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Amino-acids, Peptides, and Proteins

Volume 10

A Review of the Literature Published during 1977

Senior Reporter
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Preface

This tenth Report reviews papers relevant to the chemistry of amino-acids, peptides, and proteins published in the main journals during 1977. As in previous volumes, Reporters have been encouraged to include discussion of important papers seen in current journals. This volume therefore also includes references to the early 1978 literature and even to papers known to be in the press at the time of writing. It is hoped that this will increase the topicality and value of the Report.

Chapter 5 includes a section on vasoactive peptides, a subject not covered in Volume 9. This section therefore covers the literature for 1976 and 1977.

It is with great sadness that we record the deaths this year of two of the foremost and most respected British peptide and protein chemists. Dr. J. I. Harris died in an accident in South Wales in April, and Professor G. W. Kenner died, also under tragic circumstances, in North Wales in June. Both made major contributions to the subject over a period of more than twenty-five years. Ieuan Harris was one of the pioneering figures in the development and application of sequencing methods to peptide hormones and proteins. His work included total amino-acid sequence determination of several enzymes of more than 300 residues and was carried out with a degree of thoroughness and double checking which left little room for error. George Kenner was also a pioneering figure, but primarily in the field of synthesis. His work was characterized by strict application of the rigorous principles of organic chemistry which led him to favour solution methods rather than the solid-phase approach. This Report includes discussion of the philosophy and present status of his work on enzyme synthesis.

Ieuan Harris was 53 and George Kenner 56 years old. Both leave major research programmes presently incomplete. More happily, this volume records completion of a collaborative project, synthesis by Kenner and his colleagues of the 34 amino-acid residue hormone big gastrin according to a sequence determined by Harris and Runswick.

In another vein, it is a pleasure to thank once more all the many contributors to this Report whose names are listed opposite.

R. C. SHEPPARD

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1C: Chemical Modification of Proteins
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Abbreviations

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

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

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

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

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

1
Amino-acids

BY G. C. BARRETT

1 Introduction

The usual steady increase in the number of papers eligible for citation in this Chapter continues to press the question whether to be more selective in the material reported or to restrict the description of the work reported. Even more papers are cited this year, and this represents a certain amount of extra selectivity. The main areas of literature expansion, in the biological and metabolic studies of amino-acids, continue to be largely excluded from this Chapter.

Textbooks and Reviews.—An unusually large number of reviews have appeared recently covering the occurrence and biosynthesis of D-amino-acids,[1a] the chemistry of β-amino-acids [1b] and of cyclic α-imino-acids,[1c] free radicals formed in condensation reactions of sugars with amino-acids,[2a] αβ-unsaturated and related amino-acids in peptides,[2b] and cross-linking residues in proteins.[2c] Amino-acids present in potato,[3] unusual amino-acids in fungi,[4] and the biological significance of *N*-methylated lysine and arginine derivatives,[5] are topics in recent reviews.

Reviews of amino-acid chemistry [6] and of the distribution of non-protein amino-acids [7] provide material which is largely complementary to that in this Report.

Other reviews and textbooks are cited in the various sections of this Chapter.

2 Naturally Occurring Amino-acids

Occurrence of Known Amino-acids.—The variations found in the proportions of amino-acids in fossils of different age are probably due to variable rates of decomposition of the protein amino-acids, but may indicate stages in molecular evolution.[8] There are several other papers in the earth sciences literature dealing

[1] 'Chemistry and Biochemistry of Amino-acids, Peptides, and Proteins', ed. B. Weinstein, Dekker, New York, 1977, Vol. 4, (*a*) J. S. Davies, p. 1; (*b*) C. N. C. Drey, p. 241; (*c*) A. B. Mauger, p. 179.

[2] (*a*) M. Namiki, T. Hayashi, and Y. Ohta, *Adv. Exp. Med. Biol.*, 1977, **86B**, 471; (*b*) E. Gross, *ibid.*, p. 131; (*c*) M. Friedman, *ibid.*, p. 1; N. P. Stimler and M. L. Tanzer, *ibid.*, p. 675; R. A. Anwar, G. E. Gerber, and K. M. Baig, *ibid.*, p. 709.

[3] R. L. M. Synge, *Potato Res.*, 1977, **20**, 1; A. M. C. Davies, *ibid.*, p. 9.

[4] J. L. C. Wright and L. C. Vining, *Filamentous Fungi*, 1976, **2**, 475.

[5] E. Tyihak, B. Szende, and K. Lapis, *Life Sciences*, 1977, **20**, 385.

[6] E. A. Bell and D. I. John, in 'MTP International Review of Science, Series Two', p. 1. vol. 6, ed. H. N. Rydon, Butterworths, London, 1976.

[7] A. Kjaer and P. O. Larsen, in 'Biosynthesis', A Specialist Periodical Report, ed. J. D. Bu'Lock, The Chemical Society, 1977, Vol. 5, p. 120.

[8] C. Ivanov and R. Stoyanova, *Doklady Bolg. Akad. Nauk*, 1977, **30**, 1129.

with the natural occurrence of amino-acids, but excepting those inferring fossil age from racemization (see Section 5), they are mostly of a routine analytical nature.

Further examples of the existence of D-amino-acids in pea seedlings (see Vol. 9, p. 2) are of aspartic and glutamic acids.[9] Other simple amino-acids (leucine, tyrosine, and phenylalanine) exist as their amides, together with 5-hydroxylysine and canavanine, in *Ladino* clover seeds.[10] Simple derivatives of familiar amino-acids which have been found in new locations are *O*-acetylserine (in *Nicotiana tabacum*),[11] 3-*N*-oxalyl-2,3-diaminopropionic acid in seeds of *Crotalaria*,[12] *Acacia*,[13] and *Lathyrus sativus*,[14] accompanied by the 2-*N*-oxalyl isomer in *Lathyrus*,[14] and 2-amino-3-ureidopropionic acid (*alias* albizzine) in *Dialium*.[15] The organic component which envelopes the silicaceous cell walls of diatoms includes 4-hydroxy- and 3,4-dihydroxy-L-proline.[16]

Less-common amino-acids in plants, reported in the recent literature, include cyclopentenylglycine in seeds of *Hydnocarpus anthelminthica*,[17] N^G-methylarginine and the N^GN^G-dimethyl analogue in seeds of broad bean,[18] octopine, octopinic acid, lysopine, and histopine in *Agrobacterium tumefaciens*-induced sunflower crown gall tumours,[19] and *S*-methylmethionine (vitamin U).[20] A useful review of unusual amino-acids in edible mushrooms, including *cis*-3-amino-L-proline in *Morchella esculenta*, L-2-aminohex-4-ynoic acid and its *threo*- and *erythro*-3-hydroxy- analogues with L-3-(3-carboxy-4-furyl)alanine in *Tricholomopsis rutilans* (see also Vol. 7, p. 3), and γ-propylidene-L-glutamic acid in *Mycena pura*, has appeared.[21] 2*S*,3*R*-2-Amino-3-hydroxypent-4-ynoic acid is a toxic amino-acid present in the fungus *Sclerotium rolfsii*.[22]

Although no attempt can be made to cover the full literature on microbial production of amino-acids, room is found for representative papers.

Auxotrophic mutants of *Pseudomonas aeruginosa* accumulate L,L-2,6-diaminopimelic acid,[23] and α-methylene-γ-aminobutyric acid, the enzymic decarboxylation product of γ-methylene-L-glutamic acid, occurs in *Mycena pura*[24] (*cf.* Vol. 8, p. 3). The purer realms of the biosynthesis literature include the conversion of L-lysine into ε-hydroxy-lysine by cell-free extracts of *Aerobacter aerogenes*,[25] and

[9] T. Ogawa, M. Kimoto, and K. Sasaoka, *Agric. Biol. Chem.*, 1977, **41**, 1811.
[10] T. Kasai, K. Furukawa, and S. Sakamura, *Agric. Biol. Chem.*, 1976, **40**, 2489.
[11] I. K. Smith, *Phytochemistry*, 1977, **16**, 1293.
[12] M. Y. Qureshi, D. J. Pilbeam, C. S. Evans, and E. A. Bell, *Phytochemistry*, 1977, **16**, 477.
[13] C. S. Evans, M. Y. Qureshi, and E. A. Bell, *Phytochemistry*, 1977, **16**, 565.
[14] F. L. Harrison, P. B. Nunn, and R. R. Hill, *Phytochemistry*, 1977, **16**, 1211.
[15] P. S. Peiris and A. Sirimawathie Seneviratne, *Phytochemistry*, 1977, **16**, 1821.
[16] D. Sadava and B. E. Volcani, *Planta*, 1977, **135**, 7.
[17] U. Cramer and F. Spener, *European J. Biochem.*, 1977, **74**, 495.
[18] T. Kasai, M. Sano, and S. Sakamura, *Agric. Biol. Chem.*, 1976, **40**, 2449.
[19] J. D. Kemp, *Biochim. Biophys. Res. Comm.*, 1977, **74**, 862; E. Hack and J. D. Kemp, *ibid.*, 1977, **78**, 785.
[20] A. A. Bezzubov and N. N. Gessler, *Priklady Biokhim. Mikrobiol.*, 1977, **13**, 301.
[21] S. Hatanaka, Y. Niimura, K. Taniguchi, F. Kinoshita, and H. Katayama, *Mushroom Science*, 1976, **9** (Part I), 809 (*Chem. Abs.*, 1977, **86**, 103 099).
[22] H. C. Potgieter, M. M. J. Vermeulen, D. J. J. Potgieter, and H. F. Strauss, *Phytochemistry*, 1977, **16**, 1757.
[23] F. Saleh and P. J. White, *J. Gen. Microbiol.*, 1976, **96**, 253.
[24] S. Hatanaka and K. Takishima, *Phytochemistry*, 1977, **16**, 1820.
[25] G. J. Murray, G. E. D. Clark, M. A. Parniak, and T. Viswanatha, *Canad. J. Biochem.*, 1977, **55**, 625.

L-ornithine into L-Δ^1-pyrroline-5-carboxylic acid by ornithine aminotransferase.[26] Since rumen ciliate protozoa can convert proline, ornithine, or arginine into δ-aminovaleric acid, the α-amino-acids must be on the biosynthetic pathway to the δ-amino-acid.[27] The controversy (see Vol. 9, p. 2) continues concerning the significance of the existence of saccharopine and 2-amino-adipic acid in higher plants to the lysine biosynthetic pathway.[28] The production of amino-acids by immobilized enzymes has been reviewed.[29]

Higher organisms are represented in papers reporting the presence of more than ten quaternary amines, including δ-valerobetaine, γ-butyrobetaine, and the betaines of glycine, valine, and homoserine, in the ovary of the shellfish *Callista brevishiphonata*,[30] and a similar mixture in the adductor muscle of the fan mussel *Atrina pectinata*.[31] The spruce budworm *Choristoneura fumiferana* contains *N*-phosphorylarginine.[32]

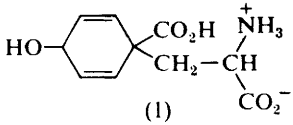
Methylated amino-acids identified as constituents of proteins provide new material for structure–function hypotheses. Ribosomal proteins of *Escherichia coli* carry *N*-terminal *N*-methylalanine, *N*-methylmethionine,[33, 34] and *N*-trimethylalanine [34] residues, and include a γ-methylglutamyl residue.[35] Cytochromes from *Crithidia oncopelti* and *Candida krusei* contain *NN*-dimethylproline [36] and N^{ϵ}-trimethyllysine [37] residues, respectively. N^{G}-Dimethylarginine occurs in sizeable amounts in non-histone nuclear proteins from rat-liver nuclei.[38] Majusculamides A and B contain *N*-methyl-*O*-methyl-D-tyrosine and *N*-methyl-L-valinamide residues.[39]

New Natural Free Amino-acids.—Sunflower plants infected with *Agrobacterium tumefaciens* develop crown gall tumours from which novel acidic amino-acids histopine [N^{α}-(1-carboxyethyl)-L-histidine] [19] and N^{α}-(1,3-dicarboxypropyl)-L-ornithine [40] have been isolated. Plant sources for other new amino-acids are *Gymnocladus dioicus*, a legume whose seeds have already proved to contain several uncommon amino-acids and from which L-*cis*-5-hydroxypipecolic acid has been isolated.[41] Related species *Morus alba* and *Lathyrus japonias* also contain this amino-acid.[42] The *trans*-configuration has been assigned to 4-

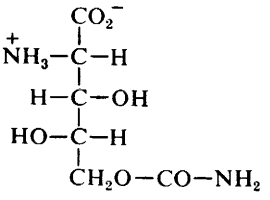
[26] R. J. Smih, S. J. Downing, and J. M. Phang, *Anal. Biochem.*, 1977, **82**, 170.
[27] R. Onodera, W. Tsutsumi, and M. Kandatsu, *Agric. Biol. Chem.*, 1977, **41**, 2169.
[28] R. Nawaz and H. Soerensen, *Phytochemistry*, 1977, **16**, 599.
[29] 'Methods in Enzymology', Vol. 44 (1976).
[30] T. Yasumoto and N. Shimizu, *Nippon Suisan Gakkaishi*, 1977, **43**, 201 (*Chem. Abs.*, 1977, **86**, 117 886).
[31] T. Hayashi and S. Konosu, *Nippon Suisan Gakkaishi*, 1977, **43**, 343.
[32] D. J. Durzan and J. A. Pitel, *Insect Biochem.*, 1977, **7**, 11.
[33] R. Chen, J. Brosius, B. Wittmann-Liebold, and W. Schaefer, *J. Mol. Biol.*, 1977, **111**, 173; R. Chen and U. Chen-Schmiesser, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4905.
[34] F. Lederer, J. H. Alix, and D. Hayes, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 470.
[35] S. J. Keene, M. L. Toews, and J. Adler, *J. Biol. Chem.*, 1977, **252**, 3214.
[36] G. W. Pettigrew and G. M. Smith, *Nature*, 1977, **265**, 661.
[37] D. J. Wilbur and A. Allerhand, *F.E.B.S. Letters*, 1977, **74**, 272.
[38] L. C. Boffa, J. Karn, G. Vidali, and V. G. Allfrey, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 969.
[39] F. J. Marner, R. E. Moore, K. Hirotsu, and J. Clardy, *J. Org. Chem.*, 1977, **42**, 2815.
[40] J. L. Firmin and R. G. Fenwick, *Phytochemistry*, 1977, **16**, 761.
[41] J. Despontin, M. Marlier, and G. Dardenne, *Phytochemistry*, 1977, **16**, 387.
[42] S. Hatanaka and S. Kaneko, *Phytochemistry*, 1977, **16**, 1041.

carboxy-L-proline, isolated from *Chondria coerulesceus*.[43] Lentinic acid, the *N*-(γ-L-glutamyl) derivative of $MeSO_2CH_2(SOCH_2)_3CH(NH_2)CO_2H$, has been isolated from *Lentinus edodes*.[44]

New amino-acids of interest in biosynthetic studies are β-hydroxy-N^{ϵ}-trimethyl-lysine, identified as an intermediate in the biosynthesis in *Neurospora crassa* of carnitine,[45] and 'pretyrosine' (1), on the pathway to L-tyrosine in blue-green algae *Pseudomonas aeruginosa*.[46] Aromatic amino-acids in *Cortinarius brunneus*



(1)



(2)

and in *Pachymatisma johnstoni* include 4-hydroxy-3-methoxy-L-phenylalanine[47] and 6-bromohypaphorine[48] (6-bromo-L-tryptophan N^{α}-trimethyl betaine), respectively.

Diastereoisomers of 2-amino-4-keto-3-methylpentanoic acid isolated from *Bacillus cereus* 439 are of particular interest as vitamin B_{12} antimetabolites.[49] The *N*-terminus of each of the nucleoside peptide antibiotics, the polyoxins, is 5-*O*-carbamoyl-2-amino-2-deoxy-L-xylonic acid (2; 'polyoxamic acid').[50] Full details are available of the isolation of 3-(2,5-*SS*-dicysteinyl-3,4-dihydroxyphenyl)-alanine from the tapetum lucidum of alligator eye (see Vol. 8, p. 4).[51]

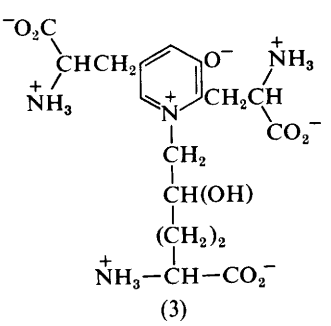
Higher homologues of the amino-acids are represented in *N*-β-alanyldopamine, from wings of *Papilio xuthus*,[52] and 4-acetamido-2-butenoic acid ($MeCONHCH_2CH_2CH{=}CHCO_2H$) from *Fusarium graminearum*.[53]

New Amino-acids from Hydrolysates.—In a previous section, the occurrence of unusual, but known, amino-acids in peptides and proteins has been surveyed, and this section is exclusively concerned this year with residues at cross-link sites in proteins.[2b, 2c]

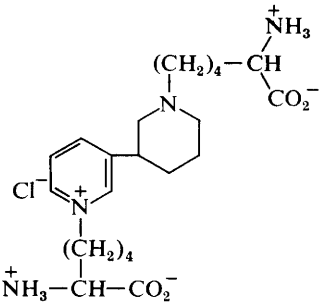
Analogues of the familiar lysine-based cross-links desmosine and allysine have arisen in protein studies, with the identification of hydroxyallysine as an intermediate in the formation of collagen cross-links,[54] ε-(γ-glutamyl)lysine as a

[43] G. Impellizzeri, M. Piatelli, S. Sciuto, and E. Fattorusso, *Phytochemistry*, 1977, **16**, 1601.
[44] K. Yasumoto, K. Iwami, H. Mizusawa, and H. Mitsuda, *Nippon Nogei Kagaku Kaishi*, 1976, **50**, 563 (*Chem. Abs.*, 1977, **86**, 185 866); G. Höfle, R. Gmelin, H.-H. Luxa, M. N'Galamulume-Treves, and S. I. Hatanaka, *Tetrahedron Letters*, 1976, 3129.
[45] R. A. Kaufman and H. P. Broquist, *J. Biol. Chem.*, 1977, **252**, 7437.
[46] N. Patel, D. L. Pierson, and R. A. Jensen, *J. Biol. Chem.*, 1977, **252**, 5839.
[47] G. Dardenne, M. Marlier, and A. Welter, *Phytochemistry*, 1977, **16**, 1822.
[48] W. D. Raverty, R. H. Thomson, and T. J. King, *J.C.S. Perkin I*, 1977, 1204.
[49] D. Perlman, K. I. Perlman, M. Bodanszky, A. Bodanszky, R. L. Foltz, and H. W. Matthews, *Bio-org. Chem.*, 1977, **6**, 263.
[50] S. Funuyama and K. Isono, *Biochemistry*, 1977, **16**, 3121.
[51] S. Ito and J. A. C. Nicol, *Biochem. J.*, 1977, **161**, 499.
[52] Y. Umebachi and H. Yamashita, *Comp. Biochem. Physiol. B*, 1977, **56**, 5.
[53] R. F. Vesonder, L. W. Tjarks, A. Ciegler, G. F. Spencer, and L. L. Wallen, *Phytochemistry*, 1977, **16**, 1296.
[54] R. C. Siegel, *J. Biol. Chem.*, 1977, **252**, 254.

cross-link in the keratin fraction of human stratum corneum,[55] and the novel desmosine relatives pyridinoline (3) and anabilysine (4), the fluorescent material from bovine Achilles tendon collagen,[56] and the cross-link residue in glutaraldehyde-treated ovalbumin,[57] respectively.



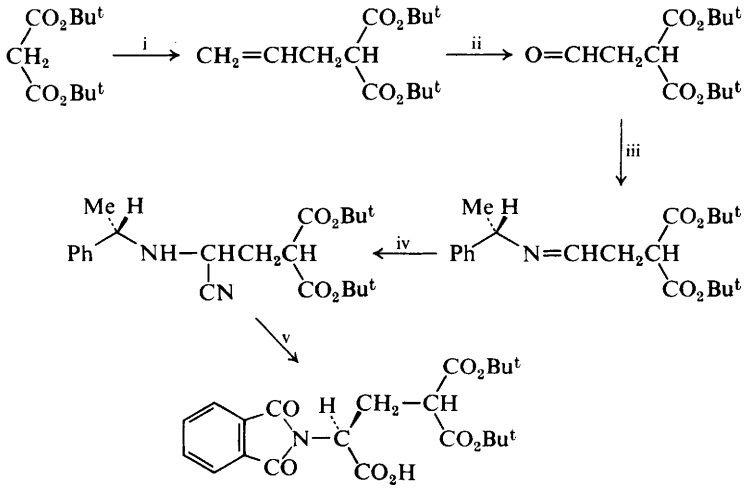
(3)



(4)

3 Chemical Synthesis and Resolution of Amino-acids

Asymmetric Synthesis.—The general possibilities for asymmetric synthesis of α-amino-acids illustrated in recent Volumes of this Report are developed further in studies published in 1977. Routes based on chiral Schiff bases give variable asymmetric yields, but



Reagents: i, allyl bromide; ii, O_3; iii, (−)-PhCHMeNH$_2$; iv, HCN; v, separation of diastereoisomers and subsequent steps according to previously-established procedure (Vol. 9, p. 5)

Scheme 1

[55] J. L. Abernethy, R. L. Hill, and L. A. Goldsmith, *J. Biol. Chem.*, 1977, **252**, 1837.
[56] D. Fujimoto, K. Akiba, and N. Nakamura, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 104.
[57] P. M. Hardy, G. J. Hughes, and H. N. Rydon, *J.C.S. Chem. Comm.*, 1977, 759.

L-γ-carboxyglutamic acid has been obtained as its *N*-phthaloyl γγ-di-t-butyl ester derivative in 100% optical purity (Scheme 1).[58] The alternative approach, alkylation of the Schiff base formed between a chiral ketone and an α-amino-acid ester, has been studied for the asymmetric synthesis of α-methyl-α-amino-acids from DL-alanine t-butyl ester.[59] A variant of this procedure, alkylation of the cobalt(III) complex of *N*-salicylideneglycine, has been used for the synthesis of L-glutamic acid from methyl acrylate;[60] electrochemical reduction was used in this case[60] and in an extraordinary example, cathodic reduction of *syn*- or *anti*-phenylglyoxylic acid oximes leading to *R*(−)-phenylglycine predominantly at cathodic potentials below 1.4 V, and to enantiomeric excesses of the *S*-isomer at potentials above this value,[61] when strychnine is present.

Prochiral acylaminoacrylates and cinnamates give moderate asymmetric yields of corresponding *N*-acylamino-acids by hydrogenation in the presence of chiral phosphine–rhodium complex catalysts.[62–64] This system is now more a test-bed for new homogeneous catalysts and no additional interest in amino-acid synthesis has emerged from the most recent papers.

Higher homologous amino-acids for which asymmetric syntheses have been reported are 3-aminobutanoic acid (Michael addition of a chiral amine to crotononitrile followed by hydrolysis and catalytic reduction),[65] and *S*-homoproline and *S*-homopipecolic acid *via* the corresponding chiral lactams.[66]

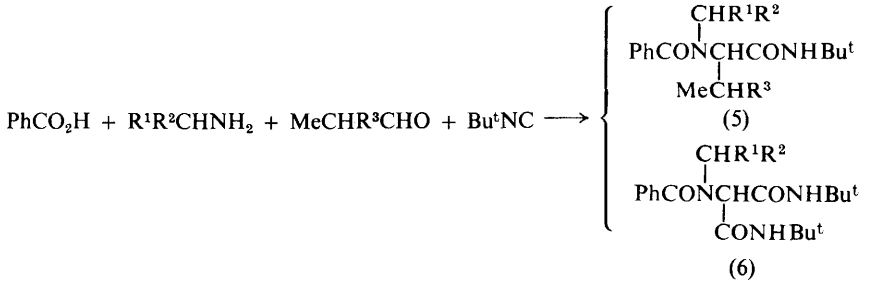
General Methods of Synthesis of α-Amino-acids.—The preceding section has served to preview some standard synthetic methods, but a broad view of synthetic methods, both those of long standing and others undergoing current evaluation, is attempted here.

Direct methods of assembly of α-amino-acids, either by alkylation of glycine derivatives[59,60,67] (including α-hydroxy- and -methoxyglycines[68]) and alanine derivatives[59,67] or by the carboxylation of aliphatic amines[69] are of special interest. Ureidoalkylation of arenes must by now be one of the methods of choice for the synthesis of aryl-substituted phenylglycines[68a] and certain aliphatic amino-acids,[68c] while carbanion alkylation involving glycine-derived Schiff bases[59,60] shows signs of conforming to the requirements of reliable high-yield procedures so that these routes, too, may more credibly enter the standard repertoire (but unwanted di-alkylation can be troublesome[59]). The most interesting paper in this area[69] describes γ-radiolytic carboxylation of amines in aqueous

[58] M. Oppliger and R. Schwyzer, *Helv. Chim. Acta*, 1977, **60**, 43.
[59] T. Oguri, T. Shioiri, and S. Yamada, *Chem. Pharm. Bull.*, 1977, **25**, 2287.
[60] Y. N. Belokon, T. F. Saveleva, and M. B. Saporovskaya, *Izvest. Akad. Nauk. S.S.S.R., Ser. khim.*, 1977, 428.
[61] M. Jubault, E. Raoult, J. Armand, and L. Boulares, *J.C.S. Chem. Comm.*, 1977, 250.
[62] M. D. Fryzuk and B. Bosnich, *J. Amer. Chem. Soc.*, 1977, **99**, 6262.
[63] K. Achiwa, *Chem. Letters*, 1977, 777.
[64] R. Glaser and J. Blumenfeld, *Tetrahedron Letters*, 1977, 2525; R. Glaser and S. Geresh, *ibid.*, p. 2527; R. Glaser, M. Twaik, S. Geresh, and J. Blumenfeld, *ibid.*, p. 4635; R. Glaser, J. Blumenfeld, and M. Twaik, *ibid.*, p. 4639.
[65] M. Furukawa, T. Okawara, and Y. Terawaki, *Chem. Pharm. Bull.*, 1977, **25**, 1319.
[66] T. Wakabayashi, K. Watanabe, and Y. Kato, *Synth. Comm.*, 1977, **7**, 239.
[67] J. J. Fitt and H. W. Gschwend, *J. Org. Chem.*, 1977, **42**, 2639.
[68] (*a*) D. Ben-Ishai and Z. Bernstein, *Tetrahedron*, 1977, **33**, 3261; (*b*) D. Ben-Ishai, J. Altman, and N. Peled, *ibid.*, p. 2715; (*c*) D. Ben-Ishai, R. Moshenberg, and J. Altman, *ibid.*, p. 1533.
[69] A. Davison, N. T. Barker, and D. F. Sangster, *Austral. J. Chem.*, 1977, **30**, 807.

formate buffers; $EtNH_2$ gives a mixture of alanine and β-alanine, isomeric aminopropanes give all possible mono-carboxylation products, while proline is formed from either 1,4-diaminobutane or pyrrolidine (accompanied by ornithine or 3-carboxyproline, respectively).

Use of isocyanides is illustrated in a study of the Ugi synthesis, exploring the influence of reactant concentrations on the proportions of the four-component condensation product (5) and of the side-product [6; an interesting puzzle is provided by the fact that (6) is not formed when the aldehyde component is omitted] when used for the synthesis of amino-malonic acid derivatives (Scheme 2).[70] β-Branched amino-acid derivatives result from the Michael addition of carbanions to isocyanoacrylate esters.[71]



Scheme 2

Reduction of α-keto-acid oximes,[61, 72] phenylhydrazones,[73] or Schiff bases [60, 74] gives corresponding α-amino-acid derivatives. Other standard general methods which have been used are alkylation of diethyl acetamidomalonate,[49, 114, 130, 147, 151, 159, 160a, 172] the hydantoin synthesis,[57, 75] including the synthesis of hydantoins in moderate yields from anodic oxidation of an alcohol with ammonium carbonate and KCN,[75] substitution reactions of α-halogenopropionates [76] and γ-bromobutyrates,[95] and alkylation of glycines,[125] α-isocyano- and -nitro-propionates,[99] and azlactones.[148]

Prebiotic Synthesis; Model Reactions.—A still larger number of papers has appeared on this topic, partly due to studies of the scope for organic synthesis in models of the present environments on other planets, as opposed to primordial Earth (*e.g.* Mars, Jupiter). Apart from the chemistry of hydrogen cyanide polymers, there are few significant new additions to synthetic methods arising from these studies. The pioneers in this field have reviewed the origin of organic compounds on Earth and in meteorites.[77]

Studies of gas-phase reactions of a familiar type but in a novel context – the ammonia-rich atmosphere of Jupiter – have shown that HCN and higher alkanes

[70] A. Gieren, B. Dederer, G. George, D. Marquarding, and I. Ugi, *Tetrahedron Letters*, 1977, 1503.
[71] U. Schöllkopf and R. Meyer, *Annalen*, 1977, 1174.
[72] J. Pospisek and K. Blaha, *Coll. Czech. Chem. Comm.*, 1977, **42**, 1069.
[73] I. Tabakovic, M. Trkovnik, and M. Dzepina, *Croat. Chem. Acta*, 1977, **49**, 497.
[74] K. Nakamura, A. Ohno, and S. Oka, *Tetrahedron Letters*, 1977, 4593.
[75] E. P. Krysin, V. V. Tsodikov, and V. A. Grinberg, *Elektrokhimiya*, 1976, **12**, 1590.
[76] Y. Nakajima, R. Kinishi, J. Oda, and Y. Inouye, *Bull. Chem. Soc. Japan*, 1977, **50**, 2025.
[77] S. L. Miller, H. C. Urey, and J. Oro, *J. Mol. Evol.*, 1976, **9**, 59.

can be formed by photolysis of $NH_3 : H_2 : He : CH_4$ (1 : 15 : 2 : 3)[77] and that electric discharge in a similar mixture containing water can lead to amino-acids when cyanide ions are also present.[78] An unexpected result,[79] the formation of porphyrin-like pigments in such systems, has been reported, and the continuing investigations of another group of workers have demonstrated further the formation of amino-acids and urea from glow-discharge electrolysis of aqueous ammonia in the presence of elemental carbon,[81] or of bicarbonate, or formate ions.[82]

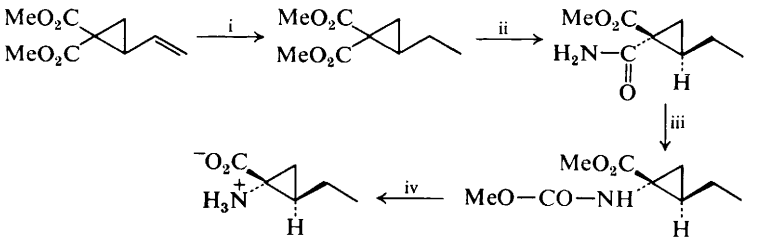
Hydroxylamine–formaldehyde mixtures have been shown [82–85] to be capable of yielding about 40 amino-acids in aqueous solution at pH 5.5, at 105 °C with [82] or without kaolin.[83] Transition metal molybdates are important in influencing the relative amounts of alanine, aspartic acid, β-alanine, and particularly proline, at the expense of glycine and serine.[84] These studies are relatively unusual in this area in not involving some external energy supply (electromagnetic or acoustic) but the production of glycine and alanine from hydroxylamine-formaldehyde in high-intensity ultrasound [85] was reported a little earlier. If simple monosaccharides are regarded as oligomers of formaldehyde, the reported formation of amino-acids in aqueous solutions of sugars in the presence of nitrates under N_2,O_2, or CO_2, in u.v. light,[86] at first sight a refreshing new approach, becomes more easily related to conventional studies in this area. A more extraordinary detail from this study, however, is that exclusion of nitrate does not bring amino-acid synthesis to a halt, amino-groups in glutamic acid and lysine formed under these conditions originating from atmospheric nitrogen.[86]

Aqueous solutions of HCN exposed to ^{60}Co γ-radiation form polymers from which glycine, alanine, valine, serine, threonine, aspartic and glutamic acids, amongst other compounds, are formed by hydrolysis.[87] ^2H-Labelling studies show that poly(aminomalononitriles) formed from HCN–water mixtures under u.v.-irradiation are the major sources of α-amino-acids formed by hydrolysis of the reaction product.[88] The same intermediate may be involved in the pathway from NH_3–CH_4–H_2O electric discharge reaction mixtures to α-amino acids,[88] and Matthews, Minard, and co-workers argue convincingly that the lower energy of the reaction pathway on which this intermediate lies gives the hypothesis still more support.[88]

Protein and Other Naturally Occurring Amino-acids.—Several examples of the use of standard general methods of synthesis of α-amino-acids, as well as unusual

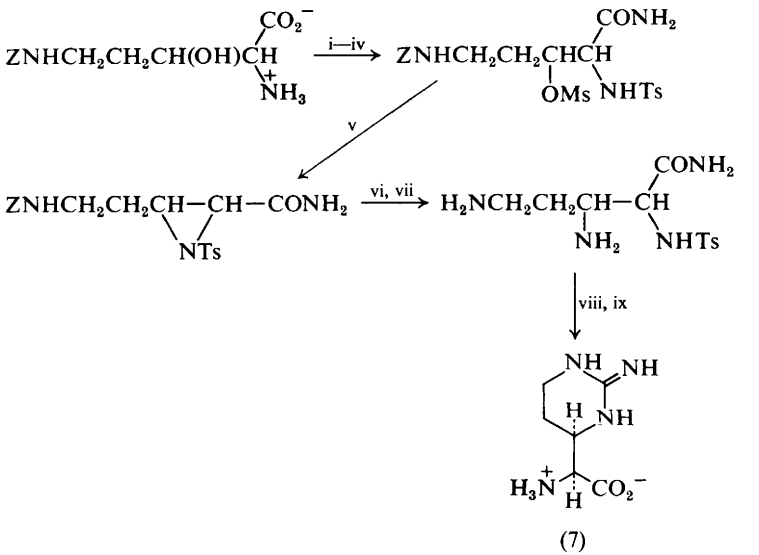
[78] J. P. Ferris, C. Nakagawa, and C. T. Chen, *Life Sci. Space Res.*, 1977, **15**, 95.
[79] V. I. Kalinichenko, V. B. Bondarev, M. V. Gerasimov, L. M. Mukhin, and E. N. Safonova, *Doklady Akad. Nauk. S.S.S.R.*, 1977, **236**, 245.
[80] C. I. Simionescu, B. C. Simionescu, R. Mora, M. Leanca, and E. Ioanid, *Compt. rend.*, 1977, **284**, 743.
[81] (*a*) K. Harada and S. Suzuki, *Naturwiss.*, 1977, **64**, 484; (*b*) *Nature*, 1977, **266**, 275.
[82] H. Hatanaka and F. Egami, *Bull. Chem. Soc. Japan*, 1977, **50**, 1147.
[83] M. Ventilla and F. Egami, *J. Mol. Evol.*, 1977, **9**, 105.
[84] H. Hatanaka and F. Egami, *J. Biochem.*, 1977, **82**, 499.
[85] A. Sokolskaya, *Origins Life*, 1976, **7**, 183.
[86] M. A. Khenokh and M. V. Nikolaeva, *Zhur. Evol. Biokhim. Fiziol.*, 1977, **13**, 105 (*Chem. Abs.*, 1977, **86**, 184 776); *Studia Biophys.*, 1977, **63**, 1.
[87] M. A. Sweeney, A. P. Toste, and C. Ponnamperuma, *Origins Life*, 1976, **7**, 187.
[88] C. Matthews, J. Nelson, P. Varma, and R. Minard, *Science*, 1977, **198**, 622.

methods, are illustrated in this section. A simple synthesis of DL-proline from pyrrolidine, giving an overall 45% yield, involves successive *N*-chlorination, dehydrochlorination, and addition of HCN to the resulting 1-pyrroline followed by hydrolysis.[89] A one-pot synthesis of 4-hydroxyproline from glyoxal and oxaloacetic acid with NH_4OH under physiological conditions, followed by reduction with sodium borohydride, gives a 40% yield.[90] Dieckmann cyclization of an *N*-(2-methoxycarbonylethyl)glycine ester represents another approach to the same ring system, and has been used for the synthesis of the stereoisomer of 3-hydroxy-5-methylproline recently shown to be a constituent of Actinomycin Z_1 (see Vol. 8, p. 5).[91]



Reagents: i, tosyl hydrazide, diglyme; ii, NH_3 in MeOH; iii, Br_2–NaOH/MeOH; iv, H_2O

Scheme 3



(7)

Reagents: i, TsCl; ii, CH_2N_2; iii, NH_3; iv, MsCl; v, Et_2NH; vi, NH_3; vii, H_2–Pd; viii, BrCN; ix, HBr

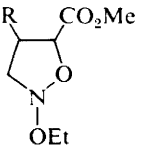
Scheme 4

[89] U. Schmidt and H. Poisel, *Angew. Chem., Internat. Edn.*, 1977, **16**, 777.
[90] S. G. Ramaswamy and E. Adams, *J. Org. Chem.*, 1977, **42**, 3440.
[91] A. B. Mauger, O. A. Stuart, E. Katz, and K. T. Mason, *J. Org. Chem.*, 1977, **42**, 1000.

