors and that the 600 nm band is due to a $\sigma S \rightarrow d(Cu)$ charge transfer transition.

The bis(dipeptidato)cobaltate(III) complexes have been known for several years, however, the absolute configuration of these complexes is at present

[192] H. Kozlowski, *Inorg. Chim. Acta*, 1978, **31**, 135.
[193] Y. Sugiura, *Inorg. Chem.*, 1978, **17**, 2176.
[194] A.G. Lappin, C.K. Murray, and D.W. Margerum, *Inorg. Chem.*, 1978, **17**, 1630.
[195] Y. Sugiura and Y. Hirayama, *J. Amer. Chem. Soc.*, 1977, **99**, 1581.

unknown. Gillard and Mitchell[196] have observed, using ^{1}H n.m.r., that in one diastereoisomer of $[Co(L\text{-Phe-Gly})_2]^-$ both of the methylene protons of the *C*-terminal CH_2 group are shifted upfield by the ring current of the aromatic ring, while in the other diastereoisomer only one of the protons is shifted upfield. An examination of molecular models allows the absolute configuration to be determined. The technique should be applicable to many other complexes.

The aqueous solution equilibria of the diastereoisomeric dipeptides L-Ala-L-Ala, D-Ala-L-Ala, L-Leu-L-Tyr, and D-Leu-L-Tyr and their copper(II) and nickel(II) have been studied potentiometrically. The hydrophobic nature of the side-chains are regarded as being important in determining the conformations and equilibria of the diastereoisomeric complexes.[197]



(8)

The cyclic pentapeptide (8) *cyclo*-(Gly$_1$-Pro-Gly$_2$-D-Ala-Pro) has been synthesized.[198] The peptide has been found to bind the divalent cations Mg^{II}, Mn^{II}, Ca^{II}, Ba^{II}, and Li^{I} strongly, and thus establishes a new class of cyclic peptide complexing agents, *viz.* the cyclic pentapeptides. Complexes with the stoicheiometry 1 peptide : 1 cation, and 1 peptide : 2 cations have been detected. The application of physical techniques to the study of metallopeptides is becoming very common. Thus ^{1}H n.m.r. spectroscopy has been used to study palladium(II) complexes of Gly-L-Phe and L-Phe-Gly,[199] the zinc(II) and cadmium(II) complexes of Leu-Gly,[200] cobalt(II)–glutathione complexes[201] nickel(II)–glutathione complexes,[202] palladium(II)–glycyl-L-tryosine,[203] silver(I) interactions with glycyl-L-methionine and L-methionyl-

[196] R.D. Gillard and P.R. Mitchell, *J.C.S. Chem. Comm.*, 1978, 428.
[197] A. Kaneda and A.E. Martell, *J. Amer. Chem. Soc.*, 1977, **99**, 1586.
[198] L.G. Pease and C. Watson, *J. Amer. Chem. Soc.*, 1978, **100**, 1279.
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[200] B. Jezowska-Trzebiatowska, L. Latos-Grazynski, and H. Kozlowski, *J. Inorg. Nuclear Chem.*, 1977, **39**, 1269.
[201] B. Jezowska-Trzebiatowska, G. Formicka-Kozlowska, and H. Kozlowski, *Bull. Acad. polon. Sci., Sér. Sci. chim.*, 1976, **24**, 987.
[202] B. Jezowska-Trzebiatowska, L. Latos-Grazynski, and H. Kozlowski, *Chem. Phys. Letters*, 1976, **42**, 242.
[203] H. Kozlowski and M. Jezowska, *Chem. Phys. Letters*, 1977, **47**, No. 3.

glycine.[204] Combined n.m.r. and e.p.r. studies of copper(II)–glutathione,[205] nickel(II) and copper(II) complexes of Thr-Lys-Ala-Ala[206] and palladium(II) and copper(II) complexes of L-Ala-L-Ser[207] have been reported.