Out-of-the-way methods are mandatory for 1-aminocycloalkanecarboxylic acids, as illustrated in Scheme 3 for the synthesis of coronamic acid.[92] More complex ring systems are present in L-capreomycidine (7 in Scheme 4) and discadenine -[6-(3-methyl-2-butenylamino)purin-3-yl]butyrine (Vol. 9, p. 4),[93] and a new synthesis of the former amino-acid has been reported (Scheme 4),[94] as well as a first synthesis of the latter from the purine and ethyl α-phthalimido-β-bromobutyrate.[95]

A reliable procedure has been worked out[96] for the preparation of N^4-ethyl-L-asparagine (see also Vol. 8, p. 14). Serine is obtained[74a] by 18-crown-6-catalysed reaction of azide ion with methyl 3-hydroxy-2-bromopropionate followed by reduction, but isoserine is also formed when the same reaction is used, but without catalysis.[74a]

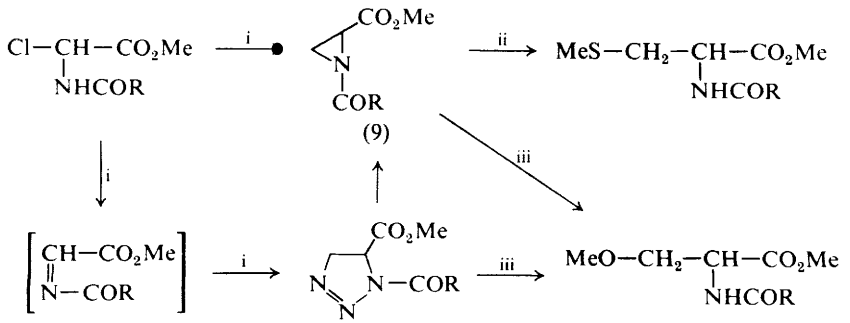
Among higher homologous amino-acids which occur in peptide antibiotics,4-amino-2-hydroxybutanoic acid and its 3-methyl derivative have been synthesized



(8)

from the isoxazolidones (8; R = H and R = Me, respectively), readily obtained from the nitrone CH_2=NO(OEt) and acrylates RCH=$CHCO_2$Me.[97]

Derivatives of *O*-methylserine and *S*-methyl cysteine may be obtained from glycine *via* *N*-benzyloxycarbonylaziridine-2-carboxylates (9 in Scheme 5).[98]



Reagents: i, CH_2N_2; ii, MeSH; iii, MeOH

Scheme 5

[92] A. Ichihara, K. Shiraishi, and S. Sakamura, *Tetrahedron Letters*, 1977, 269.
[93] T. Nomura, Y, Tanaka, H. Abe, and M. Uchiyama, *Phytochemistry*, 1977, **16**, 1819,
[94] T. Shiba, T. Ukita, K. Mizuno, T. Teshima, and T. Wakamiya, *Tetrahedron Letters*, 1977, 2681.
[95] M. Uchiyama and H. Abe, *Agric. Biol. Chem.*, 1977, **41**, 1549.
[96] R. W. Dineen and D. O. Gray, *Org. Prep. Proced. Internat.*, 1977, **9**, 39.
[97] H. Sato, T. Kusumi, K. Imaye, and H. Kakisawa, *Bull. Chem. Soc. Japan*, 1976, **49**, 2815.
[98] Z. Bernstein and D. Ben-Ishai, *Tetrahedron*, 1977, **33**, 881.

α-Alkyl Analogues of the Protein Amino-acids.—The property of powerful reversible inhibition of amino-acid decarboxylases by α-methyl analogues of some α-amino-acids stimulated the search for convenient synthetic methods. Alkylation of methyl α-isocyanopropionate or the α-nitro analogue with acetoxymethylimidazole has been employed for the synthesis of α-methylhistidine,[99] while similar alkylation of an alanine Schiff base [100, 101] has been developed into a satisfactory new synthesis of α-methylornithine (see also Vol. 7, p. 10). Full experimental details have been published [102] of the synthesis of α-methyl-α-amino-acids by cathodic reduction of an alanine Schiff base in the presence of an alkyl halide, followed by hydrogenolysis.[102]

A novel example of the oxo-Wittig rearrangement, resulting in the conversion of *N*-benzyloxycarbonyl-L-proline into α-benzylproline,[103] may be applicable to other amino-acid derivatives; it involves successive treatment with $LiPr^{i}_{2}N$ and benzyl chloride.

α-Alkoxy-, α-Alkylamino-, and α-Alkylthio-analogues of the Protein Amino-acids.—A novel oxidative alkoxylation procedure, in which an *N*-acylamino-acid is treated with dicyclohexylcarbodi-imide in an alcohol, involves the corresponding oxazol-5(4*H*)-one as intermediate, but has yet to be shown to be applicable other than in the favourable case of *N*-phenylacetyl phenylglycine.[104] Another novel synthesis involving the reaction of the αβ-dehydro-amino-acid with thallium(III) acetate gives a mixture of corresponding αβ-dimethoxy-α-*N*-acylamino-acid ester diastereoisomers.[105]

Further development of methods discussed in recent Volumes of this series deals with the formation of 2-acetoxy-2-acylamino-acids from corresponding acylaminomalonic acid mono-esters by anodic oxidation [106] and a surprising synthesis under these conditions of 3-acetoxy-2-acylamino-alkanoic acids from corresponding β-alkylaspartates,[106] also full details of the synthesis of α-hetero-atom-substituted α-amino-acid derivatives from *o*-chloranil–oxazol-5(4*H*)-one adducts.[107] A review of α-mercapto-α-amino-acids has appeared.[108]

Side-chain Halogenated Analogues of the Protein Amino-acids.—While fluorine-substituted protein amino-acids in particular are important as potential or actual enzyme inhibitors, halogenoalkyl amino-acids more generally provide useful intermediates for the synthesis of other compounds.

Conversion of hydroxyalkyl amino-acids into halogenoalkyl analogues has been achieved using PCl_5 [109] or Ph_3P–CBr_4 [110] respectively for the preparation of *erythro*-

[99] M. Miyoshi, K. Matsumoto, and T. Miyahara, Japan Kokai, 76/115 474 (*Chem. Abs.*, 1977, **86**, 155 651).
[100] P. Bey and J. P. Vevert, *Tetrahedron Letters*, 1977, 1455; 1978, 1215.
[101] G. Stork, A. Y. W. Leong, and A. Tonzin, *J. Org. Chem.*, 1976, **41**, 3491.
[102] T. Iwasaki and K. Harada, *J.C.S. Perkin I*, 1977, 1730.
[103] P. A. Crooks, R. H. B. Galt, and Z. S. Matusiak, *Chem. and Ind.*, 1976, 693.
[104] K. Tajima, *Chem. Letters*, 1977, 279; *Noguchi Kenkyusho Jiho*, 1977, **20**, 24 (*Chem. Abs.*, 1978, **88**, 62 318); Japan Kokai, 77/53 832 (*Chem. Abs.*, 1977, **87**, 117 674).
[105] M. P. Paradisi and G. P. Zecchini, *Tetrahedron*, 1977, **33**, 1729.
[106] T. Iwasaki, H. Horikawa, K. Matsumoto, and M. Miyoshi, *J. Org. Chem.*, 1977, **42**, 2419.
[107] J. M. Riordan, M. Sato, and C. H. Stammer, *J. Org. Chem.*, 1977, **42**, 236.
[108] U. Schmidt, *Pure Appl. Chem.*, 1977, **49**, 163.
[109] A. Srinivasan, R. W. Stephenson, and R. K. Olsen, *J. Org. Chem.*, 1977, **42**, 2256.
[110] T. Wieland, D. Schermer, G. Rohr, and H. Faulstich, *Annalen*, 1977, 806.

β-chloro- and -bromo-α-aminobutyric acid derivatives from threonine. Similar substitution reactions have been accomplished for hydroxyproline.[110] Less direct methods are involved for certain fluoro-substituted amino-acids; 4,4-difluoro-L-proline has been prepared from hydroxy-L-proline *via* 4-oxoproline-dioxopiperazine using SF_4–HF as reagent,[111] while a synthesis of 2-^{2}H-3-fluoro-D-alanine uses fluoropyruvic acid as starting material.[112] ββ-Difluoroaspartic acid, and the correspondingly substituted asparagine, have been prepared by fluorination of di-t-butyl oxaloacetate and conventional elaboration of the oxime of the product.[113] Long routes to 5,5-difluorolysine [114] (starting from 2-acetylaminomalonic acid ester) and to trifluoro-DL-alanine (starting from ethyl 2-iodo-2-trifluoromethylpropanoate formed by radical addition of CF_3I to $CH_2{=}CHCO_2Et$) [115] have been announced.

Aliphatic Amino-acids Carrying Hydroxy-groups in Side-chains.—Copper complexes of glycine Schiff bases have been used for the synthesis of β-hydroxyalkyl-α-amino-acids by alkylation by aldehydes;[116, 117] α-hydroxymethylserine,[116] and threonine, phenylserine, and β-hydroxytryptophan [117] have been obtained in recent studies of this well established route. An interesting outcome of one of these studies [117] is the formation of 3-methoxycarbonylproline from the salicylylglycine ethyl ester–copper(II) complex and methyl acrylate.

A straightforward route to DL-*cis*- and -*trans*-3-hydroxyprolines,[118] and a stereoselective synthesis of *threo*-3-hydroxy-4-amino-acids *via* pyrrolidin-2-ones,[119] have been reported.

Amino-acids with Unsaturated Side-chains.—Two main areas of interest, the propensity of 1-alkenyl- and -alkynyl-homologues of the protein amino-acids to act as powerful irreversible inhibitors of amino-acid decarboxylases, and the existence of dehydro-amino-acids (particularly αβ-unsaturated α-amino-acids) in certain naturally-occurring peptides,[108] have stimulated increased efforts towards efficient synthesis of the amino-acids concerned. Pride of place in this section should go to the novel route to dehydro-amino-acid imines involving an ene reaction between *N*-benzylidene amino-acid esters (of valine, phenylalanine, or isoleucine) and diethyl azodicarboxylate (Scheme 6).[120] Triazolidines are also formed. Other routes to dehydro-analogues of protein amino-acids which are represented in recent papers are already well-established (dehydrochlorination of an *N*-chloro-*N*-acylamino-acid ester,[121, 122] thermolysis of β-alkylsulphinyl-

[111] F. N. Shirota, H. T. Nagasawa, and J. A. Elberling, *J. Medicin. Chem.*, 1977, **20**, 1176.
[112] G. Gal, J. M. Chemerda, D. F. Reinhold, and R. M. Purick, *J. Org. Chem.*, 1977, **42**, 142.
[113] J. J. M. Hageman, M. J. Wanner, G. J. Koomen, and U. K. Pandit, *J. Medicin. Chem.*, 1977, **20**, 1677.
[114] F. N. Shirota, H. T. Nagasawa, and J. A. Elberling, *J. Medicin. Chem.*, 1977, **20**, 1623.
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[121] A. J. Kolar and R. K. Olsen, *Synthesis*, 1977, 457.
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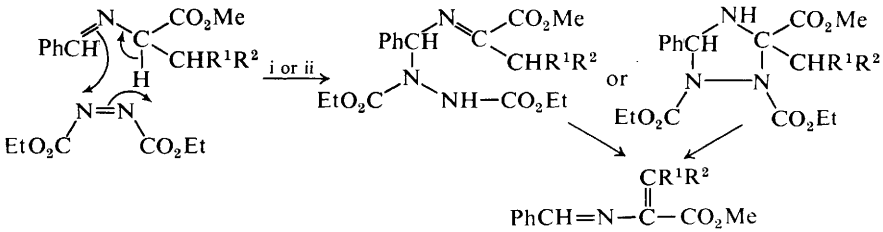

Reagents: i, 130 °C, 48 h; ii, boiling benzene or toluene, 0.5—24 h

Scheme 6

amino-acids in the presence of a phosphine or phosphite as sulphenic acid acceptor,[123] base-catalysed elimination of β-chloroalkyl amino-acid derivatives,[109] and rearrangement of acylimines formed by treatment of *o*-chloranil–oxazol-5(4*H*)-one adducts with base [107]).

Side-chain dehydrogenation of *N*-benzyloxycarbonyl-L-tryptophan by *Chromobacterium violaceum* [124] involves *syn*-elimination leading to the *Z*-isomer.

βγ-Unsaturated amino-acids may be synthesized by successive alkylation and carboxylation of the silylated propargylamine Schiff base $PhCH{=}NCH_2C{\equiv}CSiMe_3$;[125] α-ethynyl- and α-vinyl-dopas have been prepared in this way.[125] Vinylglycine has been synthesized previously, but only in modest yield, and a reliable alternative synthesis from acrolein cyanohydrin *via* 2-bromobut-3-enoic acid has been established.[126] Homologues, *e.g.* isodehydrovaline $CH_2{=}CMeCH(\overset{+}{N}H_3)\text{-}CO_2^-$, are obtainable from corresponding α-nitroacrylates [$Me_2C{=}CHCO_2Me + HNO_3 \rightarrow Me_2C{=}C(NO_2)CO_2Me$] by base isomerization.[126]

Aromatic and Heterocyclic Amino-acids.—A number of specifically interesting syntheses can be cited here; simple new amino-acids prepared by standard methods are listed later. Phenylalanine yields a mixture of *o*, *m*, and *p*-tyrosines and dihydroxyphenylalanines by reaction in acetate buffer (pH 6.0) with ascorbic acid in the presence of Cu^{2+} ions.[127] Tyrosine is converted into dopa by horseradish or mushroom peroxidase.[128] An alternative synthesis of cyclodopa from dopa methyl ester using potassium iodate for effecting the cyclization involves an iodoquinonimine intermediate.[129]

3-(5-Hydroxy-6-oxo-1,6-dihydro-2-pyridyl)-DL-alanine, a 2(1*H*)-pyridone isomer of mimosine, and its 1-hydroxy-2-oxo-1,2-dihydro-4-pyridyl isomer have been synthesized.[130]

***N*-Substituted Amino-acids.**—Studies of a conventional type are represented in the synthesis of side-chain mono-, di-, and tri-methyl arginines from ornithine and

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[124] M. E. Gustafson, D. Miller, P. J. Davis, J. P. Rosazza, C. Chang, and H. G. Floss, *J.C.S. Chem. Comm.*, 1977, 842.
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[129] G. Büchi and T. Kamikawa, *J. Org. Chem.*, 1977, **42**, 4153.
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correspondingly *N*-methylated *S*-methylisothiouronium iodide,[131] and of N^{β}-alkyl-β-amino-alanines.[132]

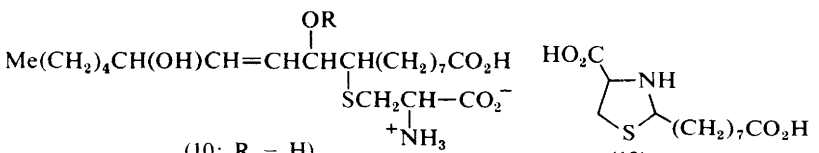
Secondary amines formally derived from two α-amino-acids have been known for many years, and have become of renewed interest recently because of their occurrence as metabolites of crown gall tumours.[19, 40] Diastereoisomer mixtures formed from α-ketoglutaric acid by condensation with L-arginine followed by borohydride reduction[133, 134] have been separated into the natural product nopaline, and its isomer isonopaline; all four isomers of octopine, formed similarly from D- or L-arginine and pyruvic acid, have been obtained.[135] Lysinoalanine, a structurally-similar secondary amine, is in equilibrium with lysine and dehydroalanine in aqueous solution.[136, 137]

Modification of the amino-acid amine function can be brought about directly in certain cases, *e.g.* the preparation of 1-nitro-proline, -pipecolic acid, and -sarcosine,[138] by peroxytrifluoroacetic acid oxidation of the *N*-nitroso-imino-acids,[138] but *N*-hydroxy-amino-acids are best prepared from an α-keto-acid and hydroxylamine followed by sodium cyanoborohydride reduction.[139]

α-Aza-amino-acids.—New results on analogues in which the α-CH group of the protein amino-acids is replaced by a nitrogen atom are the synthesis[140] of N^{α}-ethoxycarbonyl-α-aza-ornithine phenyl ester, and the unusually high tendency for *N*-acyl derivatives to cyclize to oxadiazolones.[141]

α-Amino-acids containing Sulphur or Selenium.—Optically-active cysteine derivatives are available in optical yields up to 54% by addition of a thiol to methyl α-phthalimidoacrylate or to a 4-methyleneoxazolone in the presence of a cinchona alkaloid.[142] A stereospecific synthesis of (2*S*,3*R*)-2-amino-3-mercaptobutyric acid employs Boc-D-allothreonine methyl ester as starting material.[143]

The preparation of *S*-substituted cysteines generally involves routine methods, but the reaction of cysteine with linoleic acid hydroperoxide in ethanol to give (10)—(12)[144] is of particular interest. L-Cysteine gives 2*S*,5*S*-, and 2,5-*SS*-di-



(10; R = H)
(11; R = Et)

(12)

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[141] C. J. Gray, J. C. Ireson, and R. C. Parker, *Tetrahedron*, 1977, **33**, 739.
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[143] J. L. Morell, P. Fleckenstein, and E. Gross, *J. Org. Chem.*, 1977, **42**, 355.
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cysteinyldopa and a small (1%) yield of the hitherto unknown 6-*S*-cysteinyldopa through mushroom tyrosinase co-oxidation with dopa.[145]

Selenocystine continues to find use for the synthesis of selenium analogues of the well-known sulphur amino-acids, this time in combination with formaldehyde to give DL-selenaproline.[146]

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.*
3(2′, 3′, or 4′-Fluorophenyl)-DL-alanine	147
3-(2′, 3′, or 4′-Trifluoromethylphenyl)-DL-alanine	147
3-(2′-Chloro-5′-trifluoromethylphenyl)-DL-alanine	147
3-(4′-Chloro-5′-trifluoromethylphenyl)-DL-alanine	147
3-(2′,5′-Difluorophenyl)-DL-alanine	147
3-(3′-Carboxy-4′-hydroxyphenyl)-DL-alanine	148
3-(3′-Carboxy-4′-aminophenyl)-DL-alanine	148
2-(3′-Aminophenyl)glycine	148
2-(3′-Hydroxymethylphenyl)glycine	148
2-(3′-Aminomethylphenyl)glycine	148
2-(3′-Carboxyphenyl)glycine	148
2-(3′-Carboxy-4′-hydroxyphenyl)glycine	148
3-(1′-Tetralyl)alanine	149
3-[5′-(5,6,7,8-Tetrahydroquinolinyl)]alanine	149
2-(1′-Tetralyl)glycine	150
2-(5,6,7,8-Tetrahydroquinolin-5-yl)glycine	150
3-Methyl-DL-histidine	151
3-Ethyl-DL-histidine	151
3-n-Hexyl-DL-histidine	151
3-(3-*p*-Hydroxyphenyl-1,2,4-oxadiazolyl)-DL-alanine	152
4-(Tetrazol-5′-yl)-2-aminobutyric acid	153
S-(Uridin-5-yl)cysteine	154
N-Phthalazinyl-DL-lysines	154*a*

Labelled Amino-acids.—Syntheses have been recorded of (*R*)-and (*S*)-[2-^{2}H]-glycine derivatives, and the following labelled protein amino-acids: (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]serine,[156] (*methyl-R*) and (*methyl-S*)-[*methyl*-^{2}H,^{3}H]methionine,[157] selectively deuteriated histidine, tyrosine, phenylalanine, and tryptophan,[158]

[145] S. Ito and G. Prota, *Experientia*, 1977, **33**, 1118.
[146] C. De Marco, R. Coccia, A. Rinaldi, and D. Cavallini, *Ital. J. Biochem.*, 1977, **26**, 51.
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[158] H. R. Matthews, K. S. Matthews, and S. J. Opella, *Biochim. Biophys. Acta*, 1977, **497**, 1.

(2*S*,3*R*)-[3-^{2}H]tyrosine,[156] [5,7-$^3H_2$] and [4,6-$^3H_2$]tryptophan,[159] and (2*S*,3*S*)- and (2*S*,3*R*)-[3-^{14}C,3-^{3}H]analogues,[124] phenyl-deuteriated or tritiated phenylalanines,[160a] and [*methyl*-$^2H_3$]-DL-threonine.[160b] A general method for the preparation of [2-^{2}H]amino-acids from the ^{1}H-analogues [161] employs Ac_2O and ^2H_2O, based on the well-known lability of the ring hydrogen atom of the 2-methyloxazol-5(4*H*)-one formed in this reaction. The list of ^{3}H-labelled amino-acids is lengthened by reports of [$^3H_2$]ruthenium oxide–alumina treatment of taurine,[162] and of similar exchange processes with proline.[163] However, a particularly interesting study of direct exchange with valine and isoleucine, involving microwave discharge activation of $^3H_2$, has appeared;[164] ^{3}H atoms formed in this way cause general, but not random, exchange. The α-position is least readily exchanged in solid L-valine (to the extent of 7.1%), but with net retention of configuration; 32.7% exchange occurs at the β-position, and 60.2% at the γ-carbon atom, and β-exchange involves inversion of configuration.[164b] Small amounts of tritiated glycine were formed by side-chain cleavage in these experiments.[164] Addition as well as ^{3}H–^{1}H exchange is observed in ^{3}H-atom attack on 3,4-dehydroproline and L-2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid.[164]

(2*R*,3*S*)-[*U*-^{14}C,3-$^3H_1$]- and (2*R*,3*R*)-[*U*-^{14}C,2,3-$^3H_2$]cysteine, together with (2*R*)-[*U*-^{14}C,3,3,3′,3′-$^3H_4$]cystine, have been employed in studies of penicillin G biosynthesis.[165] ^{14}C-Labelled *O*-succinyl-L-homoserine has been synthesized.[166]

Several papers have appeared describing the synthesis of ^{11}C-carboxyl-labelled amino-acids.[167] Other amino-acids labelled with short-lived isotope (^{13}N-labelled alanine [168] and asparagine)[169] and ^{15}N-labelled alanine [170] and other protein amino-acids [171] have been reported, while the synthesis of DL-[2-^{13}C,3′-^{15}N,2′,5′-$^2H_2$]histidine [172] represents something of a jamboree of labelling approaches.

Cysteine-[^{35}S]sulphonic acid [173] and *p*-[^{128}I]iodophenylalanine [174] have been prepared.

Resolution of Amino-acids.—Detailed studies of the preferential adsorption of the

[159] G. P. Gardini and G. Palla, *J. Labelled Comp. Radiopharm.*, 1977, **13**, 339.
[160] (*a*) A. Kolodziejczyk and A. Arendt, *Roczniki Chem.*, 1977, **51**, 659; (*b*) K. Fuksova and J. Benes, *Radiochem. Radioanalyt. Letters*, 1977, **30**, 187.
[161] D. A. Upson and V. J. Hruby, *J. Org. Chem.*, 1977, **42**, 2329.
[162] R. E. Hrsuka, R. J. Huxtable, and H. I. Yamamura, *Analyt. Biochem.*, 1977, **79**, 568.
[163] L. J. Altman and N. Silberman, *Analyt. Biochem.*, 1977, **79**, 302.
[164] (*a*) R. L. E. Ehrenkaufer, A. P. Wolf, W. C. Hembree, and S. Lieberman, *J. Labelled Comp. Radiopharm.*, 1977, **13**, 359, 367; (*b*) R. L. E. Ehrenkaufer, W. C. Hembree, S. Lieberman, and A. P. Wolf, *J. Amer. Chem. Soc.*, 1977, **99**, 5005.
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[166] R. C. Greene, *Analyt. Biochem.*, 1977, **78**, 182.
[167] B. W. Wieland, L. C. Washburn, R. R. Turtle, R. L. Hayes, and T. A. Butler, *J. Labelled Comp. Radiopharm.*, 1977, **13**, 202; L. C. Washburn, B. W. Wieland, T. T. Sun, and R. L. Hayes, *ibid.*, p. 203; S. Reiffers, E. Beerling, W. Vaalburg, W. Ten Hoeve, M. G. Woldring, and H. Wynburg, *ibid.*, p. 198.
[168] L. Spolter, M. B. Cohen, C. C. Chang, and N. S. MacDonald, *J. Labelled Comp. Radiopharm.*, 1977, **13**, 204.
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[174] P. Gielow, *Internat. J. Appl. Radiation Isotopes*, 1977, **28**, 326.

D-enantiomer from solutions of DL-amino-acid derivatives by (−)-quartz [175] show that a protonated amino-group favours adsorption and enhances the enantioselectivity. While the objective of this study is related to possible mechanisms for the predominance of L-amino-acids in life processes, other adsorbents are far more effective for routine resolution of amino-acids. Amino-acids bound to polystyrene [176] or polyacrylamide [177] provide a stationary phase for the resolution of DL-amino-acids, when copper(II) or nickel(II) ions are present. Further details have been published [178] (see Vol. 9, p. 17) of the resolution of an acidic amino-acid (DL-aspartic and glutamic acids) by preferential complexation of one enantiomer with copper(II) perchlorate and an enantiomer of a basic amino-acid (arginine, lysine, or ornithine); the reverse process, in which a racemic basic amino-acid is resolved using an enantiomer of an acidic amino-acid, has also been established,[178] and the procedure has been extended to the resolution of DL-histidine with copper(II) perchlorate and L-asparagine.[179] A related study [180] using cobalt(III) complexes of amino-acid Schiff bases describes the moderate enrichment of the relative amount of one enantiomer in a solution of a DL-amino-acid.

Chromatographic separation of diastereoisomers formed between a chiral reagent and a DL-amino-acid is illustrated for *N*-(*d*-camphor-10-sulphonyl)-amino-acid *p*-nitrobenzyl esters [181] and (−)-α-methoxy-α-methyl-1-naphthaleneacetyl-amino-acid methyl esters [182] using h.p.l.c. The analytical use of g.l.c. for the same purpose, using either the diastereoisomer separation principle or the use of chiral stationary phases, is discussed in Section 6 of this Chapter.

Resolution of *N*-acetyl-*p*-methoxyphenylglycine as its ammonium salt provides another example of the preferential crystallization procedure,[183] while time-honoured diastereoisomeric salt separation procedures have been used for the resolution of *N*-benzyloxycarbonyl γγ-di-t-butyl γ-carboxy-DL-glutamate,[184] and in several other studies.[92, 112, 113]

Novel approaches employing enzyme systems are involved in the asymmetric hydrolysis of DL-5-indolylmethylhydantoin to L-tryptophan,[185] and the formation of L-lysine from DL-α-amino-ε-caprolactam.[186] Both these procedures are bacterial syntheses, while a more conventional application, the preferential hydrolysis of the 2*S*-diastereoisomer of methyl (2*RS*,4*S*)-2-acetylamino-4-methylhexanoate, involves α-chymotrypsin catalysis.[187]

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[179] T. Sakurai, O. Yamauchi, and A. Nakahara, *J.C.S. Chem. Comm.*, 1977, 718.
[180] Y. Fujii, M. Sano, and Y. Nakano, *Bull. Chem. Soc. Japan*, 1977, **50**, 2609.
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4 Physical and Stereochemical Studies of Amino-acids

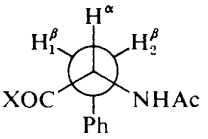
Crystal Structures of Amino-acids and their Derivatives.—Scope still exists for new *X*-ray studies with simple amino-acid derivatives, and *N*-formyl-L-methionine,[188] 2′-hydroxy-DL-phenylalanine,[189] 1-methyl-3-carbamoylpyridinium *N*-acetyl-L-tryptophanate,[190] DL-homocysteic acid,[191] *N*-pivalyl-*N*′-methyl-L-glutaminyl methylamide,[192] the L-valine aza-homologue AcNHCHPr^{i}NHCONHMe,[193] L-histidine hydrochloride,[194] zinc(II) and cadmium(II) complexes of *S*-methyl-L-cysteine,[195] and DL-aspartic acid hydrochloride [194a] have come under scrutiny. More unusual compounds subjected to *X*-ray study are (2*S*,3*R*)-2-amino-3-hydroxypent-4-ynoic acid, a toxic α-amino acid from the fungus *Sclerotium rolfsii*,[22] and coronatine, an acyl derivative of the aminocyclopropane carboxylic acid in Scheme 3.[194b]

Assignment of configuration at sulphur to diastereoisomers of *S*-adenosyl-L-methionine and of *S*-carboxymethyl-L-methionine has been reported.[196]

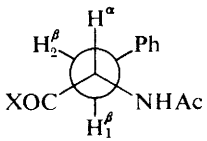
The knowledge of the crystal structure of an amino-acid leads to speculation about its conformation revealed in this way, especially any differences compared with conformations it adopts in proteins, and an example of a continuing trickle of papers of this type deals with isoleucine and alloisoleucine salts.[197] Neutron diffraction analysis permits the placing of hydrogen atoms, and L-histidine monohydrochloride monohydrate [195a] is the latest of the protein amino-acids to be studied in this way.