The c.d. spectra of copper(II) complexes of poly-L-lysine, poly-L-ornithine and poly-L-diaminobutyric acid have been studied[208] as models for copper(II)-proteins. Further c.d. spectral studies have been reported for copper(II) and nickel(II) complexes of tetra- and pentapeptides.[209] Raman spectral evidence for phenolate co-ordination in copper(II)-poly(L-lysine, L-tyrosine) has been given.[210]

An interesting development has been the use of volume effects to study the interaction of copper(II) with dipeptides.[211] A volume increase of *ca.* 11 cm^3 mol^{-1} results from the binding of copper(II) to the terminal amino-group and the carbonyl oxygen of the peptide, while a volume increase of *ca.* 20 cm^3 mol^{-1} results when the copper is bound to the terminal amino-group, the peptide nitrogen and carboxylate oxygen of the peptide. The technique may be of value in studying metal–protein interactions. The influence of copper(II) on proton nuclear relaxation rates of poly-L-histidine has been studied,[212] the complex is an effective catalyst for the oxidation of ascorbic acid, *p*-hydroquinone, and several other organic substrates. Mössbauer studies of $R_2Sn(Gly\text{-}Gly)$ (R = Ph, Me, Bu^n, or Oct^n) have been described.[213]

The co-ordination of adenosine and ATP to the palladium(II) complex of glycyl-L-aspartic acid has been studied. At intermediate pH values the Pd^{II} favours *N*1 over *N*7 co-ordination in both the nucleoside and nucleotide.[214] The preparation and oxidation of manganese(II) complexes of L-cysteine and some dipeptides has been described,[215] and the nickel(II) complex of 2-mercaptopropionylglycinamide studied.[216]

A new reagent 3,4-dicarboxyphenylisothiocyanate reacts specifically at the *N*-terminus of a peptide or a protein to give a dicarboxythiocarbamyl derivative.[217] The adjacent carboxy-groups serve as a strong binding-site for lanthanides at pH5; the resulting complexes will be of value for structural n.m.r. studies.

The crystal structure of L-asparaginato-D-aspartato-cobalt(III) monohydrate has been determined, the amide group of the L-asparaginate ligand is co-

[204] B. Jezowska-Trzebiatowska, T. Kowalik, and H. Kozlowski, *Bull. Acad. polon. Sci., Sér. Sci. chim.*, 1977, **25**, 797.
[205] B. Jezowska-Trzebiatowska, G. Formicka-Kozlowska, and H. Kozlowski, *J. Inorg. Nuclear Chem.*, 1977, **39**, 1265.
[206] G. Formicka-Kozlowska, H. Mozlowski, and B. Jezowska-Trzebiatowska, *Inorg. Chim. Acta.*, 1977, **25**, 1.
[207] H. Kozlowski and Z. Siatecki, *Chem. Phys. Letters*, 1977, **54**, 498.
[208] M. Palumbo, A. Cosani, M. Terbojevich, and E. Peggion, *J. Amer. Chem. Soc.*, 1977, **99**, 939.
[209] J.J. Czarnecki and D.W. Margerum, *Inorg. Chem.*, 1977, **16**, 1997.
[210] L. Tosi and A. Garnier, *Inorg. Chim. Acta*, 1978, **29**, L261.
[211] S. Katz and R.G. Shinaberry, *Bioinorg. Chem.*, 1978, **8**, 215.
[212] R.E. Wasylishen and J.S. Cohen, *J. Amer. Chem. Soc.*, 1977, **99**, 2480.
[213] R. Barbieri, L. Pellerito, and F. Huber, *Inorg. Chim. Acta*, 1978, **30**, L321.
[214] H. Kozlowski, *Inorg. Chim. Acta*, 1977, **24**, 215.
[215] H. Gunter and K.E. Schwarzhaus, *Z. Naturforsh.*, 1976, **31B**, 448.
[216] Y. Sugiura, T. Takeyama, and H. Tanaka, *Chem. Letters*, 1976, **5**, 491.
[217] J.H. Bradbury, J.R. Howell, R.N. Johnson, and B. Warren, *European J. Biochem.*, 1978, **84**, 503.

ordinated through the nitrogen atom of the iminol tautomer.[218] The copper(II) complexes of *N*-acetyl-L-alanine and their interaction with monodentate heterocyclic amines has been reported.[219] Interest in copper(III) complexes of peptides continues. Electrode potentials have been determined for 40 Cu^{III}–Cu^{II} peptide couples (including peptide amides) in aqueous solution.[220] The potentials are very sensitive to the ligand and span a range from 1.02 to 0.45V. The values of E^0 decrease with an increase in the number of deprotonated-peptide groups. The triple deprotonated peptide complexes and the highly *C*-substituted tripeptide complexes of copper have effective potentials at physiological pH such that O_2 oxidation to copper(III) is thermodynamically feasible.

The influence of alkyl side-chains with hydroxy- or thioether-groups on the stability of binary and ternary copper(II)–dipeptides has been investigated potentiometrically by Siegel and co-workers,[221] and the possible structures of the binary and ternary complexes discussed.