N.M.R. Spectroscopy.—A review [198] includes coverage of the conformational behaviour of amino-acids in solution as revealed by n.m.r. studies.

Continuing studies of side-chain conformational behaviour concern several of the protein amino-acids. Selective deuteriation assists the interpretation of ^{1}H-n.m.r. data in this area, with [γ-^{2}H]leucine being shown to adopt preferentially the conformer with side-chain gauche to the amino-group, and *trans* to the



(13)



(14)

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[195] P. De Meester and D. J. Hodgson, *J. Amer. Chem. Soc.*, 1977, **99**, 6884.
[196] J. W. Cornforth, S. A. Reichard, P. Talalay, H. L. Carrell, and J. P. Glusker, *J. Amer. Chem. Soc.*, 1977, **99**, 7292.
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[198] W. A. Thomas, *Ann. Reports N.M.R. Spectroscopy*, 1976, **6B**, 1.

carboxy group.[199] A similar study of L-[β-^{2}H]phenylalanine reveals the importance of solvent in determining conformation, with the most crowded conformer (13) actually predominating in a non-polar solvent, while the proportion of (14), which would be presumed to be the preferred conformation, increases with increasing solvent polarity.[200] Long-chain *O*-alkyltyrosines also provide an unexpected result, with the most crowded conformer being the second most abundant of the three possible staggered forms.[201] Aggregation of these derivatives favours their adoption of the least crowded conformation.[201] ^{1}H-N.m.r. and c.d.–pH titration studies of histidine and its derivatives show that the side-chain conformation of this amino-acid is determined by neighbouring charged groupings;[202] the ratio of the two imidazole tautomers of histidine varies with pH, and this fact, as shown by three-bond ^{13}C–^{1}H coupling constants [203] and ^{15}N-n.m.r. of ^{15}N-enriched histidine derivatives,[204] needs to be taken into account in interpretation of pH titration data for histidine. Conformational information derived from n.m.r. data has been reported for 1-aminocyclohexanecarboxylic acid derivatives,[205] and *cis–trans* ratios for the tertiary amide bond in *N*-acetyl-L-proline methylamides as a function of solvent (the *cis*-form is favoured in polar solvents) have been determined.[206]

More specialized n.m.r. studies dealing with amino-acids have been reported, in some cases developing instrumental techniques (*e.g.* wide-line n.m.r. lineshape analysis [207]), but relaxation time data for proline in water–glycerol mixtures [208] and for solid amino-acids [209] provide information on dynamic behaviour. Double nuclear resonance of ^{14}N,^{2}H-labelled glycines in various crystalline modifications has been studied.[210] Other less sophisticated physical studies provide acid dissociation constants for di-amino-acids [211] and exchange rates of the tryptophan N^{indole} proton with water as a function of pH and temperature.[212] A particularly interesting study [213] employs ^{35}Cl-n.m.r. for studying the interaction of Cl^- ions with arginine, histidine, or lysine as a function of pH.

Interaction of D- or L-tryptophan with human serum albumin has been deduced [214] to involve the benzo moiety and the amino-group as 'binding' sites.

O.R.D. and C.D. Spectra.—Advances in instrumentation, particularly the

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[209] E. R. Andrew, W. S. Hinshaw, M. G. Hutchins, R. O. I. Sjoeblom, and P. C. Canepa, *Mol. Phys.*, 1976, **32**, 795.
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[211] T. L. Sayer and D. L. Rabenstein, *Canad. J. Chem.*, 1976, **54**, 3392.
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[214] J. P. Monti, M. Sarrazin, C. Briand, and A. Crevat, *J. Chim. Phys. Phys.-Chim. Biol.*, 1977, **74**, 942.

penetration to shorter wavelengths which is possible in some prototypes, continue to provide new data on amino-acids, and the o.r.d. and c.d. of different conformations of L-proline to 160 nm have been calculated, to assist the interpretation of experimental data.[215] Vibrational c.d. spectra of D- and L-alanine in ^2H_2O have been measured, illustrating the potential of the technique for the study of solution behaviour.[216]

α-Trimethylammonio-acid amides appear to show more complex c.d. behaviour in the wavelength region 200—260 nm than would be expected for the amide chromophore.[217] Routine studies with chromophorically-substituted amino-acids continue, recent papers describing attempts to establish correlations between sign of Cotton effect and absolute configuration for *N*-acetoacetyl-,[218] *N*-2,4-dinitrophenyl-,[219] and *N*-salicylidene-amino-acids.[220]

Mass Spectrometry.—A larger number of papers than usual has come under scrutiny for inclusion in this Section, due partly to the efforts of newcomers who have adopted techniques established by pioneer specialists, but more because of the possibilities in structure determination using newer, milder, ionization techniques (chemical ionization and field ionization).

After recent success (see Vol. 9, p. 21) in obtaining data on zwitterionic amino-acids, new results have been reported on the in-beam electron-impact mass spectra (e.i.m.s.) of amino-acids.[221] Chemical ionization mass spectrometry (c.i.m.s.) of α-amino-acids, using NH_4^+ for ionization, appears particularly promising, with M + 1 peaks obtained in each of 19 cases, these being base peaks in the spectra of all but two of the compounds.[222] Peaks at *m/e* 101 and 116 seen in the mass spectra of methionine, ionized either by electron impact or by pyrolysis followed by electron impact, are formed by different pathways.[223] The base peak in the mass spectrum of lysine methyl ester at *m/e* 84 is generated by sequential loss of the methoxycarbonyl radical from the parent ion, followed by elimination of NH_3.[224]

For routine analysis, an amino-acid is converted into one of a range of derivatives of sufficient volatility that thermal fragmentation is avoided in the mass spectrometer, and *N*-trifluoroacetylamino-acid n-butyl esters,[225–227] *N*-penta-

[215] F. S. Richardson and S. Ferber, *Biopolymers*, 1977, **16**, 387.
[216] M. Dien, P. J. Gotkin, J. M. Kupfer, A. G. Tindall, and L. A. Nafie, *J. Amer. Chem. Soc.*, 1977, **99**, 8103.
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[227] K. R. Leimer, R. H. Rice, and C. W. Gehrke, *J. Chromatog.*, 1977, **141**, 121.

fluoropropionyl,[228] *N*-succinyl,[229] *N*-benzoyl,[225] *N*-trifluoroacetyl-L-prolyl,[225] and *N*-pentafluorobenzoyl analogues,[225] with (−)-menthyl esters in place of n-butyl esters in some cases,[225] have been used for ultramicrodetermination of amino-acids. Amino-acid phenylthiohydantoins can be identified at levels down to 3 nmoles,[230] and derivatives formed between amino-acids and fluorescamine can be identified by c.i.m.s.[231] Uses in the analysis of amino-acids in physiological samples have been found for c.i.m.s. in the identification of L-dopa, α-methyl-L-dopa, and their metabolites,[228] and in the quantitative analysis of amino-acids in blood specimens.[232]

Field ionization mass spectra can be obtained with 50 nmole samples of ^{15}N-labelled amino-acids.[233]

Other Physical and Theoretical Studies.—Results of spectroscopic studies not covered in a preceding section are discussed here, also miscellaneous physico-chemical studies often providing data of value in accounting for the biological roles of amino-acids.

Raman spectroscopic studies of a familiar type with *N*-acetylamino-acid methyl amides [234] deal with the conformational behaviour of the compounds in solution, compared with their structures in the solid state. Polarized Raman and far i.r. spectra of glycine crystalline modifications have been measured [235] and dielectric relaxation spectra of α- and β-alanine.[236] E.s.r. and ENDOR studies of *X*-irradiated single crystals of amino-acids [237] and of *N*-acetyl-L-cysteine [238] are reported by several research groups. The e.s.r. spectra of 1,4-disubstituted pyrazine cation radicals formed in reaction mixtures containing amino-acids and sugars have been interpreted.[2a, 239]

Under the heading 'miscellaneous physico-chemical studies', papers deal with the thermodynamics of dissolution of two crystalline polymorphs of DL-2-aminobutanoic acid, enthalpies of formation of glycine and L-alanine,[241] enthalpies of interaction of sodium chloride with amino-acids in aqueous solution,[242] activity coefficients of γ-amino-butyric acid and glycylglycine in aqueous sucrose

[228] C. R. Freed, R. J. Weinkam, K. L. Melmon, and N. Castagnoli, *Analyt. Biochem.*, 1977, **78**, 319.
[229] D. C. DeJongh, G. Faus, M. S. B. Nayar, G. Boileau, and L. Brakier-Gingras, *Biomed. Mass Spectrom.*, 1976, **3**, 191.
[230] I. V. Nazimov, N. B. Levina, I. A. Bogdanova, and B. V. Rosynov, *Bio-org. Khim.*, 1977, **3**, 192.
[231] J. J. Shieh, K. Leung, and D. M. Desiderio, *Analyt. Letters*, 1977, **10**, 575.
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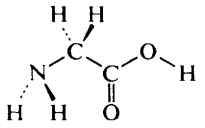
[240] M. H. Abraham, E. Ah-Sing, R. E. Marks, R. A. Schulz, and B. C. Stace, *J.C.S. Faraday Trans. I*, 1977, **73**, 181.
[241] N. N. Song, R. Sabbah, and M. Lafitte, *Thermochim. Acta*, 1977, **20**, 371.
[242] J. W. Larson, W. J. Plymale, and A. F. Joseph, *J. Phys. Chem.*, 1977, **81**, 2074.

solutions,[243] apparent molal heat capacities of amino-acids, interpreted in terms of interactions between neutral or charged amino and carboxy groups,[244] and the viscosity of solutions of glycine or DL-alanine in dimethylformamide–water mixtures.[245] Studies of possible relevance to primordial events have identified the site of adsorption of amino-dicarboxylic acids (aspartic acid, glutamic, α-amino-pimelic, and α-aminoadipic acids) to hydroxylapatite as the α-carboxy group; L-arginine is adsorbed if the solid is pre-treated with phosphate buffer.[246] Activated charcoal scarcely adsorbs amino-acids from aqueous solutions, with the notable exceptions of tryptophan, phenylalanine, and methionine.[247]

Molecular orbital computation studies include important areas of amino-acid behaviour. Glycine adopts the structure (15) with bifurcated hydrogen bonds,



(15)

when achieving the lowest energy conformation of its neutral form;[248] structural formulae for amino-acids depicted with localized positive charges may not be realistic,[249] and this is a matter of importance in deducing the electrical structures of binding sites of neurotransmitters, including γ-aminobutyric acid and acetylcholine.[249] Correlation between molecular mechanics calculations and *X*-ray and n.m.r. data is included in deducing the ranking of conformations available to *N*-acetylproline methyl ester.[250] Interaction energies involved in the formation of amino-acid–water complexes have been calculated.[251]

5 Chemical Studies of Amino-acids

Racemization.—Applications of racemization kinetics for the estimation of the age of fossils and relatively much younger mammal teeth and bones, as well as ancient wood samples, have been reviewed in recent Volumes of this Report. Knowledge of the age of a sample from ^{14}C data, together with the temperature-dependence of the racemization rate constant for a given amino-acid, allows an estimate to be made of the average temperature to which a sample has been subjected from the time it was laid down to the present time, and an average temperature for the last *ca.* 2200 years of 279 ± ~6 K has been estimated from the degree of racemization found for aspartic acid, glutamic acid, proline, and phenylalanine in sequoia heartwood.[252] A rather smaller racemization rate

[243] H. Uedaira, *Bull. Chem. Soc. Japan*, 1977, **50**, 1298.
[244] S. Cabani, G. Conti, E. Matteoli, and A. Tani, *J.C.S. Faraday Trans. I*, 1977, **73**, 476.
[245] S. Phang, *Austral. J. Chem.*, 1977, **30**, 1605.
[246] M. Kresak, E. C. Moreno, R. T. Zahradnik, and D. I. Hay, *J. Colloid Interface Sci.*, 1977, **59**, 283.
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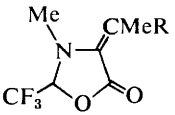
[250] D. F. De Tar and N. P. Luthra, *J. Amer. Chem. Soc.*, 1977, **99**, 1232.
[251] E. Clementi, F. Cavallone, and R. Scordamaglia, *J. Amer. Chem. Soc.*, 1977, **99**, 5531.
[252] M. H. Engel, J. E. Zumberge, and B. Nagy, *Analyt. Biochem.*, 1977, **82**, 415.

constant (2.1×10^{-5} y^{-1}) for aspartic acid in this source, in comparison with that of the same amino-acid in mammalian samples (see Vol. 8, p. 20), should be noted. Bada's group have shown that the D : L-ratio for aspartic acid in human lens protein is directly related to age,[253] and have reviewed the role of aspartic acid racemization in the ageing process.[254] A correction[255] to an earlier conclusion based on L : D-ratios of proline and hydroxyproline in a wood sample (see Vol. 9, p. 23) is required because of an incorrect assignment of a ^{14}C-calibration sample, and not because of any shortcomings in the basis of the racemization dating procedure.

The racemization of threonine reaches an equilibrium position at about 20% epimerization at the α-position, and the threonine–allothreonine ratio determined for fossil foraminifera cannot be used in geochronology.[256]

Further application of amino-acid racemization data in areas such as those described above is likely to lead to less confident conclusions until the influence of the many parameters involved in amino-acid racemization is better understood. In a review of the applications which have been made, more caution is advocated.[257] The factors which influence the racemization rates of amino-acids in aqueous solution have been listed as ionic strength, pH, nature of buffer, and buffer concentration.[258]

General Reactions.—A number of improvements to standard methods of substitution or modification of amino- and carboxy-groups of amino-acids have been published. *N*-Protected amino-acids can be esterified under mild neutral conditions by treating their caesium salts with alkyl halides.[259] While *N*-trifluoroacetyltyrosine can be prepared conveniently using 1,1,1-trifluoro-3,3,3-trichloroacetone



(16)

in DMSO,[260] *N*-methylvaline or *N*-methylisoleucine gives the product (16; R = Me, Et respectively) of oxidative cyclization of the intermediate *N*-trifluoroacetyl derivatives on treatment with trifluoroacetic anhydride.[261] Formation of 5-(*N*-trifluoroacetamido)thiazoles from *N*-thiobenzoylamino-acid amides and trifluoroacetic anhydride has been reported.[262] Cyclization of *N*-benzyloxycarbonyl-L-α-amino-acids with PCl_5 gives 2-benzyloxyoxazol-5(4*H*)-ones,[263] not

[253] P. M. Masters, J. L. Bada, and J. S. Zigler, *Nature*, 1977, **268**, 71.
[254] P. M. Helfman, J. L. Bada, and M.-Y. Shou, *Gerontology*, 1977, **23**, 419.
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[260] C. A. Panetta, *Org. Synth.*, 1977, **56**, 122.
[261] W. A. Koenig and U. Hess, *Annalen*, 1977, 1087.
[262] G. C. Barrett, J. Hume, and A. A. Usmani, in 'Solid Phase Methods in Protein Sequence Analysis', ed. A. Previero and M.-A. Coletti-Previero, Elsevier – North-Holland Biomedical Press, 1977, p. 57.
[263] J. H. Jones and M. J. Witty, *J.C.S. Chem. Comm.*, 1977, 281.

the isomeric benzyloxycarbonylaziridinones as claimed earlier (see Vol. 6, p. 29).

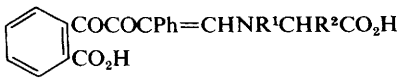
Formic anhydride, formed *in situ* by the reaction of formic acid with dicyclohexylcarbodi-imide in pyridine, is an effective reagent for the formylation of α-amino-acid t-butyl esters,[264] readily converted into α-isocyano-esters by reaction with phosgene,[265] for use in the Ugi four-component condensation reaction leading to N^{α}-acyl-N^{α}-alkylamino-acid amides.

While normal Dakin–West ketonic products AcNHCHRCOMe are formed from aliphatic amino-acids and acetic anhydride at 100 °C, higher temperatures (140—150 °C) lead to β-acetoxyenamine derivatives $Ac_2NCR{=}CMeOAc$.[266]

The nitrosation of imino-acid derivatives has been reviewed.[267] The reaction of L-histidine with nitrous acid in hydrochloric acid gives the corresponding 2-chloro-3-(4′-imidazolyl)propionic acid with retention of configuration.[268] Also reported in this paper[268] is the synthesis of N^{π}-methylhistidine by reaction of protected N^{1m}-benzylhistidine with trimethyloxonium tetrafluoroborate followed by de-protection.

The reactions of amino-acids with aldehydes include processes of importance in metabolism, food science, and analysis. The Maillard reaction of methionine or tryptophan with glucose in aqueous solution proceeds at maximum rate at pH 11, suggesting catalysis by base.[269] Pyrroles and furans are formed in reactions of fructose or rhamnose with alanine or γ-aminobutyric acid at pH 3.5.[270] Simpler reaction products are formed between amino-acids and phenylglyoxal, glyoxal, and methylglyoxal at pH 7 at 15 °C, arginine reacting faster than other amino-acids, especially as the pH is raised.[271]

A series of papers dealing with *o*-phthalaldehyde–amino-acid–thiol condensation products has appeared. The 1-alkylthio-2-alkylisoindole structure assigned to the fluorescent product (see Vol. 9, p. 24) has been confirmed by synthesis,[272a] and the stability of the fluorescence of the 1-alkylthio-compound suggests that the analytical use of this system would be improved by replacing the currently-used mercaptoethanol by ethanethiol.[272b] Lower relative fluorescence results from this reaction when non-protein amino-acids lacking an α-proton are involved.[273] Further studies of another increasingly widely used procedure for fluorescence detection of amino-acids, reaction with fluorescamine, have been described (see Vol. 8, p. 19); imino-acids give products (17) showing maximum long-wavelength



(17)

[264] M. Waki and J. Meienhofer, *J. Org. Chem.*, 1977, **42**, 2019.
[265] R. Urban, D. Marquarding, P. Seidel, I. Ugi, and A. Weinelt, *Chem. Ber.*, 1977, **110**, 2012.
[266] S. I. Zavyalov and G. I. Ezhova, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1977, 219.
[267] R. Bonnett and P. Nicolaidou, *Heterocycles*, 1977, **7**, 637.
[268] H. C. Beyerman, L. Maat, A. Noordan, and A. van Zon, *Rec. Trav. chim.*, 1977, **96**, 222.
[269] E. Dworschak and F. Orsi, *Acta Aliment. Acad. Sci. Hung.*, 1977, **6**, 59.
[270] P. E. Shaw and R. E. Berry, *J. Agric. Food Chem.*, 1977, **25**, 641.
[271] K. Takahashi, *J. Biochem.*, 1977, **81**, 395.
[272] S. S. Simons and D. F. Johnson, (*a*) *J.C.S. Chem. Comm.*, 1977, 374; (*b*) *Analyt. Biochem.*, 1977, **82**, 250.
[273] J. R. Cronin and P. E. Hare, *Analyt. Biochem.*, 1977, **81**, 151.

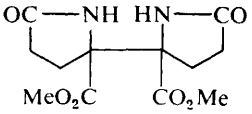
absorption in the range 300—320 nm.[274] Several papers have appeared[275] advocating the use of 4-*NN*-dimethylaminonaphthylazobenzene-4′-isothiocyanate as a colour reagent for amino-acids, giving purple arylthiohydantoins.

Oxidation and reduction of amino-acids are represented in a number of analytical applications (see Section 6) and in reports of the oxidation of amino-acids to CO_2 and nitriles (unsuitable for analytical use, since results are not reproducible within ± 5%),[276] and the electrochemical[277] and hydride reduction[278] of amino-acids. The 2-amino-alkanol formed by the treatment of an L-α-amino-acid with any one of a number of familiar reducing agents is essentially optically-pure,[278] even though wide variations in α_D are observed in the product, depending on the route used. It is concluded[278] that these variations are due to impurities, but since these are stated to be present in amounts smaller than 2%, they must possess extraordinarily large optical rotations.

Alkylamines formed by heating glycine at 240 °C with alumina (simulated primitive earth conditions) include methyl, ethyl, n-propyl, n-butyl, dimethyl, and diethylamines,[279a] while alanine, γ-aminobutyric acid, norvaline, norleucine, sarcosine, and small amounts of *N*-methylamino-acids are formed when the reaction mixture also contains basic manganous carbonate.[279b]

Specific Reactions of Individual Protein Amino-acids.—While some of the reactions covered here are relevant to biological roles of amino-acids (see following section), they mostly reflect the chemistry of side-chain functional groups.

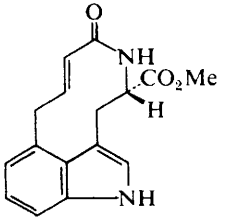
Permanganic acid oxidation of *N*-acylprolines gives corresponding pyroglutamates and side-chain protected ornithines give glutamates;[280] these are unusual products since amino-acids are generally oxidized to aldehydes and ammonia in this reaction. Photo-oxidation of methyl-DL-pyroglutamate in benzene gives a mixture of *meso*- and (±)-oxidative dimerization products (18).[281] Iodine–DMSO oxidation of L-cystine is not possible owing to poor solubility,[282] but with the addition of 12M-HCl as catalyst, stoicheiometric oxidation to cysteic acid, isolated as a 1 : 1-molecular compound with DMSO, is achieved in this system. H_2O_2-Oxidation of L-cystine, lanthionine, or L-homocystine in the presence of hydrochloric acid gives a mixture of sulphonic acids, sulphoxides, and sulphones.[283] Electrochemical reduction of cystine and oxidation of cysteine at a



(18)

[274] V. Toome, B. Wegrzynski, and J. Dell, *Biochem. Biophys. Res. Comm.*, 1976, **71**, 598.
[275] J. Y. Chang and E. H. Creaser, *J. Chromatog.*, 1977, **132**, 303; J. Y. Chang, *Biochem. J.*, 1977, **163**, 517.
[276] D. S. Mahadeveppa and N. M. M. Gade, *J. Indian Chem. Soc.*, 1977, **54**, 534.
[277] R. Saxena and M. C. Saxena, *Monatsh.*, 1977, **108**, 829.
[278] G. S. Poindexter and A. I. Meyers, *Tetrahedron Letters*, 1977, 3527.
[279] C. Ivanov and N. Slavcheva, (*a*) *Doklady Bolg. Akad. Nauk.*, 1977, **30**, 727; (*b*) *Origins Life*, 1977, **8**, 13.
[280] I. Muramatsu, Y. Motoki, K. Yabuuchi, and H. Komachi, *Chem. Letters*, 1977, 1253.
[281] N. Obata and K. Niimura, *J.C.S. Chem. Comm.*, 1977, 238.
[282] O. G. Lowe, *J. Org. Chem.*, 1977, **42**, 2524.
[283] S. H. Lipton, C. E. Bodwell, and A. H. Coleman, *J. Agric. Food Chem.*, 1977, **25**, 624.

hanging-mercury-drop electrode at several pH values has been investigated by cyclic voltammetry.[284] Selective *S*-methylation of cysteine by aqueous trimethyl phosphate is an unusual result since the other protein amino-acids are unaffected, except histidine and tryptophan to slight extents.[285] L-Cystine appears to give sulphenyl cations by HBr cleavage,[286] since the 3′-(*S*-cysteinyl) derivative is formed with L-tyrosine. In spite of a precedent for the formation of sulphenyl cations, the sulphenyl bromide appears to be a more likely intermediate in this reaction. Cysteinyl-dopa gives highly fluorescent 3,4-dihydroisoquinolines with either formaldehyde or glyoxylic acid,[287] analogous to corresponding products formed by dopa and dopamine. Di-dansylation of tyrosine can be achieved by reaction with dansyl chloride in bicarbonate buffers (pH 9.5), giving remarkably photolabile derivatives,[288] and electrophilic t-butylation of tryptophan gives the 2′,5′,7′-tri-t-butyl derivative (but the N^{im}-t-butyl derivative is the major product);[289] photoalkylation of tyrosine and tryptophan with chloroacetamide,[290] and intramolecular photocyclization[291] of tiglyl-L-tryptophan ethyl ester to give (19),



(19)

mimicking a step in alkaloid biosynthesis, are some of the more interesting papers with more than a little relevance to analytical, synthetic, and biological studies with aromatic amino-acids, as is the finding[292] that aromatic amino-acids, particularly histidine, are degraded to HCN by the action of amino-acid oxidases.

The yellow polymer formed by the reaction of L-lysine with methylglyoxal has been formulated as a series of 3-hydroxypyrrole moieties bridged by vinylene or 1,4-dihydroxy-2-oxobutylene groups.[293]

Treatment of *N*-benzyloxycarbonyl-L-glutamic anhydride with diazomethane gives *N*-benzyloxycarbonyl-α-diazomethyl-γ-methyl-L-glutamate, and not the isomer as previously claimed, which is best prepared from the corresponding α-methyl-L-glutamate.[294]

[284] M. T. Stankovitch and A. J. Bard, *J. Electroanalyt. Chem. Interfacial Electrochem.*, 1977, **75**, 487.
[285] K. Yamauchi, T. Sugimae, and M. Kinoshita, *Tetrahedron Letters*, 1977, 1199.
[286] S. Ito and G. Prota, *J.C.S. Chem. Comm.*, 1977, 251.
[287] G. Agrup, A. Bjorklund, B. Falck, S. Jacobsson, O. Lindvall, H. Rorsman, and E. Rosengren, *Histochemistry*, 1977, **52**, 179.
[288] P. L. Felgner and J. E. Wilson, *Analyt. Biochem.*, 1977, **80**, 601.
[289] E. Wünsch, E. Jaeger, L. Kisfaludy, and M. Loew, *Angew. Chem.*, 1977, **89**, 330.
[290] T. Hamada and O. Yonemitsu, *Chem. Pharm. Bull.*, 1977, **25**, 271.
[291] N. G. Anderson and R. G. Lawton, *Tetrahedron Letters*, 1977, 1843.
[292] E. K. Pistorius, H. S. Gewitz, H. Voss, and B. Vennesland, *Biochim. Biophys. Acta*, 1977, **481**, 384.
[293] A. Bonsignore, G. Leoncini, G. Andiso, L. Zetta, and P. Ferrati, *Ital. J. Biochem.*, 1977, **26**, 162.
[294] C. T. Clarke and J. H. Jones, *Tetrahedron Letters*, 1977, 2367.

Specific Reactions of Amino-acids Related to Biochemical Processes.—Some items cited in the preceding section could equally well have found a place here, although binding studies of aliphatic amino-acids with riboflavin,[295] L-cysteine with vitamin B_{12} (effect of micelles),[296] tryptophan and arginine derivatives with nucleosides,[297] and lysine, histidine, and cysteine derivatives with ATP,[298] clearly have a place in this section.

Methylmercury is formed by the photolysis of aliphatic amino-acids in the presence of mercury(II) chloride, pointing to a mechanism for the biogenesis of this pollutant.[299]

The mechanism of Schiff base formation between pyridoxal-5″-phosphate and DL-alanine in aqueous solution involves a carbinolamine intermediate.[300]

Effects of Electromagnetic Radiation on Amino-acids.—This title is used to collect papers concerned with photochemical and radiolytic studies of amino-acids. Under the former heading, continuing studies of tryptophan and substituted phenylalanine derivatives includes flash photolysis of tryptophan in aqueous solution,[301, 302] and of *N*-acetyltryptophanamide,[303] eosin-sensitized photo-oxidation of tyrosine and other substituted phenylalanines, studied by steady-state kinetic and flash photolytic methods,[304] and photolysis of *N*-acetyl-*p*-nitrophenylalanine ethyl ester in aqueous solutions, to give a small (4%) yield of the azoxy-analogue.[305] Fluorescence excitation and excitation polarization spectra of tryptophan at −58 °C in propylene glycol [306] contribute to knowledge of conformational properties of tryptophan residues in peptides and proteins.

γ-Radiolysis studies (^{60}Co radiation) of amino-acids [307] include specific studies of tyrosine (which yields dopa in aqueous solution) [308] and histidine.[309] Amino-acids with alkyl or benzyl-type side-chains are particularly resistant to γ-radiolysis in aqueous solutions.[310] Radiolytically-generated hydrogen atoms degrade methionine in aqueous solution to α-aminobutyric acid, but have no effect on phenylalanine.[311] Hydrated electrons formed by radiolysis of aqueous solutions react with solutes to form radicals, which undergo further transformations; a kinetic study of this initial step has been carried out for tryptophan.[312] Radicals

[295] N. A. Garcia, J. Silber, and C. Previtali, *Tetrahedron Letters*, 1977, 2073.
[296] F. Nome and J. H. Fendler, *J. Amer. Chem. Soc.*, 1977, **99**, 1557.
[297] V. I. Bruskov and V. N. Bushuev, *Biofizika*, 1977, **22**, 26.
[298] I. Ovary, S. Fazekas, V. Szekessy-Hermann, I. Kulovics, and P. Juhasz, *Acta Agron. Acad. Sci. Hung.*, 1977, **26**, 23.
[299] K. Hayashi, S. Kawai, T. Ohno, and Y. Maki, *J.C.S. Chem. Comm.*, 1977, 158.
[300] B. H. Jo, V. Nair, and L. Davis, *J. Amer. Chem. Soc.*, 1977, **99**, 4467.
[301] J. F. Baugher and L. I. Grossweiner, *J. Phys. Chem.*, 1977, **81**, 1349.
[302] B. Finnstrom, *Chem. Scripta*, 1976, **10**, 184.
[303] R. F. Evans, C. A. Ghiron, R. R. Kuntz, and W. A. Volkert, *Chem. Phys. Letters*, 1976, **42**, 415.
[304] F. Rizzuto and J. D. Spikes, *Photochem. Photobiol.*, 1977, **25**, 465.
[305] E. Escher, *Helv. Chim. Acta*, 1977, **60**, 339.
[306] B. Valeur and G. Weber, *Photochem. Photobiol.*, 1977, **25**, 465.
[307] T. Oku, *Nippon Daigaku Nojuigakubu Gakujutsu Kenkyu Hokoku*, 1977, **34**, 81, 93 (*Chem. Abs.*, 1977, **87**, 85 207, 85 208).
[308] K. R. Lynn and J. W. Purdie, *Internat. J. Radiation Phys. Chem.*, 1976, **8**, 685.
[309] J. Kopoldova and S. Hrncir, *Z. Naturforsch.*, 1977, **32C**, 482.
[310] N. A. Duzhenkova and A. V. Savich, *Khim. Vys. Energ.*, 1977, **11**, 168.
[311] L. K. Mee, S. J. Adelstein, C. M. Steinhart, and N. N. Lichtin, *Radiation Res.*, 1977, **71**, 493.
[312] M. Faraggi and A. Bettelheim, *Radiation Res.*, 1977, **72**, 81.

formed by irradiation of crystalline amino-acids yield e.s.r. spectra, referred to in a preceding section; a method for studying the effects of ^{60}Co-γ-radiation on solid amino-acids depends on measurement of the accompanying light emission.[313]

A sizeable crop of papers has appeared following recent reports of enantioselective photodegradation of DL-amino-acids. Walker [314] again casts doubt (see Vol. 8, p. 16) on the possibility that circularly-polarized light associated with polarized β-radiation can account for this phenomenon, and there is agreement [315] that the selective degradation is the result of ionization and not photodegradation, since too small a fraction of the energy of the radiation appears in the form of light. Further experimental proof of the greater degree of destruction of the D-enantiomer of DL-leucine by antiparallel-polarized ('natural') electrons has been obtained.[316, 317]

Right-circularly polarized light of wavelength 212.8 nm preferentially degrades the D-enantiomer of DL-leucine, resulting in a 1.98% enantiomeric excess of L-leucine after 59% of the original solid sample has been destroyed.[318] Similar results have been obtained with alanine, glutamic acid, and tartaric acid.[319]

6 Analytical Methods

Gas–Liquid Chromatography.—The main topics in the literature on g.l.c. analysis of amino-acids, as in earlier Volumes of this Report, are choice of derivative-forming procedure, instrumental aspects, and methods for determination of optical purity. There are several papers dealing with applications of g.l.c. and mass spectrometry; some are cited here, and others in the mass spectrometry section (see Section 4).

Volatile derivatives of amino-acids are formed by masking the amino- and carboxy-groups, and *N*-trifluoroacetyl n-butyl esters,[320–323] *N*-trifluoroacetyl hexafluoropropyl esters,[324] *N*-pentafluoropropionyl hexafluoropropyl esters,[325] *N*-heptafluorobutyryl isobutyl esters,[326, 327] *N*-acetyl propyl esters,[328, 329] and silylated thiohydantoins [330] have been illustrated further. Mixed disulphides can form during the routine derivatization procedure applied to cystine and homo-

[313] D. I. Thwaites, G. Buchan, K. V. Ettinger, J. R. Mallard, and A. Takavar, *Internat. J. Appl. Radiation Isotopes*, 1976, **27**, 663.
[314] D. C. Walker, *Origins Life*, 1976, **7**, 303.
[315] W. A. Bonner, *Nature*, 1976, **264**, 197; L. Keszthelyi, *ibid.*, p. 197.
[316] W. A. Bonner, M. A. Van Dort, M. R. Yearian, H. D. Zeman, and G. C. Li, *Israel J. Chem.*, 1977, **15**, 89.
[317] A. S. Garay, *Nature*, 1978, **271**, 186.
[318] J. J. Flores, W. A. Bonner, and G. A. Massey, *J. Amer. Chem. Soc.*, 1977, **99**, 3622.
[319] B. Norden, *Nature*, 1977, **266**, 567.
[320] R. D. Coffin and R. M. Thompson, *J. Chromatog.*, 1977, **138**, 223.
[321] W. Frick, D. Chang, K. Folkers, and G. D. Daves, *Analyt. Chem.*, 1977, **49**, 1241.
[322] B. M. Nair and L. A. Appelqvist, *J. Chromatog.*, 1977, **133**, 203.
[323] V. B. Dorogova, A. A. Kachaeva, and E. A. Shipilova, *Zhur. analit. Khim.*, 1977, **32**, 1465.
[324] R. Schmid and M. Karobath, *J. Chromatog.*, 1977, **139**, 101.
[325] J. D. Huizinga, A. W. Teelken, F. A. J. Muskiet, J. van der Meulen, and B. G. Wolthers, *New England J. Med.*, 1977, **296**, 692 (*Chem. Abs.*, 1977, **86**, 152 215).
[326] R. J. Pearce, *J. Chromatog.*, 1977, **136**, 113.
[327] S. L. Mackenzie and L. R. Hogge, *J. Chromatog.*, 1977, **132**, 485.
[328] I. Tunblad-Johansson, *Acta Pathol. Microbiol. Scand., Supplement*, 1977, **259**, 17.
[329] R. F. Adams, F. L. Vandemark, and G. J. Schmidt, *J. Chromatog. Sci.*, 1977, **15**, 63.
[330] F. E. Dwulet and F. R. N. Gurd, *Analyt. Biochem.*, 1977, **82**, 385.

cystine,[320] and there are stringent demands on the chemical operations used in these procedures if g.l.c.–m.s. procedures on picomole amounts of amino-acids are to give unambiguous results.[321] This sensitivity is required for quantitation of γ-amino-butyric acid in brain tissue or in cerebrospinal fluid,[324, 325] and m.s.-detection is required at these levels,[321, 324, 325, 327] or electron-capture,[324] or nitrogen-sensitive [329] detectors which are up to 200 times more sensitive than flame-ionization detectors.[329]

δ-Aminolaevulinic acid in blood plasma can be determined by conversion into the corresponding pyrrole by reaction with acetylacetone, using 6-amino-5-oxohexanoic acid as internal standard.[331]

The determination of the optical purity of an amino-acid involves either the separation of the enantiomeric pair of volatile derivatives of the type listed above, on an optically active stationary phase, or the separation of diastereoisomeric pairs formed between the *N*-trifluoroacetyl- [332–334] or *N*-pentafluoropropionyl- [335–337] or *N*-(−)-2-chloroisovaleryl-DL-amino-acid and an optically-active alcohol [333, 336, 337] or amine (L-leucine isopropyl ester [332]). These techniques have been used for determining L : D-ratios for amino-acids from fossils, meteorites,[332] and proteins; an extension of this technique for the assignment of absolute configuration to an enantiomer of an amino-acid from a natural source based on retention time data has been illustrated,[334, 337] for example in showing that amino-acids in alamethicins are of the L-configuration.[334]

Ion-exchange Chromatography.—The use of ion-exchange chromatography as the basis for the automated analysis of amino-acids in mixtures continues to stimulate the study of improved techniques, and of modifications designed to overcome problems with certain unusual amino-acids or with interfering species. Papers of a non-routine nature deal with the determination of hydroxylysine in urine,[338] γ-carboxyglutamic acid using an amino-acid analyser equipped with an anion-exchange column,[339] analysis of histidine, ornithine, tryptophan, and lysine in an improved low-salt alkaline buffer,[340] *N*-methylated basic amino-acids,[341] taurine in plasma,[342] and ^{14}C-labelled amino-acids.[343] Neutral amino-acids present in human plasma and cerebrospinal fluid may be analysed without prolonged initial preparation before ion-exchange separation.[344] A 9.5 h two-column separation of 55 ninhydrin-positive compounds present in blood and urine has been reported as

[331] J. MacGee, S. M. B. Roda, S. V. Elias, A. Lington, M. W. Tabor, and P. B. Hammond, *Biochem. Med.*, 1977, **17**, 31.
[332] J. J. Flores, W. A. Bonner, and M. A. Van Dort, *J. Chromatog.*, 1977, **132**, 152.
[333] M. A. Van Dort and W. A. Bonner, *J. Chromatog.*, 1977, **133**, 210.
[334] R. C. Pandey, J. C. Cook, and K. L. Rinehart, *J. Amer. Chem. Soc.*, 1977, **99**, 8469.
[335] H. Frank, G. J. Nicholson, and E. Bayer, *J. Chromatog. Sci.*, 1977, **15**, 174.
[336] W. A. Koenig, W. Rahn, and J. Eyem, *J. Chromatog.*, 1977, **133**, 141.
[337] W. A. Koenig, *Chem-Ztg.*, 1977, **101**, 201.
[338] T. Sato, T. Saito, M. Kokubun, M. Ito, and K. Yoshinaga, *Tohoku J. Exp. Med.*, 1977, **121**, 173 (*Chem. Abs.*, 1977, **86**, 135 825).
[339] H. Tabor and C. W. Tabor, *Analyt. Biochem.*, 1977, **78**, 554.
[340] K.-T. D. Liu, *J. Chromatog.*, 1977, **132**, 160.
[341] R. Helm, O. Vancikova, K. Macek, and Z. Deyl, *J. Chromatog.*, 1977, **133**, 390.
[342] K. H. Tachiki, H. C. Hendrie, J. Kelams, and M. H. Aprison, *Clinica Chim. Acta*, 1977, **75**, 455.
[343] R. Sylvester-Bradley, *Ann. Appl. Biol.*, 1977, **85**, 313.
[344] S. E. Moeller, *Analyt. Biochem.*, 1977, **79**, 590.

a stringent test of the Hitachi amino-acid analyser,[345] and a similar display of the sophistication of modern instruments in the separation of 145 ninhydrin-positive compounds[346] has been reported. The enantiomeric purity of *N*-methylamino-acids can be established by diastereoisomer formation by coupling *N*-benzyloxycarbonyl derivatives with N^{ϵ}-benzyloxycarbonyl-L-lysine benzyl ester, removal of protecting groups, and ion-exchange separation using the automatic amino-acid analyser;[347] although coupling and de-protection should be free of racemization, fewer steps are needed when g.l.c. is used for the analysis of enantiomer mixtures and greater accuracy is possible.

Modified instrumentation for converting an amino-acid analyser for fluorescence detection based on *o*-phthalaldehyde as reagent has been discussed,[348] and a useful modification allowing the different stages in ninhydrin colour development to be monitored by following absorbance changes at different wavelengths gives more scope for identifying the less common amino-acids tending to 'overlap' the protein amino-acids.[349]

The separation of amino-acids in systems comprising a hydrophobic solid support and water–organic solvent mixtures containing a small amount of anionic detergent is effectively an ion-exchange process, and allows the separation of 19 amino-acids by gradient elution within 30 minutes.[350]

Thin-layer Chromatography.—An important but well-established technique, such as t.l.c., tends to generate an increasing proportion of routine papers, and this is very much the case in amino-acid analysis and accounts for the relatively small proportion of the current literature cited in this section.

Minor improvements in amino-acid analysis are associated with high-performance t.l.c. of dansyl amino-acids[351] and *N*-phenylthiohydantoins;[352] in the latter case, the incorporation of a fluorescent agent in the silica gel leads to a lowering of the detection limit by some 10—20-fold. More conventional studies with *N*-(*p*-phenylazophenyl)thiohydantoins[353] and the separation of N^{α}-2,4-dinitrophenyl-lysine from other DNP-amino-acids[354] have been reported, while the resolution of racemic amino-acids on cellulose films[355] and the determination of the optical purity of D-[^{75}Se]selenomethionine by diastereoisomer formation with (−)-camphorsulphonyl chloride[356] are out of the ordinary, though not new in principle.

Comparison of the various colour reagents for amino-acids continues to favour the *o*-phthalaldehyde–alkanethiol fluorescence method as far as sensitivity is

[345] K. Murayama and N. Shindo, *J. Chromatog.*, 1977, **143**, 137.
[346] P. Adriaens, B. Meesschaert, W. Wuyts, H. Vanderhaege, and H. Eyssen, *J. Chromatog.*, 1977, **140**, 103.
[347] S. T. Cheng and N. L. Benoiton, *Canad. J. Chem.*, 1977, **55**, 911.
[348] E. Lund, J. Thomsen, and K. Brunfeldt, *J. Chomatog.*, 1977, **130**, 51.
[349] B. V. Charlwood and E. A. Bell, *J. Chromatog.*, 1977, **135**, 377.
[350] J. C. Kraak, K. M. Jonker, and J. F. K. Huber, *J. Chromatog.*, 1977, **142**, 671.
[351] N. Seiler and B. Knoedgren, *J. Chromatog.*, 1977, **131**, 109.
[352] D. Bucher, *Chromatographia*, 1977, **10**, 723.
[353] S. Datta and S. C. Datta, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 1074.
[354] A. Machida, T. Ogawa, T. Ono, and Y. Kawanishi, *J. Chromatog.*, 1977, **130**, 390.
[355] K. Bach and H. J. Haas, *J. Chromatog.*, 1977, **136**, 186.
[356] P. P. H. L. Otto and G. F. van Veen-van Staalduinen, *J. Radioanalyt. Chem.*, 1977, **35**, 37.

concerned;[357, 358] it is either ten times more sensitive than ninhydrin,[357] or of similar sensitivity since both reagents permit the identification of 50—200 pmole amounts,[358, 359] but in any case fluorescamine is less sensitive.[358] The fluorescence intensity of these derivatives can be enhanced by using aqueous DMSO as solvent.[360]

Other Separation Methods.—H.p.l.c. methods employing non-polar stationary phases such as octadecylsilica [361] for the separation of amino-acid mixtures results in the separation of components in order of increasing hydrophobicity. Examples of applications are the identification of primary amines in cerebrospinal fluid using the *o*-phthalaldehyde reagent,[362] and the separation of phenylthiohydantoins of all protein amino-acids [363] using conventional adsorbents or using covalently-bonded tripeptides.[364]

A rapid, sensitive procedure for the separation of proline from hydroxyproline is based on high-voltage paper electrophoresis.[365]

Determination of Specific Amino-acids.—The two main topic areas of this section deal with the assay of particular amino-acids by specific enzymes or by methods recognizing side-chain functional groups.

Estimation of L-alanine based on NADH formation with L-alanine dehydrogenase,[366] or by the chemiluminescence produced by the H_2O_2–luminol–ferricyanide system (the peroxide deriving from the L-amino-acid oxidase-catalysed degradation of the amino-acid) [367] has been described. Specific enzyme electrode methods [368] for L-asparagine,[369] L-phenylalanine,[370] and L-glutamic acid [371] follow previously-established methodology, while a variation involving *Bacterium cadaveris* held at the surface of an ammonia-sensing membrane electrode is advocated [372] for the determination of L-aspartic acid based on the L-aspartase activity of the living organism.

Non-enzymic methods for the assay of particular amino-acids involve reactions which have been discussed in the 'Chemical Studies' section of this Chapter in past years, or which are based on textbook amino-acid chemistry. The identification of

[357] S. Mori, Y. Nishimura, and H. Uchino, *Nippon Hojo-Hiryogaku Zasshi*, 1977, **48**, 332 (*Chem. Abs.*, 1977, **87**, 196 671).
[358] E. Schiltz, K. D. Schnackerz, and R. W. Gracy, *Analyt. Biochem.*, 1977, **79**, 33.
[359] R. Reisfeld and S. Levi, *Analyt. Letters*, 1977, **10**, 483.
[360] P. M. Froehlich and L. D. Murphy, *Analyt. Chem.*, 1977, **49**, 1606.
[361] I. Molnar and C. Horvath, *J. Chromatog.*, 1977, **142**, 623.
[362] O. M. Rennert, D. L. Lawson, J. B. Shukla, and T. D. Miall, *Clinica Chim. Acta*, 1977, **75**, 365.
[363] C. L. Zimmerman, E. Appella, and J. J. Pisano, *Analyt. Biochem.*, 1977, **77**, 569.
[364] E. J. Kitka and E. Grushka, *J. Chromatog.*, 1977, **135**, 367; G. W.-K. Fong and E. Grushka, *ibid.*, **142**, 299.
[365] D. Irwin and P. T. Speakman, *Experientia*, 1977, **33**, 976.
[366] A. W. Skillen and T. Handa, *Clinica Chim. Acta*, 1977, **77**, 431; R. B. H. Schutgens, C. T. Awie, F. A. Beemer, and W. J. M. Berntssen, *ibid.*, 1977, **80**, 1.
[367] S. N. Lowery, P. W. Carr, and W. R. Seitz, *Analyt. Letters*, 1977, **10**, 931.
[368] G. G. Guilbault, in 'Biomedical Applications of Immobilised Enzymes and Proteins', ed. T. M. S. Chang, Plenum Press, New York, 1977, Vol. 2, p. 163.
[369] R. Wawro and G. A. Rechnitz, *J. Membrane Sci.*, 1976, **1**, 143.
[370] C. P. Hsiung, S. S. Kuan, and G. G. Guilbault, *Analyt. Chim. Acta*, 1977, **90**, 45.
[371] B. K. Ahn, S. K. Wolfson, and S. J. Yao, *Bioelectrochem. Bioenerg.*, 1975, **2**, 142.
[372] R. K. Kobos and G. A. Rachnitz, *Analyt. Letters*, 1977, **10**, 751.

γ-carboxy-L-glutamic acid in bone, teeth, or prothrombin can be accomplished [373] after its release by alkaline hydrolysis (see also Vol. 8, p. 5). Assay of tryptophan by colorimetry at 2 μg levels [374] or by its fluorescence after h.p.l.c. separation,[375] and determination of cysteine or cystine down to 10^{-12} mole levels by polarography,[376] illustrate well-established techniques in this area; specific assay of di- and tri-iodothyronines and thyroxine is covered in papers too numerous to mention (they are abstracted in the 'Biochemical Methods' section of *Chemical Abstracts*), based on methods cited in this section in earlier Volumes. The inhibitory effect of methionine on the colour reaction between lactic acid and *p*-hydroxybiphenyl in H_2SO_4 has been used [377] for the spectrophotometric assay of this amino-acid; an alternative method [378] depends on g.l.c. analysis of MeSCN formed by cyanogen bromide treatment of plant samples. *S*-Methylmethionine levels in plants can be determined by degradation at pH 9.7 at 97 °C, giving homoserine and Me_2S, analysed by g.l.c.[20] The γ-aminobutyric acid content of human cerebrospinal fluid can be determined at 1 picomole sensitivity by presenting samples to membrane receptors equilibrated with the ^{3}H-labelled amino-acid and measuring the resulting distribution of labelled amino-acid.[379]

[373] P. V. Hauschka, *Analyt. Biochem.*, 1977, **80**, 212.
[374] S. M. M. Basha and R. M. Roberts, *Analyt. Biochem.*, 1977, **77**, 378.
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2
Structural Investigations of Peptides and Proteins

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

1 Introduction

As is stated annually in these Reports, the majority of proteins whose isolation was reported in 1977 were purified using the well tried and tested procedures of selective precipitation by salt or organic solvents, ion-exchange chromatography, adsorption chromatography, and gel filtration. In a few cases, where newer techniques such as hydrophobic chromatography and ligand affinity chromatography were combined, impressive purification was achieved.[1-3] The desire to give all procedures a name has led to the reappearance of some old ones in disguise. Thus, electrostatic affinity chromatography[4] (chromatography on Amberlite resin) and, perhaps more acceptable, carbohydrate recognition chromatography[5] (lectin affinity chromatography) have appeared. It is now more true than ever that limitations of space preclude even a tabulation of all the proteins isolated in the past year, and the emphasis in this year's Report is on the methodology of isolation, particularly on methods that have not yet been fully characterized. Purifications illustrating the use of these have been selected from the literature and tabulated. Aspects that appear to be generally less appreciated are discussed in the text. In addition, the purification of some selected classes of proteins is discussed.

2 Protein Isolation Methodology

Chromatography.—*General Comments on Ligand Affinity Chromatography.* The aim of ligand affinity chromatography is to achieve specific binding of a protein to a ligand in a manner that can subsequently be reversed without denaturation of the protein. Non ligand-specific binding of proteins to the column matrix such as lysozyme to agarose and dextran columns,[6] the dextran binding protein of *Streptococcus* to dextran,[7] and the folate binding protein of cows' milk to dextran

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and agarose,[8] and the displacement of these proteins with eluants frequently used in affinity chromatography can therefore complicate purification. Often hydrophobic as well as biospecific binding to ligands occurs. Elution of *Renilla reniformis* luciferase from a thiol interchange matrix required both 2-mercaptoethanol and increased salt concentration.[9] Formamide elution of haemoglobin from phenol-containing adsorbents, designed for the purification of NAD^+-dependent dehydrogenases, showed considerable hydrophobic binding to occur.[10] Other affinity columns have been shown to induce much non-biospecific binding of protein.[11–13] In an attempt to prevent subsequent loss of poly(A) polymerase, ATP-Sepharose was pretreated with bovine serum albumin to saturate hydrophobic binding sites.[14] The low degree of purification seen with chromatography of lipoxygenases on matrix-bound unsaturated fatty acids reflected the hydrophobic binding capacity of these columns.[15] Pre-chromatography on aminohexyl-Sepharose has been used to eliminate non-specific binding to ligands coupled *via* an aminohexyl spacer to Sepharose.[16]

Often it is necessary either to remove or to add cofactors to proteins for homogeneity of behaviour on affinity columns. A cytochrome P450 of bovine adrenal medulla eluted as two peaks from pregnenolone-Sepharose. One was found to contain bound NADPH and the other to lack the cofactor.[17] Dialysis to remove bound UDP from the protein synthesis elongation factor Tu [18] and $NADP^+$ from thioredoxin reductase and glutathione reductase [3] was required before successful ligand affinity chromatography: dTMP had to be removed from thymidylate kinase by passage through Dowex resin for similar reasons.[19] Thymidylate synthetase required dUMP;[20] α-3-galactosyl transferase required Mn^{2+} and UDP-galactose;[21] and a membrane galactosyl transferase required *N*-acetylglucosamine [22] for binding to their respective affinity matrices.

The specificity of binding of glycosidases to ligands has been questioned by the behaviour of takadiastase (a partially purified glycosidase mixture) and ablone liver extracts on a variety of ligands. Salt gradient elution gave the same order of displacement whatever the ligand. However, of the non-glycosidase activities investigated in this work, only taka-amylase A was bound. In contrast, the binding of concanavalin A to these adsorbents was as predicted from inhibition studies.[23] In an unsuccessful attempt to find a β-L-fucosidase, β-*N*-acetyl-D-

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glucosaminidase was found to bind to *p*-aminophenyl-1-thio-β-L-fucopyranoside-Sepharose.[24]

Choice of Matrix and Method of Attachment. Although direct coupling to CNBr-activated Sepharose remains one of the most widely used methods of insolubilization of ligands, it has the disadvantage that cationic charges are introduced into the matrix, and that these interfere with subsequent affinity procedures. Conditions for CNBr-mediated coupling to Sepharose under which ligand incorporation is maximized while minimizing the generation of a charged matrix have been described.[25] A comparison of CNBr and bifunctional oxirane coupling to Sepharose showed that the use of the bifunctional oxirane gave less non-biospecific binding to the matrix, and also that less-denaturing solvents were required to displace the ligand-bound protein. However, oxirane also gave a much lower efficiency of coupling.[26] Epichlorhydrin-crosslinked desulphated agarose, previously shown to have a reduced exchange capacity and therefore diminished non-specific binding, has continued to be used.[27] A study of three methods of immobilization of *Clostridium perfringens* neuraminidase – direct coupling to CNBr-activated Sepharose, coupling to Sepharose using adipic acid dihydrazide, and coupling to controlled pore glass glycophase following periodate oxidation – produced interesting results. All gave slow release of bound ligand, the greatest being 0.004% after 60 min. incubation at 37 °C from the CNBr-activated Sepharose. While adipic acid dihydrazide gave a higher degree of coupling than did CNBr (30 000 pg mg^{-1} support *vs.* 20 000 pg mg^{-1} support), the bound enzymic activity mg^{-1} support was no higher. The use of substrates of increasing molecular weight showed that over the range tested, CNBr-coupled neuraminidase expressed a significantly higher percentage of its bound activity than did adipic acid dihydrazide-coupled protein. Controlled pore glass-bound neuraminidase compared unfavourably in all respects.[28] The increased stability of Sepharose CL-4B over Sepharose 4B found in the presence of 6M-guanidine hydrochloride [29] and chaotrophic anions[30] suggests that it is the matrix of choice if either guanidine or chaotroph elution from an affinity column is to be used. Polyacrylamide activated with thiophosgene has been used to produce an IgG-affinity matrix. This method combines a high degree of binding with accessibility of ligand, and is regarded as producing a stable matrix with little non-specific binding.[31] The effect of the matrix on the binding of albumin to immobilized Cibacron Blue has been investigated. Sepharose and Sephacryl were found to be better matrices than cellulose and Ultragel.[32] In a novel approach, G-actin has been polymerized in the presence of Sepharose 4B to produce immobilized F-actin as an affinity ligand for myosin and heavy meromyosin. Phalloidin, which reduces the critical concentration for depolymerization of F-actin,[33] was used to stabilize the F-actin

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matrix. An eight-fold improvement in accessibility of F-actin to myosin compared with polyacrylamide-immobilized actin was found.[34]

Effect of Spacer between Matrix and Ligand. Spacer arms are frequently used to improve the accessibility of affinity ligands. Thus the turkey erythrocyte membrane β-adrenergic receptor did not bind to alprenol-Sepharose in the absence of a spacer arm. With the use of a spacer a 2000-fold purification was achieved.[35] The problem of non-specific binding to spacers has already been mentioned. The use of aminoethane instead of aminohexane as a spacer in the coupling of AMP to Sepharose, used in the purification of a protein kinase from bovine lung, eliminated non-specific binding of proteins.[36] The effect of the chemical nature of the spacer arm on affinity chromatography has been studied in the binding of dehydrogenases to AMP-Sepharose. An increase in the hydrophilicity of the spacer produced a decrease in the strength of binding of lactate and alanine dehydrogenases. This was not related to the K_I's found for the substituted AMP derivatives in free solution.[37] A higher recovery of sturgeon glyceraldehyde-3-phosphate dehydrogenase from NAD^+-Sepharose was achieved when the hydrophilic spacer, 1,3-diaminopropan-2-ol, rather than diaminohexane was used.[38] In contrast, hydrophobic rather than hydrophilic spacers were found to give better results in the purification of human transcortin on cortisol-Agarose. An optimum spacer length of 12—13 Å was also determined here.[39]

Choice of Ligand. Nucleotides remain the most widely used ligands in affinity chromatography. A large number of enzymes will bind to such matrices and more specific ligands may therefore give better results. This is illustrated by the difference in purification of bovine liver quinonoid dihydropterin reductase achieved on NAD^+-agarose (2-fold) and amethopterin-agarose (400-fold).[40] Such an improvement is not always seen, 6-phosphogluconate being of less value than $NADP^+$ as the ligand in the purification of 6-phosphogluconate dehydrogenase from sheep liver.[41] Several groups have investigated the properties of differently substituted nucleotide derivatives and their uses as affinity ligands.[3, 42–44] 2′,5′-ADP was found to be a specific ligand for $NADP^+$-dependent dehydrogenases whereas 3′,5′-ADP was specific for coenzyme A-dependent enzymes.[44] An investigation of the kinetics of interaction of various dehydrogenases with AMP-Sepharose has shown significant differences in degrees of binding with time and this may be of importance in determining conditions for both binding and elution

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of specific enzymes.[45] A simple method for determining the degree of substitution of Sephadexes with Cibacron Blue has been described.[46] The assumption that Cibacron Blue and other dyes act as affinity ligands by mimicking nucleotide conformation has been further challenged. Induced circular dichroism has shown that proteins that bind both Cibacron Blue and Congo Red undergo different conformational changes in so doing.[47] The catalytic loss of ligands from their matrices has meant that alternatives have been looked for. 6-(*p*-Amino)benzyluracil has been used instead of 5′-deoxythymidine as an affinity ligand for thymidine kinase as it is not hydrolysed by thymidine phosphorylase.[48] Similarly, histidinol, which is reduced by traces of NADH present in crude cell extracts, is less satisfactory than histamine and histidine in the purification of histidinol dehydrogenases.[49] The properties of different ligands in affinity chromatography of amino-acyl-tRNA synthetases [16] and heavy meromyosin isoenzymes [50] have also been investigated.

Protein and other Macromolecular Ligands. Natural macromolecular protease inhibitors continue to be used in the purification of proteases.[51, 52] Similarly, matrix-bound proteases have been used in the purification of specific inhibitors.[53, 54] Other proteases have been purified using matrix-bound substrates such as collagen [55] or elastin.[56] Galactosyl transferases have been purified using chromatography on α-lactalbumin-Sepharose columns.[22, 57, 58] A more ingenious approach was used in the purification of the α-3-galactosyl transferase of human serum. The enzyme was adsorbed onto blood group O erythrocyte membranes and eluted with 2′-fucosyllactose giving a 100 000-fold single-step purification.[21] *Staphlococcal* protein A,[31, 59] secretory component,[60] and complement components Clr and Cls [61] have been purified using chromatography on immobilized immunoglobulins. A protein kinase inhibitor of rabbit skeletal muscle has been purified using chromatography on Sepharose-coupled protein kinase catalytic subunit.[62] Muscle proteins have been isolated by virtue of their affinity for other muscle constituents.[50, 63]

Choice of Eluant. Much of the specificity of purification found with nucleotide ligands is dependent on a discerning manipulation of eluting conditions. Pyrophos-

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phate elution of 6-phosphogluconate dehydrogenase from NADP+-Sepharose gave only a four-fold purification whereas citrate elution gave a 40-fold purification.[41] A variety of eluants were compared in the purification of bovine liver quinonoid dihydropterin reductase.[40] In some cases, such as the elution of testosterone-binding globulin from a steroid affinity column,[64] and the elution of a 3′,5′-GMP-dependent protein kinase of bovine lung from a cGMP-Sepharose column,[65] equilibration of the eluting ligands (testosterone and cGMP) with the bound proteins was required before elution. Frequently the eluting ligand remains bound to the freed protein, as was found with Cibacron Blue elution of albumin from Cibacron Blue-Sepharose.[32] Isoelectric focusing of chicken liver dihydrofolate reductase was required to free it from bound dihydrofolate.[66] The nature of the eluant used often causes some denaturation of the eluted protein, as in the case of thiocyanate elution of albumin from Cibacron Blue-Sepharose.[32] The possibility that secondary functions may be lost while the assayed function is retained was demonstrated by the loss of the receptor-binding property of human intrinsic factor following elution from a vitamin B_{12}-Sepharose column with 7.5M-guanidine hydrochloride.[67] A useful approach to the selection of affinity and eluting ligands was illustrated by the use of competitive inhibitors of acetylcholinesterase from house-fly brain. That with a K_i of 10^{-4}M was used as the binding ligand and that with a K_i of 10^{-7}M as the eluant.[68]

The Use of Affinity Chromatography to Remove Unwanted Proteins. In many cases, the specific binding to affinity matrices of proteins that are difficult to remove by conventional means is as valuable as the specific binding of the protein being purified to an immobilized ligand. Chromatography on an affinity matrix in which all tRNAs excepting $tRNA_{Val}$ were bound followed by chromatography of residual proteins on a matrix containing $tRNA_{Val}$ only has been described. In addition to removing all tRNA synthetases excepting valyl-tRNA synthetase, the first column would have removed any protein that could bind in a non-specific manner to such a column.[69] Glycoprotein contaminants have been removed from rat serum albumin by chromatography on concanavalin A-Sepharose.[70] Concanavalin A-Sepharose has also been used to remove acid β-galactosidases from neutral β-galactosidases of human liver.[71] An ovamucoid-Sepharose column was used to remove contaminating proteases from porcine elastase II.[72] Galactosidases were removed from a bovine liver aryl-β-hexosidase preparation by affinity procedures.[73] Plasminogen was removed from plasma by chromatography on L-lysine-polyacrylamide as an initial step in the purification of the fourth component of human complement.[74] Specific antisera can conveniently be used to remove

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contaminating proteins without concern over the generally harsh conditions required to recover proteins from immunoaffinity columns. Such immunoadsorbents were used in the purification of the third component of murine complement.[75] Immobilized antisera against vitamin B_{12}-binding proteins have been used to remove those not required prior to chromatography of unadsorbed proteins on a vitamin B_{12}-Sepharose affinity column.[76] Specific anti-B1 and anti-B2 antisera were used to separate the B1 and B2 subunits of the ribonucleoside diphosphate reductase of *E. coli*.[77] Anti-C1r-Sepharose was used to remove traces of precursor and activated C1r from C1s (components of the C1 complex of human complement), thus stabilizing the precursor form of C1s.[78]

Immunoaffinity Chromatography. Both the IgG-containing ammonium sulphate fraction of serum [79–81] and purified antibodies [82, 83] have been coupled to Sepharose and used in immunoaffinity procedures. Because the affinity of antibodies for antigens is high, a major problem has been the recovery of active protein. A comparison of the efficiency of eluants in a BSA-Sepharose–anti-BSA system has been made. While chaotroph, low pH, urea, and guanidine elution all displaced similar amounts of protein, there were considerable differences in the functional titres of the recovered IgG. The highest functional titres were found with 0.2M-glycine–HCl and 5M-guanidine hydrochloride elution.[84] The importance of using antisera from early in the immune response, where the avidity of antibodies is at its lowest, is illustrated in the purification of carcinoembryonic antigen. Whereas 42%, 74%, 90%, and 94% of the bound antigen could be eluted by 1, 2, 3, and 4M-KSCN respectively from antibodies produced early in the immune response, 35% and 73% only was eluted by 3M- and 4M-KSCN from antibodies produced after intensive immunization.[85] Serum angiotensin converting enzyme was eluted from an immunoadsorbent using relatively mild denaturing conditions (3.8M-urea) following a 2-hour equilibration period.[86] Other requirements have also influenced the choice of eluant. Ia-like alloantigens from B-lymphoblastoid cell lines require deoxycholate to maintain their solubility. Alkaline elution at pH 11 from immobilized antibodies was used to prevent acid precipitation of the detergent.[79] Properdin has been purified using an immunoaffinity adsorbent that had been chemically modified to reduce the affinity of antibody for antigen. However, the eluting conditions used (0.5M-NaCl) suggest that very little if any

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antibody-determined binding remained.[87] An electrophoretic method for the desorption of antibody-bound proteins has been described, the released protein (ferritin) being collected into dialysis tubing. This procedure was rapid and gave as efficient elution as 6M-guanidine hydrochloride but with greatly increased recovery of activity.[88] Acyl carrier proteins from *Euglena gracilis* and various *Bacillaris* have been purified using anti-*E. coli* acyl carrier-protein, illustrating the use of immunoaffinity techniques in isolating related proteins. There was, however, no obvious decrease in affinity of binding and therefore ease of elution.[89] Choriogonadotrophin from human term placenta was displaced from anti-urinary choriogonadotrophin-Sepharose by both 4M-$MgCl_2$ and guanidine hydrochloride. Although only 22% of the bound protein was released by $MgCl_2$, it had a higher specific activity than the 50% that could be eluted with guanidine.[90] In an attempt to prevent non-specific binding to this last column, the antiserum was adsorbed with choriogonadotrophin-free urine prior to coupling to Sepharose. A similar approach has been used in the purification of antigen-specific blocking factors [91] and human leukocyte pyrogen.[92] Application of the sample in a high salt concentration has also been used in an attempt to prevent non-specific binding.[93] Antibodies raised against a cyanogen bromide fragment of chicken erythrocyte histone H3 have been used in the purification of the intact histone, but again there was no obvious decrease in affinity of binding.[94] The power of immunoaffinity techniques was shown by the separation of isoenzymes of the alkali light chains of myosin using a matrix-bound antibody which was specific for the difference peptide.[95] A novel use of immunoaffinity chromatography in the isolation of transmembrane proteins of phagocytic cells has been described. Cultured mouse L-cells were first ^{125}I-labelled with lactoperoxidase and then fed latex beads. The beads were taken up into phagosomes by the cells, producing an 'inside-out' orientation of the phagosome membrane. Following recovery of the latex-filled phagosomes, transglutaminase-catalysed modification with dansyl-cadaverine was performed. Dansyl-containing proteins were then isolated by chromatography on anti-dansyl-Sepharose. Transmembrane proteins were identified by the presence of ^{125}I.[96]

Lectin Affinity Chromatography. This technique has been increasingly used in the purification of glycoproteins. Coupling to Sepharose in the presence of specific monosaccharides has been used to protect the binding site.[97] Ethylene glycol has