Reactivity.—A steady number of papers continues in this area. Molecular oxygen reacts with nickel(II)–peptide complexes in aqueous solution by a facile autocatalytic process in which nickel(III) intermediates are involved.[222] With tetraglycine the formation of Ni^{III} $(H_{-3}G_4)^-$ initiates the reaction which results in the formation of carbon dioxide, triglycyl-*N*-(hydroxymethyl)amide, and glycinamide as the main products. The *N*-(hydroxymethyl)amide produced can further hydrolyse to give triglycinamide and formaldehyde. Oxygen also reacts[223] with copper(II) tetraglycine (G_4) solutions at pH 7—9 to give Cu^{III} $(H_{-3}G_4)$– (where H_{-3} refers to three deprotonated peptide nitrogens co-ordinated to copper) as an intermediate leading to the oxidation of the peptide. A dehydropeptide which hydrolyses to diglycinamide and glyoxylglycine is the major product of the peptide oxidation.

The tripeptide glycylglycyl-L-histidine contains the same potential metal binding-sites as the terminal portion of serum albumin. As a result there has been a considerable volume of research on the interactions of copper(II) ions with this tripeptide. A recent crystallographic study[224] of the *N*-methylamide derivative of the complex confirmed the earlier suggestion that the complex has a square-planar structure with the copper co-ordinated to four nitrogens of the peptide. It has now been observed[225] that the reaction of copper(II) hydroxide with glycylglycyl-L-histidine in aqueous solution leads to oxidative decarboxylation to give the α,β-didehydroglycylglycylhistaminatocopper(II) dihydrate, and the structure of this complex has been confirmed by *X*-ray crystallography.[225] The central copper(II) atom is square planar, co-ordinated to four nitrogen atoms of the ligand.

[218] M. Sekizaki, *Bull. Chem. Soc. Japan*, 1978, **51**, 1991.
[219] G. Marcotrigiano, L. Menabue, and G.C. Pellacani, *J. Inorg. Nuclear Chem.*, 1978, **40**, 165.
[220] F.P. Bossu, K.L. Chellappa, and D.W. Margerum, *J. Amer. Chem. Soc.*, 1977, **99**, 2195.
[221] H. Sigel, C.F. Naumann, B. Prijs, D.B. McCormick, and M.C. Falk, *Inorg. Chem.*, 1977, **16**, 790.
[222] F.P. Bossu, E.B. Paniago, D.W. Margerum, S.T. Kirksey, jun., and J.L. Kurtz, *Inorg. Chem.*, 1978, **17**, 1034.
[223] J.L. Kurtz, G.L. Burce, and D.W. Margerum, *Inorg. Chem.*, 1978, **17**, 2454.
[224] N. Camerman, A. Camerman, and B. Sarkar, *Canad. J. Chem.*, 1976, **54**, 1309.
[225] P. de Meester and D.J. Hodgson, *Inorg. Chem.*, 1978, **17**, 440.

The use of transition metal ions to catalyse the formation of amino-acids and peptides from such starting materials as formaldehyde and hydroxylamine has been described.[226] The amino-acids Gly, Ala, Ser, Glu, Asp, Pro, and β-Ala were detected and peptides of mol.wt. 200—1000 produced.

The reaction of triethylenetetramine with a series of deprotonated nickel(II)-dipeptideamide–cyanide complexes has been studied kinetically,[227] and the steric effects of methyl substitution assessed.

The nucleophilic displacement reactions of bis(triglycinato)cuprate(II) and bis(glycinamide)-copper(II) by triethylenetetramine have also been the subject of a kinetic investigation.[228] Further kinetic work deals with the kinetics of protonation of palladium(II)–tripeptide complexes. The dissociation begins at the carboxylate end of the ligand and proceeds *via* intermediates where rapid protonation of the peptide oxygens occurs prior to Pd–N (peptide) bond cleavage.

Cobalt–dioxygen complexes have received considerable attention as models for biological oxygen carriers. A recent paper[229] discusses the complexation equilibria and oxygenation equilibria of 1 : 1 cobalt(II) complexes of Gly-L-His, Gly-L-Asp, glycylhistamine, L-His-Gly, L-Asp-Gly, and L-His-L-His. A further paper[230] deals with the oxidative rearrangement of binuclear cobalt dioxygen complexes derived from dipeptide ligands to give mononuclear cobalt(III) complexes. This paper provides a useful summary of the current state of knowledge in this still rather contentious area.

4 Proteins

The field of metal ion–protein interactions still continues to flourish and attract the attentions of major research groups. There has been a general review on the chemical synthesis of peptides and proteins, both being potent chelators for a wide range of metal ions.[231] A second review discusses the detoxification of molluscs and crustacea exposed to heavy-metal-ion poisons; this proposes a mechanism whereby certain proteins co-precipitate with the heavy metals as intracellular granules.[232] There have been other reviews of the metal complexing characteristics of proteins[233] and protein–metal complexes involved in nitrogen fixation.[234]

Sarkar has discussed the concept of molecular design in relation to the specific metal-binding sites of proteins and of enzymes[235] and the field of electron transfer has attracted three research groups *viz.* a dissertation on metalloprotein electron transfer mechanism,[236] a discussion of the electrochemical behaviour of proteins containing co-enzyme groups and a variety of

[226] F. Egami, Proceedings of the I.C.C.C. Meeting, Prague, 1978, p. 199.
[227] G.K. Pagenkopf and W.A. Marchese, *J. Coord. Chem.*, 1978, **7**, 249.
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[229] W.R. Harris and A.E. Martell, *J. Amer. Chem. Soc.*, 1977, **99**, 6746.
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[235] B. Sarkar, *Jerusalem Symp. Quantum Chem. Biochem.*, 1977, **9**, 193 (*Chem. Abs.*, **87**, 79 706).
[236] S.E. Wherland, *Diss. Abs.*, 1977, **37**, 5075.

metals,[237] and an overview of electron transfer mechanism employed by proteins.[238]

Although the general field of using models to understand the metal binding of proteins is replete with pitfalls, in skilled hands useful information can be procured. Recent resonance Raman spectra of cobalt(II)–imidazole complexes have been used as analogues for the binding site of cobalt-substituted zinc proteins to establish fundamental trends in the visible and u.v. spectra for correlation with proteins studied.[239] Similarly, models which use rare-earth elements as substitutes for the biometals have been used to investigate the europium-binding site of a range of proteins as determined from luminescence spectra.[240] Tungsten and molybdenum have also been used in such model experiments.[241]

Heme proteins, as always, have attracted a great deal of research attention. A range of synthetic hemopolymers which mimic the reversible binding of molecular oxygen in hemoglobin and myoglobin have been composed and these seem to function well at physiological pH and temperature.[242] Previously reported model complexes containing heme functioned only at low temperatures or in non-physiological media. The presence of both zinc and manganese ions complexing to hemoglobin are reported to increase the oxygen affinity by up to 43 %. Radioisotope studies have shown that zinc(II) forms a 1 : 1 complex with carboxyhemoglobin having an equilibrium constant of 5.0×10^5 $mol^{-1}dm^3$ at room temperature. The site of the metal-ion binding to hemoglobin is unspecified.[243]

Details of the characterization of the electrochemical behaviour of the prosthetic group of hemoproteins have been published.[244] This work refers to 2,4-substituents influencing the redox behaviour of iron porphyrins. N.m.r. studies of hemoproteins continue to be investigated and there is a report of a pH dependence of the ferric horseradish peroxidase and horse heart myoglobin.[245] Similar studies on the hemoprotein P450 have appeared.[246] The structural chemistry of hemerythrin[247] and the electron spin relaxation times of iron(III) in high-spin heme-proteins have been studied.[248]

Cytochrome *c*, the electron transfer agent involved in the respiratory chain consists of an iron protoporphyrin IX unit co-ordinated to imidazole and to sulphide residues in both the reduced and oxidized forms. There is considerable interest in the redox properties of such molecules and Buckingham has been studying such phenomena by constructing simple model

[237] B.A. Kuzhetsov, N.M. Mestechkma, and G.P. Shumakovich, *Bioelectrochem. Bioenerg.*, 1977, **4**, 1.
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[243] I.-I. Wu, M.L. Borke, and N.C. Li, *J. Inorg. Nuclear Chem.*, 1978, **40**, 745.
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[245] I. Izuka, S. Ogawa, T. Inubishi, I. Yonezawa, and I. Morishima, *F.E.B.S. Letters*, 1976, **64**, 156.
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porphyrins containing various sulphur ligands such as sulphide, sulphoxide, and sulphone groupings covalently attached to tetraphenylporphins. Their binding to the iron(II) derivatives were found to vary.[249] In similar vein two papers concerning the reduction of cytochrome *c* by hydroxo(L-cysteinato)-molybdate and properties of vanadium-substituted hemeproteins have appeared.[250, 251]