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[88] P. J. Brown, M. J. Leyland, J. P. Keenan, and P. D. G. Dean, *F.E.B.S. Letters*, 1977, **83**, 256.
[89] M. L. Ernst-Fonberg, A. W. Schongalla, and T. A. Walker, *Arch. Biochem. Biophys.*, 1977, **178**, 166.
[90] C. Y. Lee, S. Wong, A. S. K. Lee, and L. Ma, *Z. physiol. Chem.*, 1977, **358**, 909.
[91] J. T. Nepom, I. Hellstrom, and K. E. Hellstrom, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4605.
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[94] D. Absolom and M. H. V. van Regenmortel, *F.E.B.S. Letters*, 1977, **81**, 419.
[95] J. C. Holt and S. Lowey, *Biochemistry*, 1977, **16**, 4398.
[96] R. M. Evans and L. M. Fink, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 5341.
[97] K. J. Clemetson, S. L. Pfueller, E. F. Luscher, and C. S. P. Jenkins, *Biochim. Biophys. Acta*, 1977, **464**, 493.

been used in conjunction with α-methyl-D-mannoside to elute lysine hydroxylase [98] and collagen glucosyl transferase [5] from concanavalin A-Sepharose columns showing that, in these cases, hydrophobic as well as carbohydrate-specific binding was involved. The nature of the carbohydrate-binding site of four *N*-acetyl-D-galactosamine-specific lectins (*Helix pomatia* A haemagglutinin, soy bean agglutinin, lima bean lectin, and *Dolichos biflorus* lectin) has been investigated and shown to correspond to the size of the monosaccharide.[99] Lectin affinity chromatography is frequently used in the purification of membrane glycoproteins which have been solubilized by and are kept in solution by detergents. The effect of several commonly used detergents on the saccharide-binding properties of lectins was therefore of interest. Non-ionic detergents, at the concentrations used for membrane solubilization, had no effect. Cationic and zwitterionic detergents, however, produced significant inhibition of binding to concanavalin A-Sepharose and soy bean agglutinin-Sepharose. In sodium deoxycholate at concentrations greater than 1% only *Ricinus communis* I agglutinin-Sepharose retained its binding capacity. While in 0.05% SDS only soy bean agglutinin lost its activity, prolonged treatment with 0.1% SDS at 23 °C led to the loss of all lectin activities.[100] Isolation of the concanavalin A receptor of human erythrocytes on concanavalin A-Sepharose has shown it to be contained in band 3.[101] Glycogen synthase I from human polymorphonuclear leukocytes has been isolated in a novel manner by using concanavalin A-Sepharose to isolate glycogen plus associated enzymes and subsequent digestion of the glycogen with amylase to release the synthase.[102]

Heparin Affinity Chromatography. Heparin-Sepharose is also being increasingly used as an 'affinity' matrix. A study of the interaction of lipoprotein lipase with heparin-Sepharose has, however, suggested that commercial heparin is polydisperse in its protein binding properties.[103] The affinity of the estradiol receptor of calf uterus for heparin-Sepharose was increased 10-fold in the presence of Mg^{2+} and estradiol, and with the addition of these ligands a significant purification was achieved.[104]

Hydrophobic Chromatography. Elution of proteins from hydrophobic ligands is generally carried out by decreasing the salt concentration, or by using solvents containing ethylene glycol or formamide, under which hydrophobic interactions are weakened, or by an increasing salt gradient. A theoretical treatment of salt effects on hydrophobic interactions in proteins, correlating 'relative surface hydrophobicity' with the salting-out effects of neutral salts, and showing that protein solubility in neutral salts should parallel elution order from

[98] T. M. Turpeenniemei, V. Puistola, H. Anttinen, and K. I. Kivirikko, *Biochim. Biophys. Acta*, 1977, **483**, 215.
[99] S. Hammarström, L. A. Murphy, I. J. Goldstein, and M. E. Etzler, *Biochemistry*, 1977, **16**, 2750.
[100] R. Lotan, G. Beattie, W. Hubbell, and G. L. Nicolson, *Biochemistry*, 1977, **16**, 1787.
[101] D. G. Barret, F. J. Sharom, A. E. Thede, and C. W. M. Grant, *Biochim. Biophys. Acta*, 1977, **465**, 191.
[102] H. Solling and V. Esmann, *European J. Biochem.*, 1977, **81**, 119.
[103] G. Bengtsson and T. Olivecrona, *Biochem. J.*, 1977, **167**, 109.
[104] A. Molinari, N. Medici, B. Moncharmont, and G. A. Puca, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4886.

hydrophobic ligands, has been published.[105] The elution of albumin from pentyl-Sepharose and ovalbumin and phycoerythrin from decyl-Sepharose by neutral salts has been studied in depth. The eluting power of salts correlated with their position in the Hofmeister series (*i.e.* with their chaotrophicity). Using c.d. techniques it was shown that salts that did not give elution (3M-NaCl, 3M-Na_2SO_4) produced no conformational change in the proteins, whereas those that did (3M-NaBr, 3M-NaSCN) produced parallel conformational changes. One exception to these findings was that in the presence of 3M-NaSCN ovalbumin became more tightly bound to decyl-Sepharose. A conformational change exposing a higher affinity hydrophobic site was suggested here.[30] The alkaline phosphatase precursor of *E. coli* has been separated from the mature enzyme by virtue of its binding to decyl-Sepharose.[106] A ligand with both hydrophobic and ionic groups has been used to produce human erythrocyte glycophorin (PAS-1).[107] The membrane adenylate cyclase from canine left ventricles has been separated from other hydrophobic proteins and detergent by chromatography on dodecyl- and cetyl-Sepharoses: more hydrophobic proteins selectively retained detergent and were not bound.[108] A complex procedure was used to purify lecithin–cholesterol acyl transferase. Dodecylamine-agarose was first saturated with plasma lipoproteins. Plasma was then passed through the column and lecithin–cholesterol acyl transferase, selectively retained by the lipoproteins, could subsequently be eluted by decreasing the ionic strength.[109]

Metal Chelate and Covalent Chromatography. The optimum conditions for thiol-disulphide interchange chromatography have been investigated.[110] Primarily developed as a technique for albumin purification, it has now been used to purify prealbumin from canine and human sera.[111] The penicillin binding protein S of *Salmonella typhimurium* has been purified by virtue of its binding to an ampicillin-affinose column. Elution was achieved by 1M-hydroxylamine at 37 °C.[112] The coupling of iminodiacetic acid to epoxy-activated Sepharose produces biscarboxymethylamino-agarose, which has a high affinity for bivalent metal ions. Saturation of the substituted agarose with Zn^{2+} has been used to purify human fibroblast interferon [113] and plasma α_2-SH glycoprotein [114] and with Cu^{2+} to purify human milk lactoferrin.[115] Organomercurial gels have been used in the fractionation of bromelain [116] and to remove other membrane proteins from the major glycoproteins PAS 1, 2, and 3 of the erythrocyte membrane.[117]

Biospecific Elution from Non-affinity Ligands. Biospecific elution is frequently used to improve the resolution of proteins bound to ion-exchange resins. Conditions

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[106] H. Inouye and J. Beckwith, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1440.
[107] R. J. Simmonds and R. J. Yon, *Biochem. J.*, 1977, **163**, 397.
[108] C. J. Homcy, S. M. Wrenn, and E. Haber, *J. Biol. Chem.*, 1977, **252**, 8957.
[109] A. G. Lacko, and T. F. Chen, *J. Chromatog.*, 1977, **130**, 446.
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[111] C. Fex, C.-B. Laurell, and E. Thulin, *European J. Biochem.*, 1977, **75**, 181.
[112] S. T. Shepherd, H. A. Chau, and P. E. Reynolds, *European J. Biochem.*, 1977, **78**, 521.
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[116] K. R. Lynn, *Analyt. Biochem.*, 1977, **77**, 33.
[117] Y. Shami, S. Ship, and A. Rothstein, *Analyt. Biochem.*, 1977, **80**, 438.

for the biospecific elution of 15 different rabbit muscle enzymes, mainly glycolytic, from phospho-cellulose or carboxymethyl-cellulose have been described.[118] The use of 'imphilytes' – ligands with hydrophobic, anionic, and cationic groups – in a similar manner has been suggested.[119] It is possible that in some cases the elution of enzymes from phospho-cellulose with inorganic phosphate can be considered biospecific.[120–122] Human hypoxanthine–guanine phosphoribosyl transferase[123] and tyrosyl-tRNA synthetase from baker's yeast[124] have been eluted from CM-Sephadex with pyrophosphate and tRNA respectively.

Electrophoretic Procedures.—These continue to be used mainly in preparations where small quantities only of protein are available. The resolving power of preparative isoelectric focusing has been systematically investigated and optimum conditions for the preparation of Eastern encephalitis viral proteins determined.[125] The use of separators – amphoteric substances that produce a flattening of the pH gradient in a specific region – to improve separation of specific proteins has been reported.[126] Pevikon (a co-polymer of polyvinyl chloride and polyvinyl acetate) has been described as a suitable support for large-scale preparative isoelectric focusing. This support required the presence of small amounts of Sephadex to produce a stable gradient and was regarded as being superior to both polyacrylamide and Sephadex.[127] Isotachophoresis compared favourably with isoelectric focusing in the preparative separation of α-crystallin subunits,[128] and optimum conditions for preparative isotachophoresis have been determined.[129] An improved procedure for the electrophoretic elution of proteins from polyacrylamide gels has been described.[130]

Miscellaneous Procedures.—The use of the same procedure twice in protein fractionation rarely gives a significant increase in purification. However, there have been several reports in the past year where modification of the properties of a protein by bound ligands subsequent to a specific step has given considerable improvement in the fractionation achieved with a second application of the same procedure. Thus borate, which complexes with glycoproteins, has been used as the buffer in the second application of placental β-glucuronidase to DEAE-cellulose,[131] L-serine dehydratase from *Arthrobacter globiformis*, which dimerizes in the presence of D-serine, has been purified using successive gel filtration steps in the presence and absence of D-serine,[132] and bovine heart mitochondrial creatine kinase has been purified using successive ethanol precipitations in the presence

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[119] R. J. Yon, *Biochem. J.*, 1977, **161**, 233.
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[124] H. G. Faulhammer and F. Cramer, *European J. Biochem.*, 1977, **75**, 561.
[125] S. C. Marker, P. Melby, and P. B. Jahrling, *Analyt. Biochem.*, 1977, **82**, 423.
[126] M. L. Caspers, Y. Posey, and R. K. Brown, *Analyt. Biochem.*, 1977, **79**, 166.
[127] W. I. Otavsky, T. Bell, C. Saravis, and J. W. Drysdale, *Analyt. Biochem.*, 1977, **78**, 302.
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[129] R. Houghten and A. Chrambach, *Analyt. Biochem.*, 1977, **77**, 303.
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[132] F. Gannon, E. S. Bridgeland, and K. M. Jones, *Biochem. J.*, 1977, **161**, 345.

and absence of Mg^{2+}.[133] Similar approaches have been used in the purification of the C-reactive protein of serum,[134] *N*-acetylglutamate synthase of *E. coli*,[135] and the Ca^{2+}-activatable cyclic nucleotide phosphodiesterase from bovine heart.[136] The problems associated with the purification of proteins from halophilic bacteria, which are rapidly denatured at low salt concentrations, have been eased by use of their agarose-binding properties in high ammonium sulphate concentrations. Proteins were eluted by a decreasing salt gradient.[137, 138] The resistance of ribonucleases to acid denaturation,[139] and of other proteins to heat denaturation,[61, 140] continues to give significant purification where these steps are used, although the structural and functional integrity of proteins must be questioned after such procedures. A similar qualification applies to the use of 6M-urea to elute rabbit muscle phosphorylase phosphatase complexed with glycogen from poly(L-lysine)-Sepharose under conditions where much protein is denatured,[141] although in this case the protein could be stabilized by its interaction with the glycogen. The distinctive properties of glycoproteins permit the use of unusual techniques. Thus an acidic glycoprotein (81% by weight carbohydrate) has been purified from the urine of germ-free rats using cetyltrimethylammonium bromide precipitation.[142] Macromolecular distribution near the limits of density-gradient columns and its application to the fractionation of glycoproteins have been investigated.[143] A biphasic procedure selectively isolating the major glycoprotein of rat brain myelin [144] and a single-phase procedure for proteolipids [145] have been described. A biphasic procedure has also been used in the fractionation of salt-dissociated histones.[146] A variety of organic solvents have been studied with a view to the selective extraction of very low density apolipoproteins from human serum.[147] The use of field-flow fractionation, giving separation on the basis of diffusion coefficients, as a method for protein fractionation has been proposed.[148] Magnetic ferrofluids have been incorporated into Sepharose without altering its properties as a potential affinity matrix. This was claimed to permit easy retrieval of the beads.[149] It has been suggested that routine application of successive chromatographic steps on anion and cation exchange resins at the isoelectric point of a protein should be used in its purification. The overall purification achieved for bovine and porcine aminotransferases (5-fold) when these principles were applied does not immediately recommend this theoretical approach.[150] 'Cold shock' has

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[140] W. F. Burke, jun. and J. Spizizen, *Biochemistry*, 1977, **16**, 403.
[141] D. Gratecos, T. C. Detweiler, S. Hurd, and E. H. Fischer, *Biochemistry*, 1977, **16**, 4812.
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[150] P. Petrilli, G. Sannia, and G. Marino, *J. Chromatog.*, 1977, **135**, 511.

been described as a procedure for releasing enzymes from bacteria.[151] Several routine procedures have been adapted for use as micro-techniques suitable for handling small samples of material.[152–154]

Solubilization of Membrane Proteins.—A wide spectrum of detergents continue to be used to displace membrane proteins. A study of the effect of detergent, salt concentration, and pH on the solubilization of the glycoproteins PAS-1 and PAS-2 of the erythrocyte membrane has been carried out.[155] A similar study has shown Brij detergents to be those of choice in the release of HLA antigens from lymphoblastoid cells.[156] The solubilization of *E. coli* membrane proteins using aprotic solvents showed hexamethylphosphonictriamide to release 40—60% of the total membrane protein. In the presence of 100 mM-LiCl or ammonium acetate, this release was increased to 80—90%.[157] Non-aqueous chloroform–methanol extraction of freeze-dried membranes has given considerable improvement on the use of aqueous chloroform–methanol in terms of enzymic activity recovered.[158] A method that utilizes the selective extraction of some membrane-bound proteins by phospholipid vesicles, which can then be separated from cells by centrifugation, has been used to prepare erythrocyte acetylcholinesterase and found to compare favourably with salt extraction.[159] The simplest method reported, however, was that of the *Azotobacter vinelandii* envelope protein, selectively solubilized by a distilled water wash of membrane fractions.[160] Agarose-suspension electrophoresis has been used to remove the detergent Tween 20 from solubilized membrane proteins of *Acholeplasma laidlawii*. The large micellar size and low critical micellar concentration have in the past made removal of this detergent difficult. However, the method clearly requires the stability of protein in a detergent-free medium.[161] A method that permits the recovery of Triton X-100 or deoxycholate-solubilized proteins by trichloracetic acid precipitation has been described. Addition of SDS at an approximately equivalent amount by weight as Triton X-100 or deoxycholate prevented the co-precipitation of detergent, presumably mixed micelles of SDS and Triton or deoxycholate being formed.[162]

Multienzyme Preparations.—Although economic in both time and materials, multienzyme preparations are rarely used as few groups have the necessary facilities or a requirement for more than one or two purified proteins. One exception to this generalization is in the field of protein synthesis; 21 of the 40S subunit ribosomal proteins and 21 of the 60S subunit ribosomal proteins have

[151] H. N. Ananthaswamy and J. A. Grunau, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 289.
[152] M. Krieger and J. Tobler, *Analyt. Biochem.*, 1977, **81**, 450.
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[161] G. Dresdner and H. Cid-Dresdner, *Analyt. Biochem.*, 1977, **78**, 171.
[162] K. C. Retz and W. J. Steele, *Analyt. Biochem.*, 1977, **79**, 457.

been purified from single ribosomal subunit extracts.[163, 164] Similarly, 6 initiation factors for mammalian protein synthesis have been purified,[165] all of these purifications using classical procedures. The resolution of *E. coli* B aminoacyl-tRNA synthetases on aminohexyl-Sepharose has been reported.[166] The simultaneous purification of 12 rabbit muscle enzymes and 8 chicken muscle enzymes using biospecific elution from anion-exchange resins has also been described.[167]

3 Purification of some Specific Classes of Proteins

Lectins.—The use of lectin affinity chromatography in the purification of glycoproteins has led to an increased interest in the isolation of lectins themselves. Butane-1,4-diol–diglycidyl ether has been used to couple amino-sugars, methyl-glycosides, and di- and oligosaccharides to epoxy-activated Sepharose. All but the reducing sugars were found to withstand the alkaline conditions used for coupling, and the coupled sugars could be used as affinity matrices for lectins.[168] Sodium cyanoborohydride has been used to couple disaccharides to aminoethyl polyacrylamide by reductive amination, unreacted amino groups being blocked by *N*-acetylation. Such matrices were found to be stable, and to combine good flow characteristics with a high capacity for lectins.[169] Other affinity matrices used have been polyacrylamide-entrapped guar beads (galactomannan),[170] a copolymer of the *N*-carboxyanhydride of L-leucine and hog blood group substances A and H,[171] and asialofetuin-Sepharose.[172, 173]

Antibodies.—Some workers have preferred to use purified antibodies rather than IgG-containing fractions of serum for coupling to matrices and subsequent use in affinity chromatography. These have been purified either by elution of the specific antibody from its immobilized antigen,[81] or by dissociation of an immune precipitate followed by gel filtration in the presence of the dissociant to separate the antigen and antibody.[82] Electrophoretic elution of antibodies to steroid hormones from antigen-Sepharose was found to be equivalent to elution with 1M-NH_4OH. Sepharose containing the antigen-bound antibody was placed in a tube plugged with polyacrylamide, and the antibody electrophoresed through the plug into the bottom buffer reservoir.[174] Anti-galactocerebroside antibodies were isolated by affinity-binding to liposomes. Antibodies were recovered from washed liposomes by sodium iodide or chloroform extraction.[175] Anti-galactosyl antibodies raised against group D *Streptococcus* were eluted from lactosyl-Sepharose with 0.5M-galactose.[176] Cell-column chromatography has been described as a new technique

[163] E. Collatz, N. Ulbrich, K. Tsurugi, H. N. Lightfoot, W. MacKinlay, A. Lin, and I. G. Wool, *J. Biol. Chem.*, 1977, **252**, 9071.
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[173] C. Irle, *J. Immunol. Methods*, 1977, **17**, 117.
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[176] J. H. Pazur and K. L. Dreher, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 818.

for the isolation of immunoglobulins specific for cell surface carbohydrates. Sephadex G50 was first saturated with concanavalin A. Cells were then passed through the column, and the adsorbed cells fixed by washing the column with 3% glutaraldehyde. Specific antisera were then passed through the column, and bound antibodies eluted with glycine–HCl, pH 3. Periodate treatment of a control column showed that there was no binding to non-carbohydrate determinants, but if this had been a problem, these could have been removed by prior passage through the periodate-treated column.[177]

Proteases.—A study of the requirements for affinity chromatography of trypsin and related enzymes on peptide ligands has shown that tripeptide ligands give the highest binding affinity. No increase in K_d was observed with tetra- or greater length peptides. The use of tripeptides of the form -Gly-X-Arg showed that the preferred residue in position 2 was alanine. Interaction was also found to be stronger at a pH considerably lower than that required for maximum catalysis.[178] Chromatography of various commercially available proteases on affinity ligands to remove contaminating material has given improvements in specific activity ranging from 10% for trypsin to 10-fold for *Rhizobium nereus* acid protease.[179, 180]

Cascade Enzymes of Serum.—The problems of obtaining the precursor forms of these proteins have been overcome by the rigorous use of protease inhibitors to prevent activation. Inhibitors used have been DFP, particularly for coagulation factors [181, 182] and the early components of the classical pathway of complement activation,[77, 183, 184] and EDTA for purification of Factor B of the alternative pathway of complement activation.[185] High concentrations of ε-aminocaproic acid have also been used.[184] Removal of plasminogen by passage of plasma through L-lysine-polyacrylamide was used early in the purification of complement component C4.[73] Plasticware and siliconized glassware must also be used if precursor coagulation proteins are required.[186] Thorough washing of the barium sulphate adsorbed bovine factor X enabled its isolation in precursor form without the addition of protease inhibitors.[187]

4 Tabulated Lists of Purifications.—Tables 1 and 2 list some of the proteins isolated using chromatographic methods and reported in the literature for 1977. These are not necessarily first-time purifications, but have been selected to illustrate the techniques used. Proteins have been grouped in Table 1 (ligand affinity chromatography) according to the ligand used, and in Table 2 (other chromatographic procedures) according to the method used. While the degree of purification achieved with a particular procedure is dependent on many factors

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Table 1 *General ligand affinity chromatography*

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
Nucleotides and analogues						
AMP	S	adenine phosphoribosyl transferase	yeast	Mg^{2+}–5-phosphoribosyl-1-pyrophosphate	×17	189
	A	alcohol dehydrogenase	human liver	NADH	—	190
	S	alcohol dehydrogenase	horse liver	NAD^+–citrate	—	191
	A	aldehyde dehydrogenase	rabbit liver	NAD^+	—	192
	S	aldehyde dehydrogenase	human liver	0.1M-phosphate	×10	193
	S	FMN oxidoreductase	*Beneckea harveyi*	NAD^+	×50	194
	S	glyceraldehyde-3-phosphate dehydrogenase	yeast	NAD^+	×8	37
	A	methionine-tRNA synthetase	*E. coli*	(−)-L-methioninol or (+)-L-methionine	×200	195
	S	NAD^+: protein ADP-ribosyl transferase	T4-infected *E. coli*	NH_4Cl	—	196
	S	nucleotide phosphotransferase	*E. coli*	?	×18	197
	S	phosphorylase b	lamprey skeletal muscle	AMP	—	198
2′-AMP	S	glutamate dehydrogenase	*Neurospora*	$NADP^+$ gradient	—	199
3′,5′-AMP	S	protein kinase	bovine lung	cGMP	×300	35
ADP	S	glutathione reductase	*E. coli*	NADPH	×300	3
	S	glutathione reductase	human erythrocytes	NADPH	×1100	200
	S	glutathione reductase	yeast	$NADP^+$	×134	201
	S	heavy meromyosin	rabbit muscle	ADP or pyrophosphate gradient	—	202
	S	myosin II	*Acanthamoeba*	KCl gradient	×2	203
	S	$NADP^+$-binding protein	human erythrocytes	$NADP^+$	—	204
	S	polynucleotide 5′-triphosphatase	vaccinia virus	NaCl	×19	205
	S	thioredoxin reductase	*E. coli*	NADPH	×300	3
ATP	A	creatine kinase	bovine heart	ATP gradient	—	133
	S	γ-glutamylcysteine synthetase	rat liver	ATP gradient	×2	206
	S	poly(A) polymerase	rat liver nuclei	ATP gradient	×46	14
CDP	S	sialyl transferase	bovine colostrum	CDP	×1500	207
				NaCl gradient	×30	207

cGMP	S	protein kinase	bovine lung	cGMP	×400	64
GDP	S	elongation factor Tu	*E. coli*	GDP	—	18
GTP	A	adenylate cyclase	rat liver	0.5M-Tris	—	208
UDP	S	galactosyl transferase	sheep mammary glands	EDTA	×2	56
	S	*N*-acetylgalactosamine transferase	porcine submaxillary glands	2M-NaCl	×60	209
	S	UDP-galactose-4-epimerase	bovine liver	UMP or UDP	×100	210
			bovine mammary glands	UMP or UDP	×100	210
UDP	A	UDP-glucuronyltransferase	rat liver	UDP-glucuronic acid	×40	211
	S	UDP-glucuronosyltransferase	rat liver microsomes	UDP-glucuronic acid	×8	212

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Table 1 *(cont.)*

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
NAD^+	S	glyceraldehyde-3-phosphate dehydrogenase	sturgeon	NAD^+	×15	37
	S	malate dehydrogenase	*Halobacterium*	NADH	×5	137
	S	NAD^+: protein ADP-ribosyl transferase	T4-infected *E. coli*	ND_4Cl	—	196
	A	quinonoid dihydropterin reductase	bovine liver	NAD^+, NADH	×2	39
	S	UDP-galactose-4-epimerase	bovine liver	phosphate	×2	210
			bovine mammary glands	phosphate	×2	210
$NADP^+$	S	FMN oxidoreductase	*Beneckea harveyi*	NADPH	×70	194
	S	6-phosphogluconate dehydrogenase	sheep liver	citrate	×40	40
	S	cytochrome P450 reductase	rat liver	$NADP^+$	×5	213
Blue Dextran	S	adenylosuccinate synthetase	*Azotobacter vinelandii*	IMP gradient	—	13
	S	fructose-6-phosphate kinase	*E. coli*	Mg^{2+}–ATP	×600	214
	S	3-hydroxy-3-methyl glutaryl CoA reductase	chicken liver	KCl	×250	215
	S	3-hydroxy-3-methyl glutaryl CoA reductase	rat liver	KCl	×34	216
	S	interferon	mouse	poly(I), poly(U)	—	217
Blue Dextran	S	isocitrate dehydrogenase	human heart	$NADP^+$	×16	218
	S	isocitrate dehydrogenase	*B. stearothermophilus*	EDTA gradient	×10	219
	S	nitrate reductase	yeast	NADPH	×17	220
	S	2-oxoaldehyde dehydrogenase	rat liver	KCl gradient	×5	221
	S	pyruvate dehydrogenase complex	*Azotobacter vinelandii*	0.6M-KCl	—	222
	S	pyruvate kinase	human erythrocytes	fructose 1,6-diphosphate	×18	223
Cibacron Blue	S	aldehyde dehydrogenase	yeast	1M-KCl	×3	224
	S	deoxynucleotide kinase	*Lactobacillus acidophilus*	Mg^{2+}–ATP	×20	225
	X	phosphofructokinase	*Lactobacillus acidophilus*	10mM ATP–2M-$(NH_4)_2SO_4$	×3	226
			Lactobacillus plantarum	5mM ATP	×3	226
	S	prostaglandin dehydrogenase	human placenta	KCl	×250	227
	S	pyruvate kinase	human kidney	ADP	×4	228

Nucleic acid ligands

DNA	C	DNA nicking–closing enzyme	vaccinia virus	NaCl gradient	×52	229
	C	DNA polymerases	human spleen	KCl gradient	—	230
	A	DNA unwinding enzyme II	*E. coli*	0.4M-NaCl	×17	231
	C	endonucleases	*Micrococcus luteus*	NaCl	—	232
	C	endonuclease	*E. coli*	NaCl	×250	233
DNA	C	poly(A) polymerases	vaccinia-infected HeLa cells	KCl	×5, ×4	234
	C	restriction endonuclease	*E. coli*	phosphate gradient	×6	235
	C	RNA polymerase	*Caulobacter crescentus*	KCl	—	236
	C	RNA polymerase	rat liver	$(NH_4)_2SO_4$ gradient	—	237
	C	RNA polymerase	mouse ascites fluid	$(NH_4)_2SO_4$ gradient	—	237
	S	ω-protein	*Micrococcus luteus*	4.2M-NaCl	×43	238

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Table 1 (*cont.*)

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
RNA	S	polynucleotide phosphorylase	*E. coli*	NaCl	×30	239
	S	RNA polymerase basic protein	rat liver	1M-$(NH_4)_2SO_4$	—	240
tRNA	S	tRNA-nucleotidyl transferase	*E. coli*	NaCl–EDTA	×200	241
5S RNA	A	ribosomal proteins L18, L25	*E. coli*	KCl–EDTA	—	242
Poly(A)	S	poly(A) polymerase	vaccinia virus	KCl	×9	234
Poly(C)	A	DNA polymerase	Rauscher leukaemia virus	KCl	×100	243
Poly(rC)	S	DNA polymerase	chick egg allantoic fluid	0.4M-KCl	×10	244
Poly(U)	C	DNA polymerases	avian sarcoma virus B77	KCl	×15	245
	A	polynucleotide 5′-triphosphatase	vaccinia virus	NaCl	×4	205
Saccharides						
Fucosamine	A	α-L-fucosidase	rat liver lysozomes	fucose	×262	246
Galactomannan	S	exo-β-galactofuranosidase	*Penicillium charlesii*	NaCl	×130	247
Galactopyranosylamine	S	α-D-galactosidase	soy bean meal	D-galactose	×825	248
Galactosylamine	S	neutral β-galactosidase	human liver	citrate	—	70
Lactose	P	β-galactosidase	wheat germ	galactosylglucitol	×300	249
Mannosylamine	S	acid α-D-mannosidase	bovine kidney	mannose	×4	250
Thiofucopyranoside	S	α-L-fucosidase	rat epididymis	L-fucose	×60	251
			horseshoe crab	L-fucose	×394	251
			Clostridium perfringens	L-fucose	×186	251
Thiogalactopyranoside	S	acid D-galactosidases	human liver	galactonolactone	—	252
	S	hexosaminidases A and B	human liver	gluconolactone, pH 4	×23	253
Thiogalactoside	S	β-galactosidase	*Streptococcus*	thiogalactoside or ethyleneglycol	×255	254
Thio-*N*-acetyl-glucosaminide	S	*N*-acetylglucosaminidase	*Streptococcus*	1M-NaCl	×184	254
Lectins						
Galactopyranoside	S	haemagglutinin	*Machura pomifera* seeds	pH 10	×300—×600	255
	P	lectin	peanut	D-galactose	—	170
Galactose	S	lectin	Sunn Hemp seeds	lactose	×300	256
Galactosylamine	S	lectin	peanut	galactose	—	257

Lactose	S	lectin	embryonic chick pectoral muscle	lactose	×262	258
	P	lectin	peanut	lactose	×75	169
			castor bean	lactose	×17	169
Maltose	P	concanavalin A	jack bean	methyl-mannoside	×5	169
		lectin	lentil	methyl-mannoside	×250	169
Melibiose	P	lectin	*Bandeiraea simplicifolia*	melibiose	×20	169
	P	lectin	*Bandeiraea simplicifolia*	*N*-acetylgalactose	—	259
				methylgalactose	—	259
N-Acetylchitibiose	P	lectin	wheat germ	*N*-acetylglucosamine	×125	169
N-Acetyl-galactosamine	S	lectin	*Vicia cracca*	pH gradient	×7, ×8	260
—	S	haemagglutinins	*Pseudomonas aeruginosa*	mannose, galactose	—	261

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Table 1 (*cont.*)

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
Protein ligands						
Actin	S	myosin	rabbit psoas muscle	pyrophosphate or KCl	—	33
		heavy meromyosin	rabbit psoas muscle	pyrophosphate or KCl	—	33
Activator protein	S	phosphodiesterase	rat cerebrum	Ca^{2+} chelating agent	—	262
Adenosine deaminase	S	binding protein	human kidney	6M-urea	× 14	263
Antifreeze glycoprotein	S	endogalactosaminidase	*Streptococcus*	2M-NaCl	× 400	254
Asialofetuin	S	β-galactoside-specific lectin	chick embryonic thigh muscle	lactose + thiodigalactoside	× 620	172
	S	agglutinin	peanut	?	—	173
Casein	S	protein kinase	rat liver nuclei	NaCl	× 35	264
Catalytic subunit	S	protein kinase	rabbit skeletal muscle	KCl–guanidine hydrochloride	× 358	61
Chymotrypsin	S	chymotrypsin inhibitor	*O. radiatum* (calf worm)	pH 2.9	× 156	53
Collagen	S	anti-collagen IgG	?	1M-NH_4OH	—	265
	S	collagenase–procollagenase	mouse	NaCl	× 45	54
	A	collagen-glucosyl transferase	chick embryo	collagen peptides	× 387	1
	S	collagen-glucosyl transferase	chick embryo	ethylene glycol	× 223	5
Complement component C4b	S	complement component C2	human plasma	NaCl	—	184
Elastin	S	elastase	human blood platelets	1M-sodium acetate, pH 4.5	—	55
Elongation factor Tu	S	elongation factor Ts	*E. coli*	Mg^{2+}–GDP	—	18
Ferredoxin	S	nitrite reductase	spinach	phosphate gradient	× 8	266
Histone	S	protein kinase NII	rat liver nuclei	NaCl gradient	× 4	267
IgG	P	protein A	*Staph. aureus*	pH 3	—	31
	S	protein A	methicillin-resistant *Staph. aureus* A676	0.1M-glycine–HCl, pH 3	—	58
	S	complement component C1r	human serum	EDTA	—	60
		C1s	human serum	EDTA	—	60
IgM	S	secretory component	human whey	1M-KSCN	—	59
Insulin	A	insulin receptor	rat liver membranes	4.5M-urea	—	268
Insulin B chain	S	neutral peptidase	rat kidney	Tris, pH 7.2	× 4	269
α-Lactalbumin	S	galactosyl transferase	sheep mammary glands	1M-NaCl	× 2	56
	S	galactosyl transferase	rat liver microsomes	– *N*-acetylglucosamine	× 680	22