There have been four important papers concerning myoglobin: the *X*-ray structure of myoglobin has now been refined to 2.0 Å resolution, the crystals being obtained from sperm-whale metmyoglobin,[252] the reduction by dithionite of iron(III) myoglobin using different ligands attached to the iron atom has been scrutinized using rapid wavelength scanning stopped-flow spectrophotometry,[253] and also carbon monoxide derivatives of ruthenium(II) myoglobin have been used as probes of the hemeprotein conformation in this macromolecule.[254]

The field of iron–sulphur cluster complexes attracts a great deal of interest. Much of the research in this area has originated from Holm's laboratory and has recently been reviewed.[255] Detailed compatibility studies concerning synthetic iron–sulphur clusters and those occurring in non-haem iron proteins have been made, one of the most useful applications of work on synthetic cluster compounds being the core-extrusion technique for the identification of iron sites within proteins. There has been a recent report of cores from the active site of ferredoxins and the hydrogenase of *Clostridium pasteurianum*.[256] These studies led on to synthetic analogues of the active sites of iron–sulphur proteins which were then investigated using comparative polarographic potentials of the $[Fe_4S_4(SR)_4]^{2-}$ and $^{3-}$ clusters and of *Clostridium pasteurianum* ferredoxin redox couples.[257]

When the electron transfer reactivity of spinach ferredoxin was investigated using kinetic approaches and Marcus-type analysis, it was found that electron transfer from ferredoxin to ferricytochrome *c* is a particularly inefficient process.[258] This paper also reports the influence of fluoride upon the rates. Low temperature magnetic c.d. studies of iron–sulphur proteins related to oxidized rubredoxin have appeared and analogies drawn with 2-iron ferredoxins.[259, 260] These should be read in conjunction with two general reviews on iron–sulphur proteins.[261, 262]

[249] D.A. Buckingham and T.B. Rauchfuss, *J.C.S. Chem. Comm.*, 1978, 705.
[250] J.T. Spence and G.D. Lawrence, *J. Inorg. Nuclear Chem.*, 1978, **40**, 2101.
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[260] A.J. Thomson, R. Cammack, D.O. Hall, K.K. Rao, B. Briat, J.C. Rivoal, and J. Badoz, *Biochim. Biophys. Acta*, 1977, **493**, 132.
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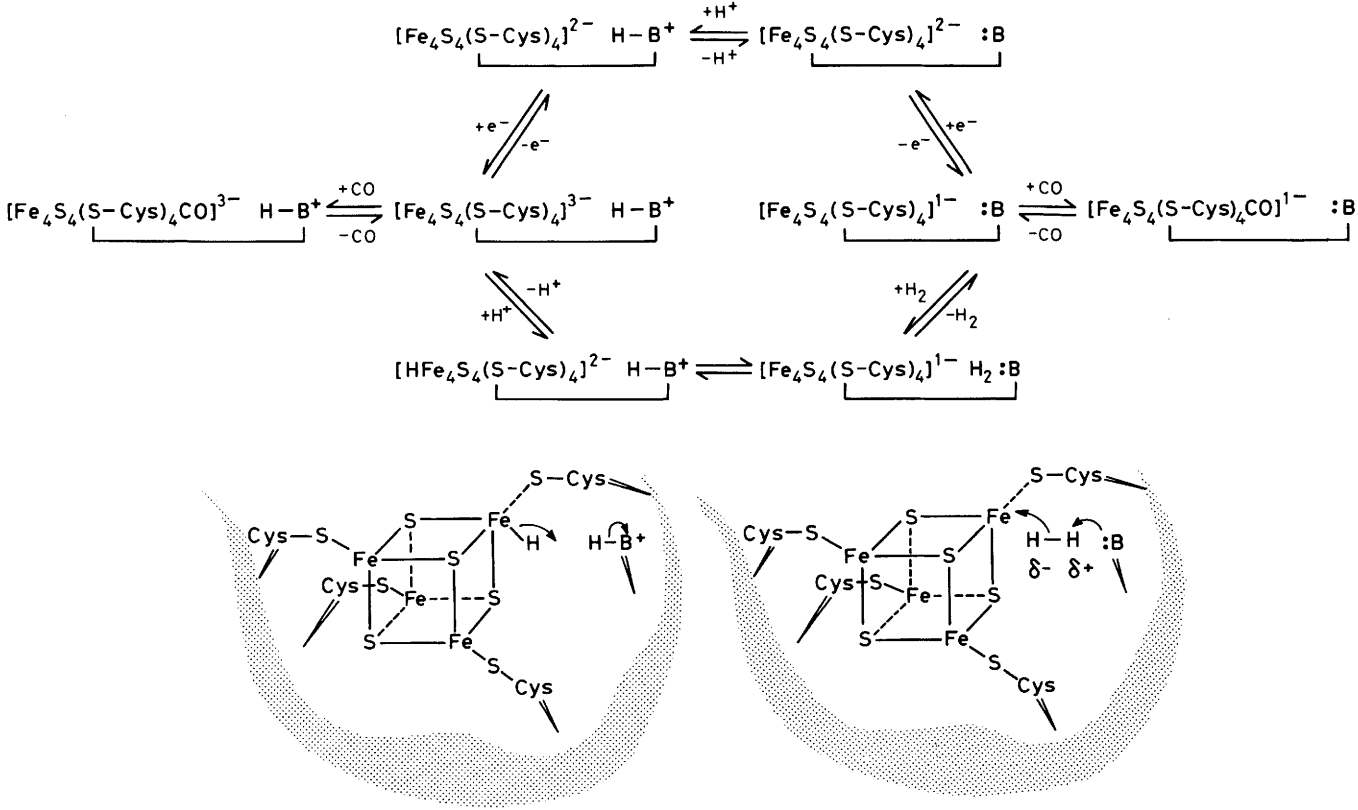


Figure 1 *The proposed mechanism of action of hydrogenase* (Reproduced by permission from *J. Amer. Chem. Soc.*, 1978, 100, 5234.)

The reversible oxidation of molecular hydrogen catalysed by bacterial enzyme hydrogenase is, in principle, one of the simplest reactions in biochemistry and yet detailed mechanistic studies have proved difficult until recently when paramagnetic carbon monoxide adducts of reduced iron–sulphur tetramers have revealed some aspects of the mechanism of the action of hydrogenase.[263] Figure 1 summarizes some aspects of the proposed mechanism. The molybdenum-binding site in nitrogenase has been modelled using EXAFS (extending *X*-ray absorption fine structure) spectroscopy and a molybdenum–sulphur cluster complex of composition $[Mo_2Fe_6S_9(SEt)_8]^{3-}$.[264]

Two related papers concerning the structural studies of transition-metal complexes of tri-ionized and tetra-ionized citrate ions have been used as models for the co-ordination of this ion to the active site of aconitase. Carbon-13 n.m.r. studies and *X*-ray crystallographic determinations are used in these investigations.[265, 266]

We turn now to a zinc-containing enzyme, carbonic anhydrase. The crystal structure of human carbonic anhydrase B complexed to its inhibitor imidazole has now been solved.[267] The imidazole is bound in a hydrophobic pocket having a nitrogen atom directly co-ordinated to the zinc ion. This does not require displacement of water or hydroxide bound to the zinc and so the metal is apparently 5-co-ordinate. Similar 5-co-ordinate zinc complexes have been suggested for horse-liver alcohol dehydrogenase. There have been many studies in which the zinc of carbonic hydrase has been substituted by both cobalt(II) and copper(II). Italian researchers[268] suggest that cobalt(II) maintains the activity of the parent enzyme whereas the copper(II) derivative, although less active physiologically, still retains many of the physical and chemical properties of the native carbonic anhydrase. This has permitted a spectroscopic investigation of the parent bovine carbonic anhydrase and further physical measurements (c.d., e.s.r., n.m.r., and Raman) on the cobalt bovine carbonic anhydrase.[269] Two recent papers from the same research school find that both the cobalt(II) substituted and indeed the copper(II) substituted bovine carbonic anhydrase are both 5-co-ordinate in solution.[270, 271]

The hydrolysis of *S*-*p*-nitrophenylthioacetate by native, cobalt(II) and copper(II) forms of carbonic anhydrase has been studied,[272] and some systems involving metal bound imidazolate, and hydroxide investigated as models for carbonic anhydrase.[273] The *in vivo* competition between this zinc protein and

[263] B.A. Averill and W.H. Orme-Johnson, *J. Amer. Chem. Soc.*, 1978, **100**, 5234.
[264] T.E. Wolff, J.M. Berg, C. Warrick, K.O. Hodgson, and R.H. Holm, *J. Amer. Chem. Soc.*, 1978, **100**, 4630.
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[273] J.M. Harrowfield, V. Norris, and A.M. Sargeson, *J. Amer. Chem. Soc.*, 1976, **98**, 7282.