		S	UDP-galactose-glycoprotein galactosyl transferase	human plasma	$-N$-acetylglucosamine	× 200, × 50	57
α-Neurotoxin		S	acetylcholine receptor	muscle cell line	carbamoyl chloride	—	270
Neurotoxin (cobra)		S	acetylcholine receptor	*Torpedo californica*	0.5M-hexamethonium	× 38	271
Plasminogen		S	anti-plasmin	human serum	6-aminohexanoic acid	× 148	272
P-Light chain		S	myosin light chain kinase	rabbit fast skeletal muscle	$(-)Mg^{2+}$, phosphate	× 2	273
Pokeweed mitogen	Pa-1	S	B-cell receptor	mouse lymphocytes	di-N-acetylchitibiose	—	274
	Pa-2	S	T-cell receptor	mouse lymphocytes	di-N-acetylchitibiose	—	274
Protamine		S	IgM	human serum	1.1M-NaCl	—	275
Protease inhibitor (potato)		S	carboxypeptidase	bovine pancreas	pH 11.4	× 6	50
				porcine pancreas	pH 11.4	× 11	50
Protease inhibitor		S	vicilin peptidohydrolase	mung bean seedlings	2M-NaCl	—	51
Retinol binding protein		S	prealbumin	rat plasma	NaCl	× 166	276
Soy bean inhibitor		A	trypsin	human blood platelets	pH 2	—	55
Transferrin		S	transferrin binding protein	rabbit reticulocyte membrane	0.5M-NaCl, pH 9.5	—	277
Troponin I		S	troponin C	various rabbit muscle tissues	8M-urea–EGTA	—	62
Trypsin		S	trypsin inhibitor	bovine cartilage	low pH	× 40	52
				bovine aorta	low pH	× 70	52

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Table 1 (*cont.*)

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
	S	trypsin inhibitor	opaque-2-corn seeds	1M-acetic acid	×7.5	278
Miscellaneous ligands						
Acridinium deriv.	S	acetylcholinesterase	*Electrophorus electricus*	decamethonium chloride	—	279
S-Adenosylhomocysteine	S	N^2-guanine-RNA-methyltransferase	chicken embryo	NaCl	×26	280
Agalacto-orosomucoid	S	*N*-acetylglucosamine-specific hepatic binding protein	chicken liver	NaCl–low pH	×180	281
Alprenol	S	β-adrenergic receptor	turkey erythrocyte membrane	NaCl–dihydroalprenol	×2000	34
Amethopterin	A	quinonoid dihydropterin reductase	bovine liver	Tris	×400	39
Aminobenzamidine	S	enterokinase	bovine duodenum	benzamidine gradient	×23	282
Androsterone deriv.	S	testosterone binding globulin	bovine serum	testosterone	×140	63
Arginine	S	clostripain	*Clostridium histolyticum*	Tris gradient	×55	283
D-Arginine	S	carboxypeptidase	*Streptomyces griseus*	NaCl gradient–low pH	—	284
Arginine methyl ester	S	plasminogen activator	human pancreatic carcinoma	0.5M-arginine	×19	285
Benzamidine	S	clostripain	*Clostridium histolyticum*	Tris gradient	—	283
	S	factor IX	bovine plasma	NaCl gradient	×11	181
	S	factor X	bovine plasma	NaCl gradient	×11	181
	S	factor XII	bovine plasma	NaCl gradient	—	182
Benzyladenine	S	cytokinin-binding protein	tobacco leaves	50mM-NaOH	—	286
Benzyloxyaniline	S	luciferase	*Renilla reniformis*	35% ethylene glycol	×5	9
Bromosulphophthalein	S	glutathione-S-transferase	porcine liver	KSCN	×6	287
Chitin	—	lytic enzyme (amidase)	T7 bacteriophage	KCl	×250	288
Coenzyme A	A	glutaryl-Co A reductase	rat liver	KCl	×5	289
Colominic acid	St	neuraminidase	*Arthrobacter*	acetate	×5	290
C-Polysaccharide	A	C-reactive protein	rabbit serum	$+Ca^{2+}$	—	291
Cycloserine	A	alanine aminotransferase	human serum	phosphate	×100	292
Cytidine derivs.	S	ribonuclease	bovine pancreas	4M-NaCl	—	293
α-Dextrin	S	pullulanase	*Aerobacter aerogenes*	β-dextrin	×70	294
Ethylglutamate	S	L-glutamate decarboxylase	rat brain	NaCl	×80	295
Flavin-mononucleotide	S	NAD(P)H-flavin oxidoreductase	*Beneckea harveyi*	FMN gradient	×35	296

Folate	S	dihydrofolate reductase	L1210 cells	folate–high pH	—	297
			Lactobacillus casei	folate–high pH	—	297
			human leukaemic leukocytes	folate–high pH	—	297
	S	folate-binding protein	goat milk	0.2M-acetic acid	×260	298
Glutathione	S	glutathione-*S*-transferase	human liver	KCl gradient	×2	299
	C	glutathione reductase	—	NADPH	—	300
	S	glyoxylase I	pig erythrocytes	glutathione gradient	×16	301
	S	glyoxylase I	mouse liver	glutathione	×57	2
Haem	P	haemopexin	human serum	low pH	—	302
Histamine	S	histidinol dehydrogenase	*Bacillus psychrophillus*	1M-imidazole, pH 9	—	48
			Bacillus subtilis	1M-imidazole, pH 9	—	48

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Table 1 (*cont.*)

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
Histidine	S	histidinol dehydrogenase	*Bacillus subtilis* HT1	0.1M-histidine, pH 9	—	48
			Bacillus caldolyticus	0.1M-histidine, pH 9	—	48
			Bacillus stearothermophilus	0.1M-histidine, pH 9	×24	48
Hydroxycholecalciferol	S	calciferol-binding protein	chick kidney	ammonium acetate	×200	11
			chick serum	ammonium acetate	×1000	11
Hydroxycobalamin	S	transcobalamin II	rabbit	increased temperature	—	303
Linolenate	A	lipoxygenase	soy bean	NaCl gradient	×16	15
			pea	NaCl gradient	×16	15
Lysine	S	plasminogen	sheep plasma	ε-aminocaproic acid	—	304
Methotextrate	S	dihydrofolate reductase	L1210 cells (resistant subline)	dihydrofolate	×167	305
			Chinese hampster ovary	dihydrofolate	×38	306
			chicken liver	dihydrofolate	×210	65
	S	thymidylate synthetase	human blast cells	Tris gradient	×90	20
Mucin glycopeptides	S	neuraminidase	*Streptococcus*	2M-NaCl	×112	254
Oxamate	S	lactate dehydrogenase	*Fundulus heteroclitus*	NADH	—	307
Palmitate	C	lipase	*Chromobacterium viscosum*	detergent	×11	308
Pepstatin	A	renin	hog kidney	pH 3.2	×370	309
Phenylalanine	S	phenylalanine ammonia lyase	sweet potato	phenylalanine	×7	310
Phenylalanine derivs.	S	chorismatemutase–prephrenate dehydrogenase	*E. coli*	pH 9–adamantaneacetate	×52	311
N-Phenylphosphenyl-phe-phe	A	neutral protease	*Bacillus cereus*	pH 9	—	312
Phenylproprionate derivs.	S	carboxypeptidase A	bovine pancreas	KCl gradient	×104	313
Poly(L-lysine)	S	pepsin	sheep abomasa	NaCl gradient	×50	314
Pregnenolone	S	cytochrome P450	bovine adrenal medulla	KCl–pregnenolone	—	17
Pyridoxamine 5′-phosphate	A	ornithine decarboxylase	SV40 transformed 3T3 mouse fibroblasts	pyridoxal 5′-phosphate	×500	315
Pyromellitic acid	S	fumarase	pig liver	L-malate	×211	12
			pig heart	L-malate	×57	12
Quaternary ammonium	S	acetylcholine receptor	mammalian skeletal muscle	gallamine triethiodide	×173	316
Quaternary ammonium	S	acetylcholine receptor	housefly brain	inhibitor	×1223	67
Silica	—	folate-binding protein	*Lactobacillus casei*	folate, pH 10	×60	317

Sulphanilamide	A	carbonic anhydrase	rat kidney	chloride, perchlorate	—	318
			rat erythrocytes	chloride, perchlorate	—	318
Sulphonamide	S	carbonic anhydrase	sheep and chicken organs	0.2M-sodium sulphate, pH 8	—	319
dTMP Deriv.	S	thymidylate kinase	human myelocytic leukaemia cells	dTMP	×650	19
TPP	S	transketolase	*Candida utilis*	TPP	—	320
D-Tryptophanyl methyl ester	S	protease	human purulent sputum	Ca^{2+}–acetic acid	—	321
	A	'chymotrypsin-like' protease	human blood platelets	pH 2–0.2M-KCl	—	55
Tyramine	S	monoamine oxidase	rat liver	KCl gradient	×2	322
			human liver	KCl gradient	×2	322
UDP-Glucose	S	collagen glycosyl transferase	chick embryo	ethylene glycol	×250	288
UDP-Glucuronic acid	A	collagen glycosyl transferase	chick embryo	collagen peptides	×37	1
Uracil deriv.	S	thymidine kinase	*E. coli*	1M-NaCl	×350	47

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Table 1 (*cont.*)

Ligand	*M*	*Protein*	*Source*	*Eluant*	*Factor*	*Ref.*
Vitamin B12	S	methylmalonyl–CoA mutase	*Proprionibacterium shermanii*	vitamin B12	× 300	323
Vitamin B12–intrinsic factor	S	intrinsic factor receptor	rat intestine	EDTA	× 335	324
	S	intrinsic factor receptor	guinea-pig ileum	EDTA	× 15 500	325
495C (Anti-allergen)	S	phosphodiesterase	guinea-pig lung	575C	× 422	326

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Table 2 *Selected chromatographic procedures*

Protein	*Source*	*Ligand*	*Eluant*	*Factor*	*Ref.*
Immunoaffinity chromatography					
Acyl carrier protein	*E. gracilis, Bacillaris*	—	glycine–HCl pH 2.8	—	88
Adenosine deaminase	human erythrocytes	—	urea gradient	×8000	327
Adrenocorticotrophic hormone precursor	ovine pituitary	—	0.1N-acetic acid–1N-NaCl–3M-guanidine	—	328
Alkaline phosphatase	human placenta	—	pH 12	×6	79
Angiotensin converting enzyme	human serum	—	3.8M-urea	×875	85
Arylsulphatase	*Klebsiella*	—	glycine–HCl pH 2.4	×15	329
Blocking factors	mouse serum	—	3M-KSCN	—	90
Carcinoembryonic antigen	human colon mucosa	—	3M-KSCN	×30	330
Choriogonadotropin	human term placenta	—	4M-$MgCl_2$	×57	89
			guanidine	×53	89
α-Fetoprotein	human	—	0.5M-NaCl–glycine–HCl pH 2.8	—	82
	ovine	—	7M-urea	×42	80
	rat	—	glycine–HCl pH 2.8	—	81
Histone H3	chick erythrocytes	—	0.1M-glycine–HCl pH 2.5	—	93
Hypoxanthine phosphoribosyl-transferase	HeLa cells	—	0.2% SDS	—	92
Ia-like alloantigen	B-lymphoblastoid cells	—	pH 11	×10	78
Interferon	human lymphoblastoid cells	—	pH 2.2	—	331
Myosin alkali light chains	chicken breast muscle	—	4M-guanidine	—	94
Pyrogen	human leukocyte	—	pH 3.2	—	91
Properdin	human serum	—	0.5M-NaCl	—	86
Release factor RF2	*E. coli*	—	6M-guanidine	—	332
Lectin affinity chromatography					
Antiplasmin	human plasma	concanavalin A	α-methylmannoside	×1.6	272

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Table 2 (*cont.*)

Protein	*Source*	*Ligand*	*Eluant*	*Factor*	*Ref.*
Arylsulphatase A	rabbit kidney cortex	concanavalin A	α-methylmannoside	×9	333
B				×37	333
Carcinoembryonic antigen	human colon mucosa	concanavalin A	α-methylmannoside	×27	84
Collagen glycosyl transferase	chick embryo	concanavalin A	α-methylmannoside	×79	5
	human foetal tissue			×30	5
Concanavalin A receptor	human erythrocyte	concanavalin A	α-methylmannoside	—	100
Erythropoietin	sheep	wheat germ agglutinin	*N*-acetylglucosamine	×100	334
α-Fetoprotein	rat	RCA I agglutinin	galactose	—	335
α-Fetoprotein	rat	concanavalin A	α-methylmannoside	—	335
Glucocerebroside-glucosidase	human placenta	concanavalin A	α-methylmannoside	×130	336
γ-Glutamyl transferase	human liver	concanavalin A	methylglucoside	×3	337
Glycogen debranching enzyme	dogfish muscle	concanavalin A	α-methylmannoside	×6	338
Glycogen synthase I	human polymorphonuclear lymphocytes	concanavalin A	glucose	×100	101
Haemopexin	human	wheat germ agglutinin	*N*-acetylglucosamine	—	339
Hexosaminidases	human kidney	concanavalin A	α-methylmannoside	×15	340
Hexosaminidases	human liver	concanavalin A	α-methylmannoside	×24	253
H-2 Antigens	mouse	*Lens culinaris* haemagglutinin	α-methylmannoside	×16	341
HLA-Antigens	human lymphoblastoid cells	*Lens culinaris* haemagglutinin	α-methylmannoside	×12	156
HLA-Antigens	B-cells	*Lens culinaris* haemagglutinin	α-methylmannoside	—	342
IgG3	human serum	*Ricinus* agglutinin	lactose	—	343
Insulin receptor	rat liver	concanavalin A	α-methylmannoside	—	268
Lysine hydroxylase	chick embryo	concanavalin A	α-methylmannoside + ethylene glycol	×450	97
α-D-Mannosidase	bovine kidney	concanavalin A	α-methylmannoside	×20	250
Prostatic acid phosphatase	human	concanavalin A	mannose gradient	×6	344

Sphingomyelinase	human brain	concanavalin A	α-methylmannoside	× 15	345
Heparin-Sepharose chromatography					
Antithrombin III	human plasma	—	NaCl gradient	× 8	346
Estradiol receptor	calf uterus	—	heparin or chaotrophs	× 118	103
Factor IX	bovine plasma	—	NaCl gradient	× 11	181
Factor XI	bovine plasma	—	NaCl gradient	× 200	347
Factor XII	bovine plasma	—	NaCl gradient pH 7.2	× 38	182
			pH 6.0	× 10	
Lipoprotein lipase	rat heart	—	NaCl	× 43	348
Lipoprotein lipase	human plasma	—	NaCl gradient then	× 800	349
			NaCl stepwise	× 63	
Salt-resistant lipase	human plasma	—	NaCl gradient then	× 500	349
			NaCl stepwise	× 22	
Lipoprotein lipase	rat plasma	—	1.3M-NaCl	× 160	350
RNA Polymerase III	wheat embryo	—	$(NH_4)_2SO_4$	× 69	351
Triacylglycerol lipase	porcine plasma	—	NaCl	× 300, × 600	352

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Table 2 (*cont.*)

Protein	*Source*	*Ligand*	*Eluant*	*Factor*	*Ref.*
Hydrophobic chromatography					
Adenosylhomocysteinase	*Lupinus luteus* seeds	aminohexyl	KCl	× 13	353
Adenylosuccinate synthetase	*Azotobacter vinelandii*	butyl	NaCl gradient	—	13
Alanyl-tRNA synthetase activator protein	rat liver	aminohexyl	NaCl	—	354
Alkaline phosphatase	human placenta	phenylalanyl	decreasing $(NH_4)_2SO_4$ gradient	× 3	79
Aminoacyl-tRNA synthetases (Ileu)	*E. coli* B	aminohexyl	KCl gradient	× 3–× 94 (× 94)	166
Aspartokinase II	*Bacillus subtilis*	leucyl	decreasing $(NH_4)_2SO_4$ gradient	× 4.3	355
Collagen glucosyl transferase	chick embryo	valyl	decreasing$(NH_4)_2SO_4$ gradient	× 33	5
4-Coumarate: CoA ligase	parsley	aminohexyl	Tris gradient	× 3	356
Cytochrome P450	yeast	aminohexyl	Emulgen 913	× 33	357
DNA Polymerase III	*E. coli*	valyl	decreasing $(NH_4)_2SO_4$ gradient	× 6	358
Fructose-6-phosphate kinase	*E. coli*	propyl	decreasing $(NH_4)_2SO_4$ gradient	× 24	214
Glucocerebrosidase	human placenta	octyl	ethylene glycol gradient	× 8	359
		decyl	ethylene glycol gradient	× 16	359

Glutathione reductase	*E. coli*	aminohexyl	NaCl gradient	×7	3
Glycosyl transferase	*E. coli*	4-aminobutyl	1M-KCl + maltose	×535	360
Glyoxylase I	mouse liver	aminoethyl	phosphate gradient	×20	2
IgG(T)	equine	phenyl	decreasing $(NH_4)_2SO_4$ gradient	—	361
Ketonemono-oxygenase	*Pseudomonas cepacia*	aminohexyl	KCl	×2.5	362
Protein kinase	rabbit muscle	hexyl	NaCl gradient	×30	363
Thioredoxin reductase	*E. coli*	aminohexyl	NaCl gradient	×7	3

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such as the level of the protein in the starting material or the stage at which the technique in question is applied (*e.g.* the specific removal of haemoglobin results in a 900-fold purification of human erythrocyte hypoxanthine phosphoribosyl transferase [188]), it is included to give an idea of the power of the particular method.

In Table 1, column 2 the following abbreviations are used: M = matrix; A = agarose; C = cellulose; P = polyacrylamide; S = Sepharose; X = Sephadex.

5 Protein Characterization Methodology

Kirschenbaum has continued his compilations of published amino-acid compositions of proteins expressed either as residues per molecule (H—Iso,[364] K—Ly [365]) or as residues per 1000 residues [366] and his compilations of Molar absorptivities of proteins (O—Z [367] and, starting again, A—Cy [368] and A—H [369]); any reader desiring such information about a specific protein is recommended first to check through these and previous lists.

Protein Determination.—A combined biuret–Folin assay for protein which combines the specificity of the biuret method with the sensitivity of Folin has been described. Heating of the protein at 100 °C for 100 min with the biuret reagents prior to the addition of Folin reagent abolished the wide variation of Molybdate Blue production previously seen with different proteins.[370] Deoxycholate–trichloracetic acid precipitation of proteins at concentrations as low as 1 μg ml^{-1} has also been used to remove interfering substances and therefore make the Folin method more generally applicable.[371] Yet more methods using Coomassie Brilliant Blue to quantitate protein have been described, with a level of detection of less than 0.1 μg protein.[372–374] Others have, however, criticized the use of Coomassie dyes to quantitate protein, finding a highly variable response with different proteins.[375, 376] A modified method using the anionic dye bromosulphalein and applicable within the range 0.5—100 μg protein has been described.[377] Exposure of proteins to sodium hydroxide has been shown to affect adversely the quantitation of proteins using Xylene Brilliant Cyanin G.[378] A fluorometric assay for proteins involving spotting of the protein-containing solution onto kieselguhr and of use over the range 0.1—1.0 μg protein has been claimed to give minimal variation between different proteins.[379] [^{3}H]dansyl chloride has been described as

[364] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **83**, 484.
[365] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **83**, 521.
[366] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **79**, 470.
[367] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **80**, 193.
[368] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **81**, 220.
[369] D. M. Kirschenbaum, *Analyt. Biochem.*, 1977, **82**, 83.
[370] T. E. Dorsey, P. W. McDonald, and A. O. Roels, *Analyt. Biochem.*, 1977, **78**, 156.
[371] G. L. Peterson, *Analyt. Biochem.*, 1977, **83**, 346.
[372] G. S. McKnight, *Analyt. Biochem.*, 1977, **78**, 86.
[373] J. J. Sedmak and S. E. Grossberg, *Analyt. Biochem.*, 1977, **79**, 544.
[374] R. W. Rubin and R. W. Warren, *Analyt. Biochem.*, 1977, **83**, 773.
[375] J. Pierce and C. H. Suelter, *Analyt. Biochem.*, 1977, **81**, 478.
[376] H. van Kley and S. M. Hale, *Analyt. Biochem.*, 1977, **81**, 485.
[377] J. McGuire, P. Taylor, and L. A. Greene, *Analyt. Biochem.*, 1977, **83**, 75.
[378] A. T. Høstmark, L. Sørensen, and R. Askevold, *Analyt. Biochem.*, 1977, **83**, 782.
[379] T. Kinoshita, F. Iinuma, K. Atsumi, and A. Tsuji, *Analyt. Biochem.*, 1977, **77**, 471.

a reagent for the quantitation of nanogram amounts of protein.[380] A convenient direct spectrophotometric method for the determination of Sepharose-bound protein using polyethylene glycol to reduce light scattering has been described.[381]

Electrophoretic Characterization.—*Apparatus and Electrophoretic Support Media.* An apparatus that allows the simultaneous performance of polyacrylamide gel electrophoresis at multiple pHs has been described. A useful appendix to this paper lists the composition of a number of buffer systems giving separation at different pHs.[382] A procedure that permits analytical polyacrylamide gel electrophoresis to be performed under anaerobic conditions was used to characterize oxygen-sensitive proteins such as nitrogenase. Since dithionite was used as an oxygen scavenger, the dithionite-resistant guaiazulene-1-sulphonate was used as a tracking dye.[383] A simple procedure that permits the running of several slab gels at one time using the same buffer reservoirs has been published. Sucrose was added to the bottom buffer to increase its density, and an organic solvent of intermediate density between this and the top buffer was used to separate the two. Organic solvents of very high dielectric constant and low solubility in water were mixed to produce the required density.[384] High agarose concentrations (4—15%) were used as the support medium in separations giving resolution of up to 30 plasma proteins.[385] The claimed simplicity of the method, particularly in dealing with such high concentrations of agarose, should be treated with caution. Liquid polyacrylamide (acrylamide polymerized in the absence of cross-linker) immobilized in agarose has been used to give improved molecular sieving characteristics to the agarose. The viscosity of liquid polyacrylamide, however, placed an upper working limit of 5% acrylamide in the gel.[386, 387]

Isoelectric Focusing. A series of papers dealing with natural pH gradients [388, 389] and the stabilization of pH gradients [390, 391] have been published. The presence of urea and Nonidet-P40 during focusing has been claimed to give increased resolution of proteins.[392] A theoretical treatment has delineated the conditions under which transient double peaks occur in pH gradient electrophoresis.[393] Isoelectric focusing in 8M-urea of proteins separated initially by polyacrylamide gel electrophoresis in sodium dodecyl sulphate has shown that the isoelectric point of the protein is not affected by its preparation in SDS, and that the SDS must therefore be removed during focusing.[394]

[380] R. M. Schultz and P. M. Wassarman, *Analyt. Biochem.*, 1977, **77**, 25.
[381] T. O. Golovina, T. V. Cherednikova, A. T. Mevkh, and N. K. Nagradova, *Analyt. Biochem.*, 1977, **83**, 778.
[382] C. Bui, V. Galea, and A. Chrambach, *Analyt. Biochem.*, 1977, **81**, 108.
[383] C. E. McKenna, H. T. Nguyen, C. W. Huang, and S. A. Smith, *Analyt. Biochem.*, 1977, **83**, 337.
[384] C. Dubertret and J. Legault-Demare, *Analyt. Biochem.*, 1977, **82**, 564.
[385] H. Hoch and C. G. Lewallen, *Analyt. Biochem.*, 1977, **78**, 312.
[386] H.-J. Bode, *Analyt. Biochem.*, 1977, **83**, 204.
[387] H.-J. Bode, *Analyt. Biochem.*, 1977, **83**, 364.
[388] N. Y. Nguyen and A. Chrambach, *Analyt. Biochem.*, 1977, **79**, 462.
[389] M. L. Caspers and A. Chrambach, *Analyt. Biochem.*, 1977, **81**, 28.
[390] N. Y. Nguyen and A. Chrambach, *Analyt. Biochem.*, 1977, **82**, 54.
[391] N. Y. Nguyen and A. Chrambach, *Analyt. Biochem.*, 1977, **82**, 226.
[392] K. Zechel, *Analyt. Biochem.*, 1977, **83**, 240.
[393] M. Dishon and G. H. Weiss, *Analyt. Biochem.*, 1977, **81**, 1.
[394] G.-I. Danno, *Analyt. Biochem.*, 1977, **83**, 189.

Slab Gels and Two-dimensional Separations. An improved method of electrophoresis in chloral hydrate has been used to analyse protein constituents of biological membranes. The use of aluminium lactate buffer enabled separation to be carried out in slab rather than tube gels.[395] A modification to a previously described system that permitted the separation of all histones in a single gel utilized thiourea and hydrogen peroxide as polymerizing catalysts.[396] Ultracentrifugation prior to loading and the use of SDS in sample preparations have given improved resolution in the two-dimensional separation of proteins described originally by O'Farrell.[397] A method for calculating confidence intervals in the identification of proteins separated by electrophoresis has been presented. This was argued as being of special value where two-dimensional separations were being compared.[398]

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. The use of SDS from different commercial sources has been shown to give differing mobilities for several viral proteins. This was especially pronounced where discontinuous buffer systems were used. The possibility that this was related to the presence of alkyl chains containing more than 12 carbon atoms was discussed. Coomassie staining intensity was also affected by the different SDS preparations.[399] Changes were found in the order of migration of polypeptides when three different commonly used systems for SDS-gel electrophoresis were compared.[400] It is worth noting that in none of these was the sample prepared by heating at 100 °C. A modified procedure for SDS-gel electrophoresis in a continuous buffer system has been claimed to give improved resolution. In addition to rigorous attention to the purity of the reagents, glycerol was included in the gel and, perhaps more noteworthy, during polymerization the gel was overlayered with buffer containing SDS and the polymerizing catalysts.[401] A critical appraisal of the effect of SDS concentration on the stacking of proteins and of the validity of R_F reference points has been made. In the optimized system described there was no SDS in the gel or the anodal well buffer, 150 μg or less in the sample, and only 0.03% in the cathodal well buffer.[402] An SDS-micro-gel technique useful for analysing proteins in the range 0.1—1 ng and claimed to be easier to use than ultra-micro techniques has been described.[403] Frequently further characterization of a protein is required, and for this it must be removed from the gel. Previously described procedures generally give high dilution of the protein. A method by which concentrated protein samples can be recovered from SDS-gels has now been described. After electrophoretic removal of the protein from the gel, it was precipitated with trichloracetic acid and the protein collected onto a nuclepore filter. The precipitate and filter were then dissolved in chloroform saturated with SDS-electrophoresis buffer, and the protein recovered by extracting the chloroform with

[395] B. Ballou and O. Smithies, *Analyt. Biochem.*, 1977, **80**, 616.
[396] C. K. Hurley, *Analyt. Biochem.*, 1977, **80**, 624.
[397] D. L. Wilson, M. E. Hall, G. C. Store, and R. W. Rubin, *Analyt. Biochem.*, 1977, **83**, 33.
[398] N. Muñiz, D. Rodbard, and A. Chrambach, *Analyt. Biochem.*, 1977, **83**, 724.
[399] H. D. Matheka, P.-J. Enzmann, H. L. Bachrach, and B. Migl, *Analyt. Biochem.*, 1977, **81**, 9.
[400] R. A. Capaldi, R. L. Bell, and T. Branchek, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 425.
[401] M. A. Porzio and A. M. Pearson, *Biochim. Biophys. Acta*, 1977, **490**, 27.
[402] M. Wyckoff, D. Rodbard, and A. Chrambach, *Analyt. Biochem.*, 1977, **78**, 459.
[403] J. S. Condeelis, *Analyt. Biochem.*, 1977, **77**, 195.

electrophoresis buffer.[404] It has been suggested that the pH at which Dowex resin is normally used to remove SDS from proteins is too high, and that better separation is achieved at pH 6.[405]

Immunochemical Techniques. Antibodies have been used to give identification of specific proteins after electrophoretic separation of a mixture of proteins in SDS. After fixing, and removal of SDS, the gel was incubated with the required antiserum, washed thoroughly, and stained with Coomassie Blue. The treated gel was then compared with a control gel which had not been exposed to antiserum, and a difference in staining intensity was looked for.[406] Proteins separated by SDS-polyacrylamide gel electrophoresis have also been identified by embedding a polyacrylamide strip in agarose and allowing the proteins to diffuse against antiserum in a manner analogous to immunoelectrophoresis.[407] A more detailed look at the use of antisera to identify proteins after separation in SDS, using an overlayer of antibody-containing agarose, and again allowing antibody and protein to diffuse against each other, found that pretreatment of antigens with 1% SDS at 100 °C for 5 min. appeared to have no effect on the precipitation. In contrast, similar treatment of the antibody resulted in total loss of precipitation. The use of 2-mercaptoethanol in sample preparation had no adverse effect, nor did the presence of 0.1% SDS in the agarose overlayer. With 1% SDS in the agarose no precipitation was seen, and if the gel had been stained with Coomassie Blue before overlayering with antibody a significant reduction in the amount of precipitation occurred.[408] These findings have interesting implications regarding the nature of antigenic determinants in proteins and either the retention of these or the ability to regain them in the presence of antibody after denaturation in SDS. The opposite approach, of separating proteins by immunochemical techniques and then characterizing them using SDS-polyacrylamide gel electrophoresis, has also been described. ^{125}I-labelled proteins were separated using two-dimensional crossed electrophoresis. Precipitin lines were then eluted with SDS-2-mercaptoethanol and subjected to electrophoresis, labelled bands being identified by autoradiography.[409] Immunochemical methods have also been used to characterize proteins separated in a non-electrophoretic manner. A novel procedure called 'immuno-ultracentrifugation' has been used to identify proteins in sucrose density gradients. Acrylamide and riboflavin were incorporated into the density gradient during its preparation. Following ultracentrifugation the gradient was photopolymerized and then the polymerized gel sliced, embedded in agarose, and antigen and antibody allowed to diffuse against each other.[410]

Electrophoretic Characterization of Specific Classes of Proteins. 'Charge-shift' electrophoresis has been described as a method by which amphiphilic proteins (intrinsic membrane proteins) can be recognized in crude mixtures. Amphiphilic

[404] G. P. Tuszynski, C. H. Damsky, J. P. Fuhrer, and L. Warren, *Analyt. Biochem.*, 1977, **83**, 119.
[405] A. S. Dion, C. J. Williams, and A. A. Pomenti, *Analyt. Biochem.*, 1977, **82**, 18.
[406] K. Olden and K. M. Yamada, *Analyt. Biochem.*, 1977, **78**, 483.
[407] S. Purszkin, W. Schook, J. Maimon, and E. Puszkin, *Biochim. Biophys. Acta*, 1977, **494**, 144.
[408] V. Neuhoff and S. Mesecke, *Z. physiol. Chem.*, 1977, **358**, 1623.
[409] B. Norrild, U. J. Bjerrum, and B. F. Vestergaard, *Analyt. Biochem.*, 1977, **81**, 432.
[410] P. T. Quinn and M. A. Fernando, *J. Immunol. Methods*, 1977, **14**, 253.

proteins were recognized by a change in mobility when electrophoresed in agarose containing either Triton X-100–deoxycholate or Triton X-100–cetyltrimethylammonium bromide. Hydrophilic proteins did not bind detergent and therefore had unaltered mobilities.[411] Amphiphilic proteins have also been recognized by performing two-dimensional crossed electrophoresis in the presence of ^{14}C- or ^{125}I-Triton X-100. The labelled Triton could either be added to the sample before electrophoresis in the first dimension or be added to the antibody-containing agarose, through which electrophoresis in the second dimension took place. Autoradiography showed amphiphilic proteins as radioactive peaks.[412] Lectins have been used in place of antibodies to detect and quantitate glycoproteins by single-dimension [413] and two-dimensional [414] crossed electrophoresis. ^{125}I-labelled lectins have also been used to 'stain' glycoproteins following electrophoretic separation on SDS-polyacrylamide gels.[415] Affinity electrophoresis of lectins on *O*-glycosyl polyacrylamide matrices has been used both to determine the dissociation constant of lectin-sugar complexes [416] and also to study their heterogeneity.[417] Commercial preparations of concanavalin A electrophoresed on α-D-mannosyl-polyacrylamide gels gave three bands. With the addition of manganese, however, only one band was seen, showing commercial preparations to be manganese deficient.