cadmium as an environmental pollutant has been alluded to by studying the cadmium-113 isomer of human carbonic anhydrase B using n.m.r. spectra.[274]

There have been two model studies on carbonic anhydrase, one chemical and one theoretical. A model of the zinc-binding site in carbonic anhydrase was attempted in the form of the synthesis of tris[4(5)-imidazolyl]carbinol, tris[2-imidazolyl]carbinol, and related ligand complexes from studies using binding constant and spectral investigations. Alas, the geometry produced does not exactly parallel that of the carbonic anhydrase.[275] Theoretical considerations of the binding of the zinc ion in carbonic anhydrase were set up in order to resolve the dichotomy between groups which suggested that CO_2 binds weakly to the fifth co-ordination-site of the metal ion in the hydrophobic region of the active site, whereas others have proposed that CO_2 binds to zinc(II) in a transient five-co-ordinate intermediate.[276] *Ab initio* molecular orbital calculations have shown explicitly that the zinc ion facilitates, to a considerable extent, the ionization of a water or imidazole bound to it, and the ionization of this water is intrinsically much more favoured than the ionization of the imidazole.

A series of three papers has announced some recent model investigations into the function of alcohol dehydrogenase.[277—279] The enzyme was isolated from liver yeast and catalysed the reaction $RCHO + NADH + H^+ \rightleftharpoons RCH_2OH + NAD^+$, the activity being dependent upon the presence of zinc ions. Although involvement of the metal ion in the catalysis is well established there is uncertainty about its function. The reports relate detailed investigation of the metal-promoted reduction of pyridine-2-aldehyde by *N*-benzyldihydronicotinamide in acetonitrile. The papers conclude that the active metal-species is not the dipositive ion, but rather the mono ionized form. Further, under certain specified conditions metal inhibition rather than catalysis occurs.

A relatively new metalloprotein being discussed in the literature is that of plastocyanin. This is one of the smaller proteins having a molecular weight of 10 500. It consists of 99 amino-acid residues and a single copper atom, and this blue copper-protein undergoes important electron-transfer functions during photosynthesis.[280] The crystal structure has been reported and it shows that the copper atom is co-ordinated by sulphur atoms of cysteine-84 and methionine-92 and by the δ-nitrogen atoms of the imidazole groups of histidine-37 and histidine-87.[281] Plastocyanin from parsley has been investigated by stop-flow techniques[282] it being suggested that there are both copper(I) and copper(II) oxidation states present, such an arrangement being appropriate for outer sphere electron-transfer.

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When ESCA was applied to plastocyanins the exceedingly high binding energy was shown to arise from extraneous sulphur present rather than from the sulphur-containing amino-acids being bonded to the metal ion.[283] This view was challenged[284] and the case for cysteine sulphur ligation at the copper site being omnipotent restated. A co-ordination model for the metal-binding site in plastocyanin and azurin has recently been suggested[285] and the relationship between these two blue copper-proteins during evolution discussed. Recent work using stopped-flow studies has commented upon the need to have two copper atoms present in this protein and suggested that one atom of copper is sufficient to explain the chemical behaviour and spectra providing the copper is bonded in a distorted tetrahedral ligand environment and the copper is not accessible to solvent water.[286] A large amount of spectral data on a range of plastocyanins from various sources has helped to build up our knowledge of this intriguing protein.[287, 288]

As co-ordination chemists turned their interests towards plastocyanins, there has been a reduction in the number of publications concerning other copper proteins but nevertheless there is still a substantial amount of material produced in this area. Weser *et al.* have discussed the effects of copper(II) salicylates upon anti-inflammatory and superoxide dismutase activity[289] and others have taken superoxide dismutase from *Bacillus stearothermophilus* and removed the metal manganese(II) and replaced it by other metals.[290] The structural implications for blue-protein copper-centres have been assessed using e.s.r. resonance spectra of copper sulphide $[Cu^{II}S_4]$ chromophores[291] and the general field of structure and electron transfer reactions of blue copper-proteins has been discussed at length.[292] The amino-acid sequences of two putative copper-site peptides from the 'blue' copper-protein, stellacyanin, have been tabulated[293] and the isolation of a non-thionein copper-bonding protein from the liver of rats injected with copper published.[294] Much of this work was discussed at a workshop centring around the structure and function of hemocyanins.[295]

An extended series of ligands and their copper(II) complexes have been prepared and studied spectroscopically as a means of commenting on the likely geometries of the co-ordination of copper proteins. Structures for the copper

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co-ordination and geometry in blue copper-proteins, for the copper in galactose oxidase, and for the copper in oxyhemocyanine have been proposed.[296] Further studies of a model system for the blue copper-proteins involved an electrochemical investigation of mercaptopropionylglycine reacting with copper(II).[297] A variety of 4- and 5-co-ordinate copper(I) and copper(II) complexes have been proposed as models for copper-containing proteins. The novel aspects of five-co-ordinate copper(I) complexes are discussed in detail.[298]

There is still a great deal of co-ordination chemistry concerning cytochrome *c* that is yet to be revealed. Proton n.m.r. studies of the heme iron co-ordination in cytochrome *c*-552 from *Euglena gracilis* and kinetic studies of the oxidation of horse heart ferrocytochrome *c* from *Pseudomonas aeruginosa* have been reported and discussed.[299, 300] A particularly detailed study of the porphyrin-protein bond of cytochrome *c*-558 from *Euglena gracilis* has been published paying particular attention to the implications of porphyrin ring–peptide chain interactions.[301] Further, the 6-co-ordinate geometry of the copper ion and the reversible 1-electron exchange have both been investigated.[302, 303]

Russian researchers have studied the reaction of calcium(II) with albumin and fibrinogen[304] and the non-co-operative calcium(II) removal and terbium-(III) substitution of carp-muscle calcium-binding parvalbumin have been investigated using circularly polarized emission spectra.[305] Potentiometric and spectroscopic studies of the equilibria in aqueous solution of copper(II)-3,6-diazaoctane-1,8-diamine and the ternary system between this ligand, copper, and human serum albumin have been used as a means of commenting on the substantial difference between this ligand (commonly called trien) and the drug D-penicillamine in the treatment of patients with Wilson's disease.[306] Fluorine-19 magnetic resonance spectra of muscle calcium-binding parvalbumin and the pH dependency of such spectra have been reported,[307] and the isolation of a high affinity zinc-binding parvalbumin from *Limulus polyphemus* described in detail.[308]

When heavy polluting metals such as cadmium and lead need to be complexed *in vivo* the high molecular weight protein metallothionein is invoked and has been studied by three research groups.[309—311]

The enzymes ribonuclease A has been bound to a mercury(II) along with

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lysozyme for comparison.[312] The synthesis of penta-ammineruthenbium–histidine complexes of ribonuclease A has been achieved in order to find agents which are sensitive to both structural and dynamic properties of proteins.[313] Molecular orbital calculations concerning the activity of carboxypeptidase in its role of peptide hydrolysis have correlated strains experienced by peptide bonds with a range of electrophilic reagents and compared several proposed modes of nucleophilic attack.[314] Concanavalin A and its cadmium, manganese, and calcium complexes have been investigated using n.m.r. and preliminary reports published.[315]

The diversity of metalloenzyme chemists never ceases to amaze: literature reports have appeared concerning the divalent cation-binding sites of yeast aldolase,[316] the dynamics of outer-sphere redox reactions involving transition metal complexes and metalloproteins in general,[317] the complexing properties of ascorbate oxidase,[318] and a review of selenoproteins in biology.[319] The iron-binding fragments from the *N*-terminal and *C*-terminal regions of human lactoferrin have been separated using Sephadex chromatography,[320] Raman resonance spectra of protocatechuate 3,4-dioxygenase studies have shed light on the co-ordination of the tyrosine residue to the ferric iron present.[321] This paper underlines an increasing trend to use Raman spectral studies in enzymology. There has been a fascinating suggestion that our olfactory perception of odour arises from chemicals binding to protein nasal receptors, or groups of receptors, which contain a transition-metal ion in their active sites.[322] It is suggested that copper(I) in particular would be particularly good from the point of view of binding the more vile smelling substances of sulphur, nitrogen, phosphorus *etc.* Since potentially chelating odorants do not seem to have different odours from their monodentate analogues it is proposed that only one site is available at the metallo-receptor. The identification of ligands by the observation of ^{199}Hg–^{1}H coupling has been described.[323] Thus the substitution of ^{199}Hg into the apoenzyme derived from superoxide dismutase allows the observation of coupling between the ^{199}Hg nucleus ($I = \frac{1}{2}$) and the protons of the co-ordinating histidyl groups.

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