Staining Techniques. A phosphorylated 10 000 dalton protein with a high serine content (14%) from frog muscle has been reported to resist staining with Coomassie Blue.[418] Another acidic protein (phosvitin) was also poorly stained with Coomassie. Cationic dyes could not be used to stain this protein as they bound to residual negative charges in the matrix. With phosvitin, however, the presence of contiguous phosphorylserine residues, which have a high affinity for trivalent metal ions, permitted Coomassie staining after saturation of the protein with trivalent aluminium ions.[419] Improved staining techniques using Coomassie Blue [420, 421] and new methods using Drimarene Brilliant Blue K-BL [422] (claimed to give a linear relationship between amount of protein and staining intensity over the range 0.5—25 μg protein) and a fluorescent maleimide [423] have been described. A single procedure for detecting both carbohydrate and protein using *p*-hydrazinoacridine was claimed to have a comparable sensitivity to Coomassie with both glycoproteins and non-glycoproteins, while also permitting one to distinguish between

[411] A. Helenius and K. Simons, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 529.
[412] O. J. Bjerrum and S. Bhakdi, *F.E.B.S. Letters*, 1977, **81**, 151.
[413] P. Owen, J. D. Oppenheim, M. S. Nachbar, and R. E. Kessler, *Analyt. Biochem.*, 1977, **80**, 446.
[414] T. C. Bøg Hansen, O. J. Bjerrum, and C.-H. Brogen, *Analyt. Biochem.*, 1977, **81**, 78.
[415] J. A. P. Rostas, P. T. Kelly, and C. W. Cotman, *Analyt. Biochem.*, 1977, **80**, 366.
[416] V. Horejsi, M. Ticha, and J. Kocourek, *Biochim. Biophys. Acta*, 1977, **499**, 290.
[417] V. Horejsi, M. Ticha, and J. Kocourek, *Biochim. Biophys. Acta*, 1977, **499**, 301.
[418] M. Barany, K. Barany, E. Gaetjens, and A. Steinschneider, *Biochim. Biophys. Acta*, 1977, **491**, 387.
[419] J. Hegenauer, L. Ripley, and G. Nace, *Analyt. Biochem.*, 1977, **78**, 308.
[420] O. Vesterberg, L. Hansen, and A. Sjösten, *Biochim. Biophys. Acta*, 1977, **491**, 160.
[421] R. W. Blakesley and J. A. Boezi, *Analyt. Biochem.*, 1977, **82**, 580.
[422] H. F. Bosshard and A. Datyner, *Analyt. Biochem.*, 1977, **82**, 327.
[423] K. Yamamoto, T. Sekine, and Y. Kanaoka, *Analyt. Biochem.*, 1977, **79**, 83.

them.[424] Detection of protein bands in SDS-gels by insoluble complex formation between SDS and a cationic detergent has also been described.[425]

Molecular Weight Determination.—Endo-β-*N*-acetylglucosaminidase H has been used to remove carbohydrate from glycoproteins and thus give an improved determination of the molecular weight of the polypeptide chain using SDS-polyacrylamide gel electrophoresis. Denaturation of the glycoprotein was required for full removal of carbohydrate.[426] Following periodate treatment, human leukocyte interferon gave a single narrow band of about 15 000 daltons rather than two broad bands at 21 000 and 15 000 daltons when subjected to SDS-gel electrophoresis.[427] Errors have been found in molecular weight determinations of multimeric proteins calculated from K_R values found by electrophoresis in gels containing different percentages of polyacrylamide, and two alternative methods for molecular weight calculation have been presented.[428] Another gel electrophoretic system using the cationic detergent cetyltrimethylammonium bromide has been described and found to give a linear log molecular weight *vs.* mobility plot. This method was claimed to be simpler to use than other cationic systems.[429] Commercially prepared polyacrylamide gradient gels have been shown to give a linear relationship between the hypothetical limiting pore size (reciprocal of the limiting gel concentration) and the cube root of the molecular weight of proteins over the range 13 500—900 000 daltons. A particular advantage of this method is that by using specific staining techniques the molecular weight of proteins can be determined without prior purification.[430] Gel filtration of proteins in 6M-guanidine hydrochloride on Sepharose CL-4B has given an impressively linear log molecular weight *vs.* K_d plot.[29] A modification to the difference sedimentation velocity method of molecular weight determination of proteins that permits its use in the range 10 000—20 000 daltons has been described.[431] Small angle *X*-ray scattering has been used in determining the molecular weight and shape of proteins ranging from 11 900 daltons (ε subunit of the chloroplast coupling factor CF-1)[432] to 9 020 000 daltons (*Helix pomatia* β-haemocyanin).[433, 434–438]

Fingerprinting of Proteins.—Limited proteolysis in SDS of proteins prepared by SDS-gel electrophoresis followed by gel analysis of the digest has been described.[439]

[424] S. D. Carson, *Analyt. Biochem.*, 1977, **78**, 428.
[425] T. Tagagi, K. Kubo, and T. Isemura, *Analyt. Biochem.*, 1977, **79**, 104.
[426] R. B. Trimble and F. Maley, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 935.
[427] W. E. Stewart, jun., L. S. Lin, M. Wiranowska-Stewart, and K. Cantell, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4200.
[428] J. K. Bryan, *Analyt. Biochem.*, 1977, **78**, 513.
[429] R. Panyim, R. Thitipongpanich, and D. Supatimusro, *Analyt. Biochem.*, 1977, **81**, 320.
[430] C. Manwell, *Biochem. J.*, 1977, **165**, 487.
[431] A. W. Rees, M. S. DeBuysere, and E. A. Lewis, *Arch. Biochem. Biophys.*, 1977, **182**, 478.
[432] U. D. Schmidt and H. H. Paradies, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 383.
[433] J. Berger, I. Pilz, R. Witters, and R. Lontie, *European J. Biochem.*, 1977, **80**, 79.
[434] I. Pilz, E. Schwarz and W. Palm, *European J. Biochem.*, 1977, **75**, 195.
[435] W. Folkhard, B. Felser, I. Pilz, O. Kraty, H. Dutler, and H. Vogel, *European J. Biochem.*, 1977, **81**, 173.
[436] H. H. Paradies, B. Zimmer, and G. Werz, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 397.
[437] P. Zipper and H. Durchschlag, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 394.
[438] B. Sjoberg, *European J. Biochem.*, 1977, **81**, 277.
[439] D. W. Cleveland, S. G. Fischer, M. W. Kirschner, and U. K. Laemmli, *J. Biol. Chem.*, 1977, **252**, 1102.

The power of modified gel systems using high acrylamide concentrations and incorporating urea has been shown in the characterization of the cyanogen bromide peptides of hypoxanthine phosphoribosyl transferase; peptides as small as 1300 daltons have been seen.[440] Radio-iodination of single polyacrylamide gel slices followed by tryptic digestion and elution of peptides from the gel has yielded impressive peptide maps.[441]

6 Polyfunctional Chains and Multienzyme Complexes

Further evidence has been presented that the *arom* enzyme system of *Neurospora* with five enzymic activities is synthesized as a single polypeptide chain.[442] The first three enzymes of *de novo* synthesis of pyrimidine nucleotides have also been isolated as a single polypeptide chain.[443] The polypeptide chain of formyl-methenyl-methylenetetrahydrofolate synthetase (combined) that contains three enzymic activities has been cleaved by limited proteolysis to a fragment that retains the formyltetrahydrofolate synthetase activity only.[444–446] The thiolase, enoyl-CoA-hydratase, and 3-hydroxyacyl-CoA dehydrogenase activities of fatty acid oxidation have been purified as a complex from *E. coli* B.[447]

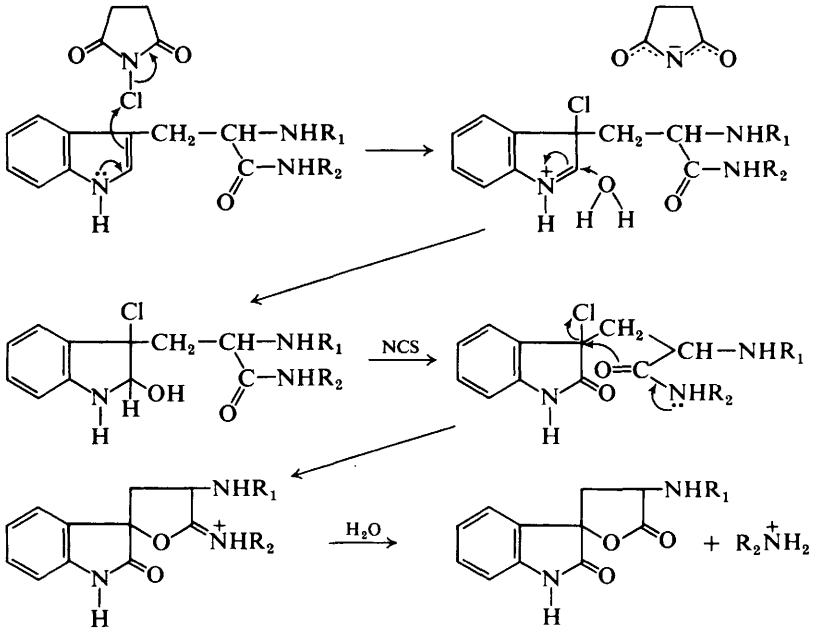
PART IB: Protein Sequencing Methods *by M. Rangarajan*

1 Chain Cleavage and Separation of Peptides

Cleavage at Tryptophan Residues.—The utilization of *N*-chlorosuccinimide as a reagent for cleaving tryptophanyl bonds has been explored by Lischwe and Sung.[1] They have developed a simple, rapid, and a highly selective procedure for cleaving peptide bonds *C*-terminal to tryptophan residues in approximately 50% yield using *N*-chlorosuccinimide (NCS)–urea. Urea is incorporated for optimum protein cleavage. The reaction conditions used here differ from those of Schechter *et al.*[2] A single addition of a 10-fold excess of the reagent cleaves approximately 50% of the Trp-X sequences in 30 minutes. Tyrosyl and histidyl moieties do not react. At 20-fold excess of reagent, methionyl residues are largely converted to methionine sulphone and cysteine residues to cysteic acid. The authors suggest that a 10-fold excess of NCS–urea is the upper limit that should be used to prevent extensive oxidation of methionyl residues to their sulphones. The method has been applied to proteins of known sequence, cytochrome *c*, and to a protein of unknown sequence, Polypeptide VII from adenovirus, to generate peptide fragments suitable for sequence analysis. The mechanisms of halogenation and cleavage by *N*-bromosuccinimide, *N*-iodosuccinimide, and NCS are

[440] S. W. Krauss and G. Milman, *Analyt. Biochem.*, 1977, **82**, 38.
[441] J. H. Elder, R. A. Pickett, jun., J. Hampton, and R. A. Lerner, *J. Biol. Chem.*, 1977, **252**, 6510.
[442] F. H. Gaertner and K. W. Cole, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 259.
[443] P. F. Coleman, D. P. Suttle, and G. R. Stark, *J. Biol. Chem.*, 1977, **252**, 6379.
[444] J. L. Paukert, G. R. Williams, and J. C. Rabinowitz, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 147.
[445] L. U. L. Tan, E. J. Drury, and R. E. Mackenzie, *J. Biol. Chem.*, 1977, **252**, 1117.
[446] L. U. L. Tan, and R. E. Mackenzie, *Biochim. Biophys. Acta*, 1977, **485**, 52.
[447] J. F. Binstock, A. Pramanik, and H. Schultz, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 492.
[1] M. A. Lischwe and M. T. Sung, *J. Biol. Chem.*, 1977, **252**, 4976.
[2] Y. Schechter, A. Patchornik, and Y. Burstein, *Biochemistry*, 1976, **15**, 5071.

discussed. It is proposed that the selectivity with respect to halogenation by NCS is due to the insignificant participation of molecular chlorine in the NCS–urea reaction. A mechanism of halogenation and cleavage at tryptophan is offered (Scheme 1).



Scheme 1

Another useful method for the cleavage of peptide bonds *C*-terminal to tryptophan residues has appeared recently.[3] Ozols *et al.* have shown that methionyl and tryptophanyl bonds are cleaved selectively by CNBr in the presence of a 1 : 1 (v/v) mixture of formic acid (88%) and anhydrous heptafluorobutyric acid (HFBA). When applied to several cytochrome b_5 preparations and equine cytochrome *c*, cleavage occurred at these two residues in high (60—90%) yields. The cleavage can be restricted to tryptophanyl bonds by the Methylene Blue-sensitized photo-oxidation of methionine residues. Photo-oxidized methionines are no longer susceptible to CNBr–HFBA cleavage, they do not interfere with sequenator analysis, and their PTH derivatives may be identified by thin-layer chromatography.

Enzyme Cleavage at Arginine Residues.—The specificities of two proteases from mouse submaxillary gland which hydrolyse peptide bonds *C*-terminal to arginine have been described.[4] When tested with proteins of known primary structure and homopolymers of arginine and lysine, it was noted that lysyl bonds were not split and there was no significant chymotryptic activity. Hydrolysis was restricted

[3] J. Ozols, C. Gerard, and C. Stachelek, *J. Biol. Chem.*, 1977, **252**, 5986.
[4] I. Schenkein, M. Levy, E. C. Franklin, and B. Frangione, *Arch. Biochem. Biophys.*, 1977, **182**, 64.

to arginyl bonds which were split differentially. The specificity and ease of isolation of these enzymes can be put to practical use in protein sequencing in conjunction with trypsin for obtaining large overlap peptides. The disadvantage of the incomplete splitting would be the failure to recover peptides quantitatively.

2 Detection of Peptides

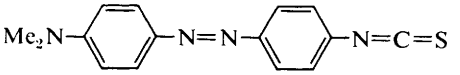
Fluorescamine, *o*-Phthalaldehyde, and Ninhydrin.—The three reagents were compared with respect to their sensitivity in detecting amino-acids and peptides on thin layers of cellulose and silica gel and their effects on the recovery and composition of peptides from such layers.[5] Results showed that the latter two reagents permitted the detection of amino-acids in the 50—200 pmol range and were superior to fluorescamine. On the other hand, ninhydrin and fluorescamine were better for peptides. Recovery of most peptides upon elution was of the order of 80% though ninydrin-treated peptides gave lower yields. Only fluorescamine treated peptides could be recovered from thin layers without the destruction of any amino-acid. Creaser and Hughes[6] have described the design and operation of a peptide analyser using *o*-phthalaldehyde (*o*-PA) for detection. It is useful for the analysis of peptides in the range 5 nmol to 10 μmol normally derived from enzymic digestion of proteins. The analyser is simple and comprises a gradient-generating device feeding volatile pyridine buffers *via* a pump to a microbore column of cation-exchange resin. Column effluent is fed through a proportioning pump to a fluoro-colorimeter. For analytical runs, the effluent is mixed with *o*-PA prior to its passage into the detector. For preparative work, the stream is split, one for reaction with *o*-PA, the other for collection. The reagent is saturated with *o*-PA, mercaptoethanol is included as otherwise there is very little reaction, and Brij 35 seems to enhance the size of the peptide peaks. Initially a 5—10 nmol sample of peptide mixture is run on a small microbore column in order to optimize the elution conditions and to allow the separation of a maximum number of peptides. Preparative separations are then performed on a larger column. Tryptic peptides from cytochrome *c* and *Salmonella* histidinol dehydrogenase were satisfactorily separated using this strategy.

Amino-acid Analysis.—A sensitive and reproducible colorimetric method for the determination of very low amounts of tryptophan has been described.[7] It is based on the oxidation of tryptophan by sodium nitrite and coupling with a leucodye. Peptides containing tryptophan are less reactive than free tryptophan and for the method to be useful proteins must first be subjected to alkaline hydrolysis.

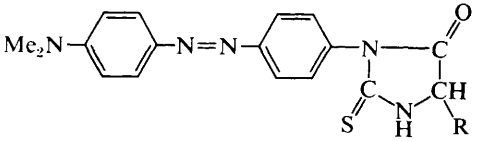
End-group Determination.—*N*-Terminal tryptophan residues in peptides cannot be identified using dansyl chloride because DNS-tryptophan is completely destroyed during hydrolysis of DNS-peptides with 6M-HCl. This is a limitation of the dansyl-Edman sequence determination procedure. Very high recoveries (95%) of tryptophan in protein hydrolysates have been obtained using 3N-mercaptoethanesulphonic acid (MES) at 110 °C for hydrolysis. A method which uses 3N-MES at 106 °C to release DNS-tryptophan from DNS-peptides has been

[5] E. Schiltz, K. D. Schnackerz, and R. W. Gracy, *Analyt. Biochem.*, 1977, **79**, 33.
[6] E. H. Creaser and G. J. Hughes, *J. Chromatogr.*, 1977, **144**, 69.
[7] S. M. M. Basha and R. M. Roberts, *Analyt. Biochem.*, 1977, **77**, 378.

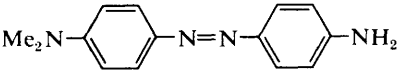
published.[8] The hydrolysis is complete in 6 h and there is no significant destruction of DNS-tryptophan in 24 h. After neutralization, the DNS-tryptophan is extracted into an organic solvent and identified by thin-layer chromatography. The identification of *N*-terminal tryptophan was successfully performed for several synthetic peptides and two thermolytic peptides of ribitol dehydrogenase from *Klebsiella aerogenes*. An extremely sensitive method for the stepwise degradation of peptides uses 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate (1) (DAA-ITC).[9] The thiohydantoins (2) formed after conversion of the thiazolinones are identified by thin-layer chromatography. Extended sequence analysis on peptides and proteins can be performed with 2—10 nmol of material. The method was tested with a hexapeptide and two proteins. There are several disadvantages; firstly, rather drastic conditions are required for quantitative coupling between



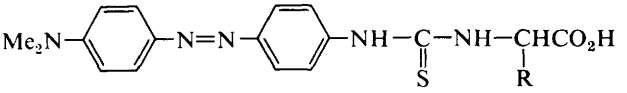
(1)



(2)



(3)



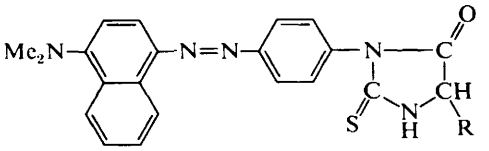
(4)

DAA-ITC and the *N*-terminal amino-acid. Secondly, the rate of hydrolysis of the DAA-ITC to 4-*NN*-dimethylamino-4′-aminoazobenzene (3) is high at the pH of the coupling reaction. This increases the amount of by-products in the reaction, and the solvents used for their extraction were found also to extract at least 15—20% of the DAA-thiocarbamyl derivatives (4) from the aqueous phase. If these problems can be overcome, the method will offer a simple and cheap alternative for sequencing micro-amounts of peptides. The separation and identification of the DAA-TH derivatives (2) has so far been performed on thin-layer polyamide plates. Leucine and isoleucine derivatives are not resolved and elution of all the thiohydantoins from the plates is difficult. A thin-layer chromatographic separation on silica gel is described which overcomes these difficulties.[10] Also, the preparation and thin-layer separation of 4-*NN*-dimethylamino-naphthylazo-

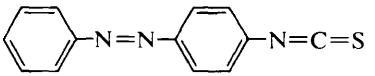
[8] J. R. Giglio, *Analyt. Biochem.*, 1977, **82**, 262.
[9] J. Y. Chang, *Biochem. J.*, 1977, **163**, 517.
[10] J. Y. Chang, E. H. Creaser, and G. J. Hughes, *J. Chromatogr.*, 1977, **140**, 125.

benzene-4′-thiohydantoins (5) of 24 amino-acids is reported.[11] They are purple and can be used as markers to aid in the identification of the red DAA-THs (2).

Another chromophoric reagent, *p*-phenylazophenyl-isothiocyanate (6), has been used for *N*-terminal sequence determination of peptides and proteins.[12] A thin-layer chromatographic separation of the corresponding thiohydantoins is reported.[13] The first five residues of pepsin were determined by this procedure.



(5)



(6)

Spinning Cup Sequencing.—The use of the more volatile pentafluoropropionic acid in the cleavage step instead of HFBA and a mixture of dichloroethane and benzene instead of chlorobutane to extract the thiazolinones is discussed by Inglis and Burley.[14] These modifications were necessary because HFBA is not readily removed after cleavage and this can cause loss of hydrophobic residues during the subsequent extraction step. Dichloroethane–benzene extraction was effective in improving yields of acidic residues during sequencing of polar peptides. Using this modified program it was possible to extend the *N*-terminal sequence of duck apovitellenin I from 35 to 73 residues.

A method has been described for the successful solubilization of the hydrophobic, insoluble, coat protein of bacteriophage f1 by coupling with 4-sulphophenylisothiocyanate in the presence of detergent (SDS).[15] The use of the detergent did not appear to cause any difficulties in automated sequencing or in detection of the residues. A fast Quadrol program was used with a single HFBA cleavage and single chlorobutane extraction with high repetitive yield through residue 47 in the 50 residue protein. In addition to improving the yield, this program is faster than the normal Quadrol program and halves the consumption of HFBA, one of the most expensive sequenator reagents. The effects of detergent on the automated sequencing of ordinarily soluble proteins have been examined using whale apomyoglobin. Repetitive yield in detergent-treated and untreated cases was identical and no differences were observed in the recovery of residues or the quality of background during detection by gas chromatography. The chemical modification described here is useful if the protein contains one or two lysines near the *C*-terminus. Preliminary studies have been carried out for the attachment of other highly polar modifying groups such

[11] J. Y. Chang and E. H. Creaser, *J. Chromatogr.*, 1977, **132**, 303.
[12] S. Datta, S. C. Datta, and R. Sengupta, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 1296.
[13] S. Datta and S. C. Datta, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 1074.
[14] A. S. Inglis and R. W. Burley, *F.E.B.S. Letters*, 1977, **73**, 33.
[15] G. S. Bailey, D. Gillett, D. F. Hill, and G. B. Petersen, *J. Biol. Chem.*, 1977, **252**, 2218.

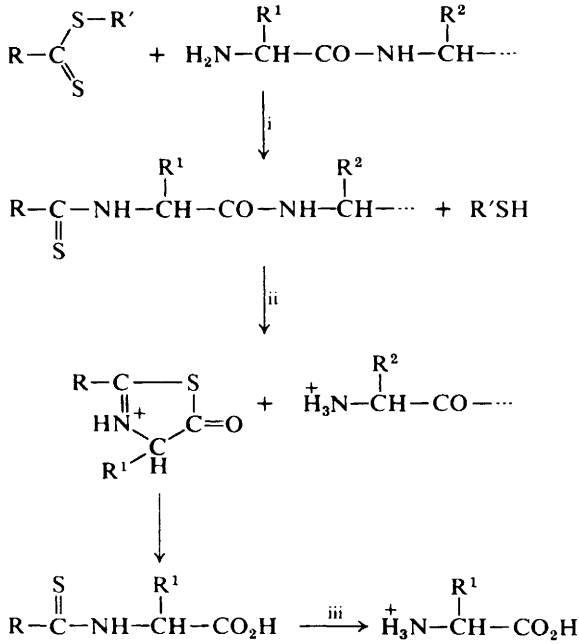
as 2-amino-1,5-naphthalene disulphonic acid to side-chain and terminal carboxyl groups of detergent-dissolved hydrophobic peptides and to the coat protein of bacteriophage f1.

The introduction of detergent-treated unmodified coat protein of f1 into the spinning cup and use of an ordinary Quadrol program allowed the first 15 residues to be determined but the repetitive yields fell rapidly. Attempts to improve the program have been unsuccessful. More success was achieved with a modified Beckman DMAA program. This program provided the addition of a thin film of detergent containing DMAA buffer immediately prior to extraction of the thiazolinone with chlorobutane. Uncorrected repetitive yields of 87% were obtained with successful identification of the first 18 residues. However, overlap was high using this protocol; the initial overlap was 35% and the sequence became out of step at residue 10. Correction for overlap raised the repetitive yield to 93%. Reduction of initial high overlap was achieved by another modification in which double coupling with PITC was performed at each cycle, but the overlap per cycle was still high and the sequence was out of step at residue 12. This is probably due to poor cleavage by HFBA.

For the automated, stepwise, degradation of small peptides, the addition of succinylated polyornithine has been recommended [14] for use with 0.5M-Quadrol buffer because it assists in holding small amounts of hydrophobic peptides in the cup. Polyquaternary amines and specifically polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) have been added to the spinning cup to prevent losses during the degradation of small peptides with a DMAA program.[16] It seems likely that polybrene provides a stable polar environment in addition to ionic bonding and results with peptides (less than 30 residues) show that it prevents mechanical loss of peptides from the spinning cup allowing complete sequencing of even the most hydrophobic peptides tested.[17]

Solid-phase Sequencing.—A simple, inexpensive, solid-phase sequencing device has been constructed which does not employ any pumps but utilizes nitrogen pressure to drive reagents to or from a reaction vessel.[18] A Tenor stepping-drum programmer is used for flexible programming so that any kind of chemical degradation may be performed simply by changing the contents of the reservoirs. Details are given for assembling the unit which the authors say will cost approximately $2500, including all parts and labour. The machine was used for a novel thioacetylation degradation procedure for peptides attached to glass beads. The outline of the steps involved in the degradation is shown in Scheme 2. The B-chain of insulin (50 nmol) was subjected to 25 rounds of automatic degradation with repetitive yields of 95%. A HPLC method has been developed for the separation and identification of thioacetyl amino-acids which should aid in identifying those residues which are lost or destroyed during acid hydrolysis. A solid-phase degradation procedure using 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate

[16] G. E. Tarr, J. F. Beecher, M. Bell, and D. J. McKean, *Analyt. Biochem.*, 1978, **84**, 622.
[17] J. D. Capra, C. E. Wilde, and D. G. Klapper, in 'Solid Phase Methods in Protein Sequence Analysis', ed. A. Previero and M.-A. Coletti-Previero, North-Holland, Amsterdam, 1977, p. 69.
[18] L. R. Doolittle, G. A. Mross, L. A. Fothergill, and R. F. Doolittle, *Analyt. Biochem.*, 1977, **78**, 491.



Reagents: i, H_2O-pyridine, pH 9; ii, anhydrous acid; iii, 6M-HCl hydrolysis. R = —Me; R′ = $-CH_2CO_2H$

Scheme 2

has been developed and *N*-terminal sequences of 2—10 nmol of lysozyme (19 residues) and glucagon (10 residues) coupled to glass beads were determined.[19] The bright red colour of the thiohydantoin derivatives allows subnanomole quantities of amino-acids to be detected.

A method is described for the quantitative measurement of 'coupling capacity' of solid supports containing amino groups, defined as the number of amino groups per unit weight of resin available under the conditions of coupling.[20] This will allow the stability of the supports to be measured over a period of time. It will also allow comparisons to be made between different batches of the same resin, and with a variety of other supports.

Coupling of proteins and peptides to iodoacetamide-activated glass through cysteine residues [20] and coupling to diazotized glass beads through tyrosine and histidine residues in aqueous alkaline media have been reported.[21] The former is specific for cysteine residues, and coupling yields of 75—90% have been obtained. Coupling yields of 40—50% were obtained with the diazotized glass beads. No sequence data on peptides immobilized in this way were presented. Several derivatives of macroporous polystyrene beads have been prepared which could serve as useful supports for solid-phase sequence analysis.[22] These beads do not

[19] J. Y. Chang, E. H. Creaser, and G. J. Hughes, *F.E.B.S. Letters*, 1977, **78**, 147.
[20] H. W. Schmitt and J. E. Walker, *F.E.B.S. Letters*, 1977, **81**, 403.
[21] J. Y. Chang, E. H. Creaser, and G. J. Hughes, *F.E.B.S. Letters*, 1977, **84**, 187.
[22] J. K. Inman, G. U. DuBois, and E. Appella, ref. 17, p. 81.

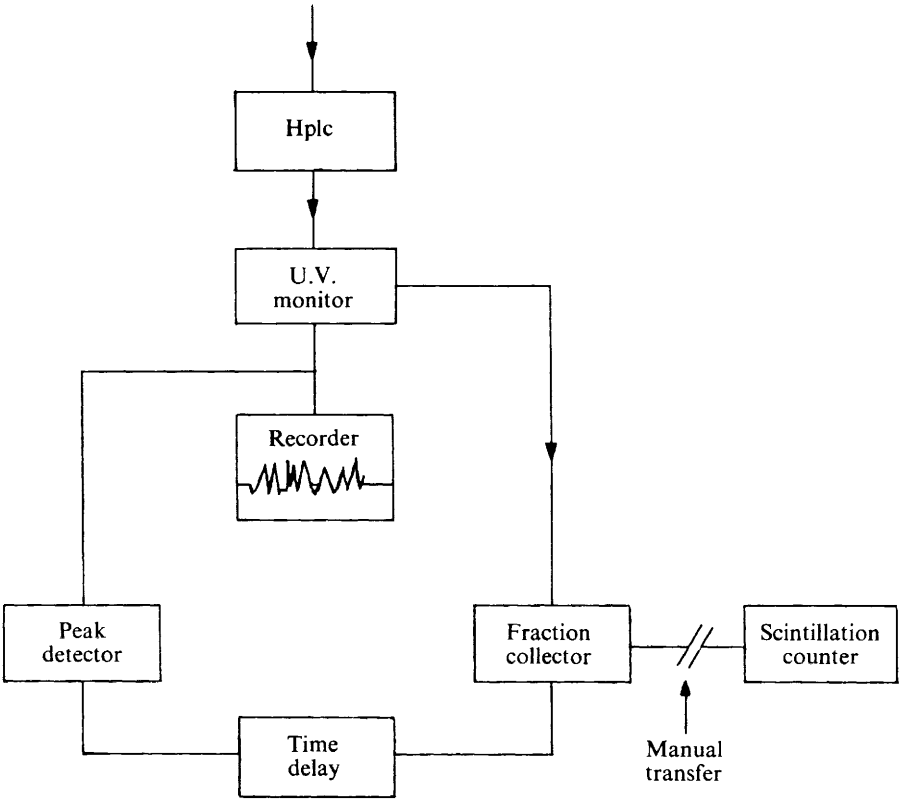
swell or shrink in solvents of differing composition unlike low-crosslinked polystyrene beads, and the use of long polyethyleneglycol 'spacer-arms' should increase the number of reactive groups available for peptide coupling. Successful solid-phase sequencing of two small proteins, the phosphocarrier protein, HPr, of *Staphylococcus aureus* and the acyl carrier protein of citrate lyase from *Klebsiella aerogenes*, was performed by coupling phenylthiocarbamyl proteins to aminated solid supports.[23]

Microsequencing.—It has not been possible to perform sequence analysis of subnanomole amounts of proteins and peptides in the spinning cup sequenator due to loss of material during liquid-phase extraction procedures. Two methods can be used to circumvent this difficulty. A synthetic 'carrier' such as succinylated polyornithine can be added to the spinning cup together with the protein to be sequenced to minimize losses during extraction. Secondly, the sensitivity of detection of the PTH amino-acids can be improved to permit the sequence analysis of subnanomole amounts of protein. Several advances have been made in the development of HPLC systems which permit resolution of all 20 PTH amino-acids and which are sensitive to 1 nmole.[24] The use of fluorescamine and *o*-PA for amino-acid detection in the amino-acid analysers permits a 50—100-fold increase in sensitivity.[25] The use of radioactive PITC for coupling also increases the sensitivity of the detection system. A method is described which enables picomole quantities of proteins and peptides attached to glass supports to be subjected to automated solid-phase sequencing procedures.[26] The coupling and cleavage reaction conditions are exactly as described earlier by Bridgen [27] except that HPLC and liquid scintillation counting are employed for PTH amino-acid detection. The ^{35}S-PTH amino-acid from the sequenator is mixed with 20 carrier PTH amino-acids and analysed by HPLC. Each peak is recovered in a separate tube and the radioactivity measured by liquid scintillation counting. A schematic diagram of a HPLC system is shown (Scheme 3). The signal from the detector is monitored by a minicomputer which is programmed to cut the peaks. A signal is sent to index the fraction collector. Insulin-B chain (80 pmol) and horse heart myoglobin (190 pmol) were subjected to the microsequencing procedure using 2 mCi and 4 mCi of ^{35}S-PITC respectively, and 12 residues and 6 residues were determined.

Phenylthiohydantoin Amino-acids.—The separation of all 20 amino-acid PTH derivatives in 20 min by HPLC has been reported.[24] At least three analyses can be performed per hour thus keeping pace with an automated sequenator. An amino-acid analyser using *o*-PA for the fluorescence detection of amino-acids obtained by back-hydrolysis of PTH derivatives has been developed.[25]

***C*-Terminal Sequencing.**—In recent years, several attempts have been made to develop suitable methods for sequencing peptides and proteins from the *C*-terminus. Some solutions to the difficulties encountered in the solid-phase *C*-

[23] K. Beyruther, ref. 17, p. 107.
[24] C. L. Zimmerman, E. Appella, and J. J. Pisano, *Analyt. Biochem.*, 1977, **77**, 569.
[25] E. Lund, J. Thomsen, and K. Brunfeldt, *J. Chromatogr.*, 1977, **130**, 51.
[26] J. Bridgen and M. J. Waxdal, ref. 17, p. 153.
[27] J. Bridgen, *Biochemistry*, 1976, **15**, 3600.



Scheme 3

terminal degradation methods with thiocyanate [28] have been suggested [29] which involve modification of side-chain carboxyl groups, changing reaction conditions when proline residues are involved, and synthesis of new solid supports. A new reaction has been described by Previero and Coletti-Previero [30] which converts the last peptidic function into an imidoether which on cleaving with alcohols releases the *C*-terminal amino-acid. There appeared to be incomplete cleavage of the *C*-terminal amino-acid when tested with model peptides immobilized by *N*-acylation with polyacryloyl chloride. Studies are under way to improve the efficiency of the reaction in the hope that it will make a useful method for solid-phase *C*-terminal sequencing.

Mass Spectrometry.—A new strategy has been described for the rapid screening of homologous proteins, in particular a *Pseudomonas* azurin.[31] The protein is digested with a non-specific proteolytic enzyme and the peptides partially purified by ion-exchange chromatography. Peptide mixtures are then sequenced by low

[28] M. Rangarajan and A. Darbre, *Biochem. J.*, 1976, **157**, 307.
[29] B. Kassel, C. Krishnamurthi, and H. L. Friedman, ref. 17, p. 39.
[30] A. Previero and M.-A. Coletti-Previero, ref. 17, p. 49.
[31] A. Dell and H. R. Morris, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 874.

resolution, electron impact mass spectrometry. Peptides are aligned by homology with a type sequence. Approximately 85% of the sequence of azurin was obtained from two digests of which 76% has been aligned with the type protein. The method has been shown to be reliable and rapid (only 4 weeks were taken to perform this study).

Mass spectrometry has also been used to identify *N*-methylalanine at the *N*-terminus of two ribosomal proteins of *Escherichia coli*, namely S11 and L33, and *N*-methyl-methionine at the *N*-terminus of protein L16.[32]

New Strategies in Protein Sequencing.—During the alignment of CNBr peptides of *E. coli* β-galactosidase, there were two fragments CB21 and CB22 which could not be ordered because no overlapping peptides were available. However, tryptic digestion of lysine-blocked β-galactosidase generated large fragments due to incomplete cleavage of arginyl bonds. If any of these large peptides contained most of the sequence of a CNBr peptide, say CB21, it would be expected to be immunologically similar to it and cross-react with anti-CB21.[33] A radioimmunoassay for CNBr peptides was developed using ^{125}I-labelled peptide and double-antibody precipitation. Such an assay was used to test for a cross-reacting peptide in the tryptic digest. There was indeed some cross-reacting material which yielded two peptides IA and IB on further purification. Amino-acid analysis and *N*-terminal sequence analysis showed that IB contained the last 31 residues of CB21 and the first 11 residues of CB22. IA differs from IB in having an additional 13 amino-acids at its *N*-terminus. This highly sensitive method could be used to monitor the purification of picomole quantities of any desired peptide in a digest. The authors have been successful in raising antibodies to peptides containing 23 or more amino-acids without the need to couple it to a protein carrier. The method should be of general use in isolating and identifying overlapping peptides from the cleavage products of other proteins and also aid in the purification of altered peptides from mutant strains.

Primary Structures

Table 1 *Amino-acid sequences reported during 1977*

Protein	*Source*	*Comments*	*Ref.*
Enzymes			
Acid protease	*Penicillium roqueforti*	*N*-terminal 33 residues	34
Acid protease	*Rhizopus chinensis*	sequence of 2 peptides containing Asp reactive with 1,2-epoxy-3-Cp-nitrophenoxy propane, 6 residues for each	35
Alcohol dehydrogenase	*Saccharomyces cerevisiae*	complete sequence, 347 residues	36

[32] R. Chen, J. Borosius, B. Wittmann-Liebold, and W. Schafer, *J. Mol. Biol.*, 1977, **111**, 173.
[33] A. J. Brake, F. Celada, A. V. Fowler, and I. Zabin, *Analyt. Biochem.*, 1977, **80**, 108.
[34] J.-C. Gripon, S. A. Rhee, and T. Hofmann, *Canad. J. Biochem.*, 1977, **55**, 504.
[35] S. Nakamura and K. Takahashi, *J. Biochem. (Tokyo)*, 1977, **81**, 805.
[36] H. Jornvall, *European J. Biochem.*, 1977, **72**, 425.

Table 1 *(cont.)*

Protein	*Source*	*Comments*	*Ref.*
Arginine kinase	lobster muscle (*Homarus vulgaris*)	partial sequence of CNBr fragment, 42 residues	37
L-Asparaginase	*Escherichia coli*	active-centre peptide containing Ser, 10 residues	38
Aspartate amino transferase	pig heart mitochondria	complete sequence, 403 residues	39
	pig heart mitochondria	complete sequence, 401 residues, 48% homology with cytoplasmic isozyme	40
	pig heart cytoplasm	active-centre peptide, 4 residues	41
	chicken heart mitochondria	active-centre peptide, 7 residues	41
Aspartate carbamoyl transferase	*E. coli* K12	*N*-terminal sequence of catalytic chain, 37 residues	42
Aspartokinase II	*E. coli* K12	two identical subunits	43
Homoserine dehydrogenase II		*N*-terminal, 9 residues *C*-terminal, 2 residues	
Carbonic anhydrase	equine erythrocyte	sequence of 242 residues determined; 84% of the sequence is identical to that of the human enzyme	44
Collagenase	fiddler crab	partial sequence of CNBr fragments	45
Cytochrome oxidase	bovine heart	complete sequence of Haem α-subunit, 109 residues	46
Dihydrofolate reductase	*Lactobacillus casei*	partial sequence around the three Trp residues	47
	Lactobacillus casei, methotrexate resistant	*N*-terminal sequence, 51 residues	48
	Lactobacillus casei, amethopterin resistant	complete sequence, 162 residues	49
	mouse lymphoma L1210, methotrexate resistant	complete sequence, 186 residues	50

[37] B. Delbuire, K.-K. Han, M. Dautrevaux, G. Biserte, F. Regnouf, and R. Kassab, *J. Biochem. (Tokyo)*, 1977, **81**, 611.
[38] R. G. Peterson, F. F. Richards, and R. E. Handschumacher, *J. Biol. Chem.*, 1977, **252**, 2072.
[39] D. Barra, F. Bossa, S. Doonan, H. M. A. Fahmy, G. J. Hughes, K. Y. Kakoz, F. Martini, and R. Petruzzelli, *F.E.B.S. Letters*, 1977, **83**, 241.
[40] H. Kagamiyama, R. Sakakibara, H. Wada, S. Tanase, and Y. Morino, *J. Biochem. (Tokyo)*, 1977, **82**, 291.
[41] H. Gehring, R. R. Rando, and P. Christen, *Biochemistry*, 1977, **16**, 4832.
[42] D. Gigot, N. Glausdorff, C. Legrain, A. Pierard, V. Stalon, W. Konigsberg, I. Caplier, A. D. Strosberg, and G. Herve, *F.E.B.S. Letters*, 1977, **81**, 28.
[43] A. Dautry-Varsat, L. Sibilli-Weill, and G. N. Cohen, *European J. Biochem.*, 1977, **76**, 1.
[44] R. P. Bray and H. F. Deutsch, *Fed. Proc.*, 1977, **36**, 739.
[45] G. A. Grant, K. O. Henderson, A. Z. Eisen, and R. A. Bradshaw, *Fed. Proc.*, 1977, **36**, 837.
[46] M. Tanaka, M. Haniu, K. T. Yasunobu, C. A. Yu, L. Yu, Y. H. Wei, and T. E. King, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 1014.
[47] J. H. Freisheim, L. H. Ericsson, K. G. Bitar, R. B. Dunlap, and A. V. Reddy, *Arch. Biochem. Biophys.*, 1977, **180**, 310.
[48] K. E. Batley and H. R. Morris, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 1010.
[49] K. G. Bitar, D. T. Blankenship, K. A. Walsh, R. B. Dunlap, A. V. Reddy, and J. H. Freisheim, *F.E.B.S. Letters*, 1977, **80**, 119.
[50] D. Stone and A. W. Phillips, *F.E.B.S. Letters*, 1977, **74**, 85.

Table 1 *(cont.)*

Protein	*Source*	*Comments*	*Ref.*
	E. coli, trimethopterin resistant	complete sequence, 159 residues	51
Dopamine hydroxylase	bovine adrenal medulla	*N*-terminal sequence, 18 residues; contains two types of chains differing only in that one lacks the tripeptide Ser-Ala-Thr	52
Fatty acid synthetase	baker's yeast	sequence of a tryptic peptide in the condensing enzyme containing a peripheral—SH group, 6 residues	53
	baker's yeast	sequence of peptides containing the palmityl-binding site, 13 residues in the acyl carrier protein, 5 residues of palmityl transferase peptide	54
Formylglycinamide ribonucleotide amido transferase	chicken liver	sequence of two peptides located at the glutamine binding site, 7 residues, and 5 residues	55
Fructose 1,6-bis-phosphatase	rabbit liver	*N*-terminal, 78 residues	56
Galactokinase	*E. coli*	*N*-terminal, 18 residues	57
	Saccharomyces cerevisiae	*N*-terminal, 12 residues the sequences do not show any homology	57
β-Galactosidase	*E. coli*	complete sequence, 1021 residues; largest protein to be sequenced so far	58
ADP-glucose phosphorylase	*E. coli* B	*N*-terminal, 46 residues containing the activator-site Lys	59
NAD-specific Glutamate dehydrogenase	*Neurospora crassa*	sequence of *C*-terminal region of peptide chain, 669 residues	60
	Neurospora crassa	partial sequence of *N*-terminal region of peptide chain, three peptides, 42, 209, and 55 residues; out of 1030 residues, 975 have been accounted for	61

[51] D. Stone, A. W. Phillips, and J. J. Burchall, *European J. Biochem.*, 1977, **72**, 613.
[52] T. Skotland, T. Ljones, T. Flatmark, and K. Sletten, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 1483.
[53] G.-B. Kresze, L. Steber, D. Oesterhelt, and F. Lynen, *European J. Biochem.*, 1977, **79**, 181.
[54] T. Schreckenbach, H. Wobser, and F. Lynen, *European J. Biochem.*, 1977, **80**, 13.
[55] S. Ohnoki, B.-S. Hong, and J. M. Buchanan, *Biochemistry*, 1977, **16**, 1070.
[56] L. H. Botelho, H. A. El-Dorry, O. Crivellaro, D. K. Chu, S. Pontremoli, and B. L. Horecker, *Arch. Biochem. Biophys.*, 1977, **184**, 535.
[57] D. H. Schlesinger, M. A. Schell, and D. B. Wilson, *F.E.B.S. Letters*, 1977, **83**, 45.
[58] A. V. Fowler and I. Zabin, *Proc. Nat. Acad. Sci.*, 1977, **74**, 1507.
[59] T. F. Parsons, C. A. Carlson, and J. Preiss, *Fed. Proc.*, 1977, **36**, 856.
[60] B. M. Austen, M. E. Haberland, J. F. Nyc, and E. L. Smith, *J. Biol. Chem.*, 1977, **252**, 8142.
[61] M. E. Haberland and E. L. Smith, *J. Biol. Chem.*, 1977, **252**, 8196.

Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Glutamate dehydrogenase	beef liver	partial sequences of peptides from cross-linked trimeric species; location of ε-amino groups involved in cross-linking	62
Glutamine synthetase	*Bacillus subtilis*	*N*-terminal, 29 residues	63
Glutathione reductase	human erythrocytes	sequence of peptide containing the catalytic disulphide of the protein, 13 residues	64
Glyceraldehyde-3-phosphate dehydrogenase	*Bacillus stearothermophilus*	complete sequence, 333 residues, highly homologous with the lobster muscle enzyme	65
Glycerol-3-phosphate dehydrogenase	rabbit muscle	sequence of 7 His-containing peptides	66
Glycogen phosphorylase b	rabbit muscle	complete sequence, 841 residues; amino-acids involved in allosteric control and in binding pyridoxal-5′-phosphate identified	67
Glycogen synthetase	rabbit skeletal muscle	partial sequence of peptide containing the phosphorylation site, 26 residues	68
	rabbit skeletal muscle	*N*-terminal sequence of two peptides at the two phosphorylation sites, 10 residues, and 3 residues	69
Histidinol dehydrogenase	*Salmonella typhimurium*	*N*-terminal, 8 residues	70
	Salmonella typhimurium *E. coli*	amino-acid sequence around the reactive thiol group, 8 residues; no sequence homology with other dehydrogenases	71
Inorganic pyrophosphatase	yeast	complete sequence	72
Invertase (external)	*Saccharomyces*	*C*-terminal sequence, 12 residues of two subunits shows them to be identical	73

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Lactate dehydrogenase, C subunit	mouse	partial sequence, peptide containing the essential thiol, 13 residues	74
Lactate dehydrogenase	dogfish muscle (*Squalus acanthius*)	complete sequence, 329 residues	75
Isoenzymes M_4	porcine	complete sequence, 331 residues	76
H_4	porcine	complete sequence, 333 residues	76
Lysozyme	rat urine	complete sequence, 130 residues	77
Muconolactone isomerase	*Pseudomonas putida*	*N*-terminal, 14 residues, and partial sequence of CNBr peptides	78
Nuclease B	*Staphylococcus aureus*	*N*-terminal, 20 residues	79
Ornithine carbamoyl transferase	*E. coli* K12	*N*-terminal sequence of catalytic chain, 36 residues	80
Pepsin	*Rhizopus chinensis*	*N*-terminal sequence extended from 27 to 39 residues	81
Peroxidase P_1 P_2 P_3 P_7	turnip	sequence around His residues: 25 residues around His proximal to Haem and 34 residues around His distally located	82
Phenylalanyl tRNA synthetase	baker's yeast	partial sequence of 8 cysteine peptides of α-subunit, and 2 cysteine peptides of β-subunit	83
Phosphofructokinase	rabbit skeletal muscle	partial sequence of peptide containing highly reactive —SH group, 5 residues	84
3-Phospho-glycerate kinase	yeast	sequence of active-site pentapeptide	85
Phospholipase A CM I CM II CM III	*Naja mossambica mossambica* venom	complete sequence, 118 residues; invariant amino-acids resemble those of phospholipases from other snake venoms	86
Phospholipase A_2	*Crotalus adamanteus* venom	complete sequence, 122 residues	87

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Phospholipase A_2	horse pancreas	complete sequence, 125 residues; homologous to snake venom phospholipase A_2	88
	porcine pancreas	reinvestigation of sequence, 124 residues; changes made in previously published sequence	89
ATP-Phosphoribosyl transferase	*Salmonella typhimurium*	*N*-terminal, 17 residues	90
RNA-Polymerase (DNA-dependent)	*E. coli*	complete sequence of α-subunit, 329 residues	91
Prochymosin	bovine	complete sequence, 365 residues; 204 amino-acids common to prochymosin and pepsinogen	92
Pyruvate carboxylase	sheep liver mitochondria	sequence of biotin-containing peptide, 24 residues	93
	chicken liver mitochondria turkey liver mitochondria	sequence of biotin-containing peptide, 19 residues	93
Ribonuclease	red deer pancreas (*Cervus elaphus*) roe deer pancreas (*Capreolus capreolus*)	reinvestigation of primary structure; *N*-terminal sequence 15 to 23 and residue 99 were corrected	94
Ribonucleases A	guinea-pig pancreas	complete sequence, 124 residues, does not contain carbohydrate	95
B	guinea-pig pancreas	complete sequence, 128 residues, glycoprotein; A and B differ at 31 positions in the sequence	95
Sulphite oxidase	chicken liver	*N*-terminal sequence of 'core' obtained by limited chymotryptic digestion, 34 residues	96
Superoxide dismutase	*Thermus aquaticus*	*N*-terminal, 40 residues; bears a close resemblance to the dimeric Mn-enzyme from *B. stearothermophilus*	97

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
	Desulphovibrio desulphuricans	*N*-terminal, 30 residues; belongs to the same family of homologous proteins constituted by the Fe- and Mn-dismutases of aerobic organisms	98
Trypsinogens A_1 A_2 A_3	African lungfish (*Protopterus aethiopicus*) pancreas	*N*-terminal, 48 residues; all 3 contain the same sequence beginning with the activation peptide	99
Tryptophanyl tRNA synthetase	*Bacillus stearothermophilus*	complete sequence, 327 residues	100
Tyrosinase	*Neurospora crassa*	*N*-terminal, 5 residues, blocked *N*-terminus	101
Hormones			
Choriogonadotropin	human	reinvestigation of *C*-terminal sequence of β-subunit, residues 115—145; position of attachment of carbohydrate to Ser established	102
Choriogonadotropin	human	reinvestigation of *C*-terminal sequence of β-subunit, residues 109—145; Ser-linked carbohydrate also located	103
β-Endorphin	bovine pituitary	complete sequence, 31 residues	104
	sheep pituitary	complete sequence, 31 residues	105
	human pituitary	complete sequence, 31 residues	106
Kininogen (low molecular weight)	bovine plasma	sequence of *C*-terminal fragment, 47 residues	107
Lutropin -subunit	ovine pituitary	partial sequence, location of 3 disulphide bonds	108
Neurophysin -I	bovine	complete sequence, 92 residues	109
-I	ovine pituitary	*N*-terminal, 36 residues	110
-II	ovine pituitary	identical to bovine except that there is micro-heterogeneity at position 18	

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Table 1 *(cont.)*

Protein	*Source*	*Comments*	*Ref.*
-I	rat pituitary	*N*-terminal, 33 residues	110
-II	rat pituitary	*N*-terminal, 33 residues	110
Neurophysin major minor	guinea-pig pituitary	*N*-terminal, 26 residues	111
Neurophysin	cod	*N*-terminal, 16 residues; considerable variation in *N*-terminal nonapeptide sequence, then followed by sequence identity with mammalian neurophysins	111
MSEL-Neurophysin	horse pituitary	complete sequence, 95 residues	112
Preproinsulin	bovine	*N*-terminal sequence of bovine mRNA-directed protein, 24 residues	113
Preprolactin	rat pituitary	*N*-terminal sequence of protein synthesized *in vitro*, 50 residues	114
Relaxin	porcine ovaries	complete sequence, 2 chains A and B joined by disulphide bridges, 22 and 30 residues	115
Thyrotropin	human pituitary	complete sequence of α- and β-subunits, 89 and 112 residues; sequence of α- identical to lutropin α-	116
Muscle proteins			
Actin	calf brain	partial sequence of CNBr peptides, 157 of the 374 residues have been identified	117
Myosin	rabbit skeletal muscle	sequence of CNBr fragment containing the 2 CysH residues whose alkylation modifies the catalytic properties of myosin, 92 residues	118
Myosin L-2 light chain	rabbit skeletal muscle	complete sequence, 168 residues	119
Myosin	bovine heart	partial sequence of CNBr fragment containing 2 essential thiols, 28 residues	120
Myosin light chains	bovine cardiac muscle	partial sequence of 2 CNBr peptides, 17 and 15 residues	121

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Myosin L-2 light chain	chicken skeletal muscle	complete sequence, 166 residues	122
Parvalbumin (major)	thornback-ray (*Raja clavata*) muscle	complete sequence, 109 residues	123
Troponin I	rabbit slow muscle	complete sequence, 184 residues	124
Troponin C	rabbit skeletal muscle	complete sequence, 159 residues	125
Troponin T	rabbit skeletal muscle	complete sequence, 259 residues	126
Histones			
Chromosomal protein A 24	calf thymus	sequence of tryptic peptide containing part of the non-histone polypeptide linked to Histone 2A Lys(119), 7 residues	127
H1	calf thymus	partial sequence of 5 peptides at the sites of phosphorylation by kinases from different stages of the cell cycle	128
H1	trout testis	complete sequence, 194 residues, largest of the histones; a pentapeptide occurs 6 times in the complete sequence	129
$H2B_{(1)}$	sea urchin sperm (*Parechinus angulosus*)	complete sequence, 144 residues; *N*-terminal third contains a repeating penta-peptide over 24 residues	130
H5	pigeon chromatin	partial sequences of 5 tryptic peptides phosphorylated by protein kinase from pig brain	131
H5	goose (*Anser anser*)	*N*-terminal, 112 residues	132
	pigeon (*Columbia livia*)	*N*-terminal, 38 residues	132
Toxins			
Cardiotoxin Analogues II	*Naja naja atra*	complete sequence, 60 residues, *N*-terminal Leu	133
IV		*N*-terminal Arg; this is the only difference between the two	

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Table 1 *(cont.)*

Protein	*Source*	*Comments*	*Ref.*
Cholera toxin	*Vibrio cholerae*	complete sequence of B subunit, 103 residues	134
	Vibrio cholerae	complete sequence of B subunit, 103 residues	135
Erabutoxin a b	snake	revision of sequence 21—22 to Glu-Ser instead of Ser-Gln	136
Neurotoxin	*Lapemis hardwickii* venom	complete sequence, 60 residues; presence of a single −SH group was shown by laser Raman spectroscopy	137
Neurotoxins I III	African cobra (*Naja mossambica mossambica*) venom	complete sequence, 62 residues for each	138
Taipoxin	taipan venom (*Oxyuranus scutellatus*)	complete sequence of γ-subunit, 133 residues, a glycoprotein	139
Toxin III	sea anemone (*Anemonia sulcata*)	complete sequence, 30 residues	140
	sea anemone (*Anemonia sulcata*)	complete sequence, 27 residues	141
Toxins CM-2h CM-4b CM-6	*Naja haje annulifera* (Egyptian cobra)	complete sequence of all three toxins, 60 residues	142
CM-2a CM-3	*Naja haje annulifera*	complete sequence, 61 residues and 60 residues	143
Toxins 9B 11 12A	*Hemachatus hemachatus* venom	complete sequence, 63, 61, and 61 residues	144
Toxin γ	*Tityus serrulatus* Lutz and Mello	*N*-terminal, 29 residues	145
Toxin FS2	black mamba (*Dendro aspis polylepis polylepis*) venom	complete sequence, 60 residues, third most abundant toxin of black mamba venom	146
Venom toxin V_{i2}	black mamba (*Dendro aspis polylepis polylepis*)	complete sequence, 57 residues; related to the basic pancreatic trypsin inhibitor; active-site Lys (15) is preserved	147

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Ribosomal proteins			
S6	*E. coli* (wild type)	complete sequence. Five different forms exist; the longest form S6-6 consists of 136 residues; the shorter forms S6-5, S6-4, S6-3, and S6-2 have 1, 2, 3, and 4 Glu less than S6-6	148
	E. coli (mutant N 729)	complete sequence; identical to S6-2	148
S7	*E. coli* K *E. coli* B	complete sequence, residues; *C*-terminal sequence of *E. coli* K protein is longer than *E. coli* B protein by 24 residues	149
S9	*E. coli* K12	complete sequence, 128 residues	150
S12	*E. coli* K12	complete sequence, 123 residues; high degree of homology among the *N*-terminal sequences of S12 from *E. coli* and *B. stearothermophilus* and *B. subtilis*	151
S13	*E. coli* K	complete sequence, 117 residues; high degree of homology exists between the *N*-terminal region of S13 of *E. coli* and S14 of *B. stearothermophilus*	152
S16	*E. coli* K	complete sequence, 82 residues	153, 154
L6	*E. coli*	complete sequence, 176 residues	155
L7/L12	*Artemia salina*	*N*-terminal, 25 residues	156
	Saccharomyces cerevisiae	*N*-terminal, 16 residues	156
L11	*E. coli* K	complete sequence, 141 residues; most heavily methylated ribosomal protein; blocked *N*-terminus is *N*-trimethyl alanine	157
L15	*E. coli* K	complete sequence, 144 residues	158
L28	*E. coli* K12	complete sequence, 77 residues	159

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Globins			
Haemoglobins:			
β-Chain	rat (*Rattus norvegicus*)	complete sequence, 146 residues	160
β-Chains	pig (*Suidae*)	complete sequence, 146 residues;	161
	llama (*Lama glama camelidae*)	differ from human β-chains in 22 and 23 residues respectively	
α-Chains	pig (*Suidae*)	complete sequence, 141 residues;	162
	llama (*Lama glama camelidae*)	differ from human α-chains in 22 and 25 residues respectively	
α-Chain	newt (*Taricha granulosa*)	complete sequence, 142 residues	163
α-Chain	tupai (*Tupaia glis*)	complete sequence, 141 residues	164
β-Chain	tupai	complete sequence, 146 residues	164
Abnormal haemoglobins:			
Austin	human	β40, Arg → Ser	165
J. Calabria	human	β64, Gly → Asp	166
J. Cubujuqui	human	α141, Arg → Ser	167
Fort de France	human	α45, His → Arg	168
Handsworth	human	α18, Gly → Arg	169
Izu (Macaca)	Japanese monkey	β83, Gly → Cys	170
J. Lome	human	β59, Lys → Asn	171
North Shore	human	β134, Val → Glu	172
North Shore–Caracas	human	β134, Val → Glu	173
M. Oldenburg	human	α87, His → Tyr	174
Port Phillip	human	α91, Leu → Pro	175
Raleigh	human	β1, Val → *N*-acetyl Ala	176

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Table 1 (*cont*)

Protein	*Source*	*Comments*	*Ref.*
Rothschild	human	β37, Trp → Arg	177
Sherwood Forest	human	β104, Arg → Thr	178
S. Travis	human	β6, Glu → Val, β142, Ala → Val	179
Waco	human	β40, Arg → Lys	180
Myoglobin	gastropod mollusc (*Busycon canaliculatum* L.)	complete sequence, 147 residues	181
	siamang (*Symphalangus syndactylus*)	complete sequence, 153 residues	182
	killer whale (*Orcinus orca*)	complete sequence, 153 residues; there are 6 differences in sequence between the killer whale and dolphin, 8 with the porpoise, and 14 with the sperm whale	183
	fruit bat (*Rousettus aegyptiacus*)	complete sequence, 153 residues	184
	Cape fox (*Vulpes chama*)	complete sequence, 153 residues	185
	Arctic minke whale (*Baleanoptera acutorostrata*)	complete sequence, 153 residues	186
	dwarf sperm whale (*Kogia simus*)	complete sequence, 153 residues	187
	Pacific common dolphin	complete sequence, 153 residues	188
Cytochromes			
b_5	calf liver	sequence of *C*-terminal segment, 55 residues	189
b_5	porcine liver microsomes	complete sequence of membranous segment, 43 residues; this fragment is at the *C*-terminus of the molecule and is essential for the insertion of cytochrome into the endoplasmic reticular membrane	190

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
b_5	horse liver	complete sequence of the membranous segment	191
c	guanaco (*Lama guanicoe*)	complete sequence, 104 residues	192
c	mouse rat guinea-pig	complete sequence of all three cytochromes, 104 residues; complete mapping identity of tryptic and chymotryptic digests	193
c	locust (*Schistocerca gregaria* Forskål)	complete sequence, 107 residues; overlap between chymotryptic and tryptic peptides at residue Tyr(97) Leu(98) was not observed	194
f	cyanobacterium (*Plectonema boryanum*)	complete sequence, 85 residues; has three Asn-Gly linkages	195
P450	rabbit liver microsomes	*N*-terminal sequence of one electrophoretically homogeneous form P450 LM_2	196
P450	rabbit liver microsomes	partial sequence of peptides cleaved at Trp residues	197
Allophycocyanin	*Cyanidium caldarium*	*N*-terminal sequence of two subunits α- and β-, 10 residues	198
Azotoflavin	*Azotobacter vinelandii*	complete sequence, 179 residues	199
Ferredoxin	*Chromatium vinosum*	correction of previously published sequence, 82 residues; sequence 50 to 52 and 53 to 57 should be mutually displaced and one Ile added between residues 57 and 58	200
	Halobacterium halobium	complete sequence, 128 residues; largest ferredoxin to be sequenced so far	201
Ferredoxin I II	horsetail (*Equisetum arvense*)	complete sequence, 95 and 93 residues	202
Flavodoxin	*Desulphovibrio vulgaris*	complete sequence, 148 residues	203

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
High potential iron–sulphur protein (Hi PIP)	denitrifying coccus genus *Paracoccus*	complete sequence, 71 residues	204
Iron protein (N_2) of nitrogenase	*Clostridium pasteurianum*	complete sequence, 273 residues	205
Stellacyanin	*Rhus vernicifera*	sequence of a glycopeptide containing Cysh, 13 residues and a pentapeptide containing His	206
	Rhus vernicifera	complete sequence, 107 residues	207
Lectins			
Agglutinin α and β	soybean peanut	*N*-terminal, 27 residues of both subunits of both proteins	208
Conglutin γ	*Lupinus angustifolius* 'Uniwhite'	complete sequence, 154 residues	209
Isolectin E_4	*Phaseolus vulgaris*	*N*-terminal, 18 residues	210
Lectins α and β	lentil (*Lens culinaris*) garden pea (*Pisum sativum*)	*N*-terminal sequence, 27 residues of both subunits	211, 212
Lectin	*Dolichos biflorus*	*N*-terminal sequence of subunits I and II, 30 residues	213
Seed protein	*Ricinus communis*	*N*-terminal, 22 residues	214
Structural proteins			
Collagen type III	foetal calf skin	Partial sequence of 1α (III) chain, 750 residues representing 75% of the chain, cysteine residues located	215
Collagen type III	human liver	*N*-terminal, 229 residues; close interspecies homology with Type III Collagen from calf aorta	216
Collagen type II	bovine nasal cartilage	Partial sequence, residues 363—551, *i.e.* 188 residues in the chain of 1050 amino-acids	217

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Collagen type II	bovine nasal cartilage	Partial sequence of CNBr fragment, 39 residues; occurrence of sequence heterogeneity	218
Collagen	chick skin	*N*-terminal sequence of three chymotryptic peptides, 132 residues	219
α-Crystallin	human eye lens	Complete sequence of B chain, 175 residues	220
α-Crystallin	eye lens of elephant, whale, hyrax, rhinoceros	Complete sequence of A chain, 173 residues; only a small number of substitutions was found among these species	221
Viral proteins			
A-Protein	coliphage MS2	Complete sequence, 393 residues; only 363 residues were ordered unambiguously in large sequence stretches; 23 amino-acids were characterized in individual peptides but could not be positioned in the chain without data from the nucleotide sequence of the corresponding gene	222
Coat protein	bacteriophage f1	complete sequence, 50 residues	223
Coat protein	phage Qβ	revised sequence, Asn instead of Asp at position 22, additional Ser between Pro(55) and Arg(56), 132 residues	224
Coat protein	alfalfa mosaic virus (strain S)	complete sequence, 217 residues	225
Core protein VII	adenovirus	*N*-terminal sequence, 40 residues of *N*-terminal Trp cleavage fragment, and 14 residues of *C*-terminal Trp cleavage fragment. Both protein VII and its precursor form proprotein VII were sequenced	226

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Cro regulatory protein	bacteriophage λ	complete sequence, 66 residues	227
Internal protein I (IPI)	bacteriophage T_4	complete sequence of IPI* and IPI, 76 and 80 residues. IPI* is derived from IPI by a specific morphogenetic cleavage of a Glu-Ala bond, IPI* is the form found in the mature head	228
Small core protein	bacteriophage ϕX 174	complete sequence, 37 residues; 6 Lys and 6 Arg are concentrated in two homologous 12-residue segments of the sequence	229
Viral coat protein	alfalfa mosaic virus	complete sequence, 220 residues	230
Blood clotting proteins			
Fibrinogen	bovine plasma	sequence of peptides released by thrombin digestion of Aα and Bβ; 35 residues adjacent to fibrinopeptide A and 15 residues beyond fibrinopeptide B, 29 residues of fragment γ	231
Fibrinogen α-chain	human plasma	complete sequence of one CNBr fragment, 56 residues; partial sequence of 4 fragments and a preliminary characterization of the largest fragment	232
	human plasma	*N*-terminal, 198 residues	233
	human plasma	partial sequence of two CNBr fragments, 5 residues; these fragments also occur in fully cross-linked fibrin	234
Fibrinogen	human	partial sequence of disulphide-containing CNBr fragments	235
Fibrin γ-chain	human	partial sequence of Arg-specific tryptic peptide, 50 residues	236

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
	human	complete sequence of two Arg-specific peptides, 99, and 50 residues	237
Fibrin β-chain	human	complete sequence, 447 residues	238
Factor VII_a	bovine plasma	*N*-terminal sequence of heavy and light chains, 4 residues, and sequence of active-site peptide, 21 residues	239
Factor IX	human plasma	*N*-terminal, 15 residues	240
Factor X	human plasma	*N*-terminal, 15 residues	240
Factor X_a (activated Stuart factor)	human plasma	*N*-terminal sequence of heavy chain, 17 residues, and sequence of active-site peptide, 25 residues	241
Factor XI (plasma thromboplastin antecedent)	bovine plasma	*N*-terminal sequence of active-site CNBr fragment, 20 residues	242
Factor XI_a	human plasma	*N*-terminal sequence of heavy chains, 17 residues, of light chains, 16 residues, and sequence of active-site peptide, 20 residues	243
Factor XII (Hageman factor)	bovine plasma	*N*-terminal, 10 residues, and sequence of a CNBr fragment, 26 residues which is homologous with the active-site of a number of plasma Ser proteases	244
Factor XII_a	bovine plasma	*N*-terminal sequence of heavy and light chains, 12 and 15 residues	245
Plasmin	human	complete sequence of B-chain, 230 residues; extensive sequence homologies with the pancreatic Ser proteases	246
Platelet factor 4	human	complete sequence, 70 residues; *N*-terminal region is negatively charged, *C*-terminal region	247, 248

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
		contains a repetitive clustering of positively charged and hydrophobic pairs of amino-acids	
Prothrombin	rat	*N*-terminal, 9 residues	249
	human plasma	*N*-terminal, 15 residues	250
Prothrombin Fragments 1 and 2	human	complete sequence of non-thrombin half of prothrombin, 155, and 118 residues	251
Prethrombin 2	human	complete sequence, 308 residues	252
Protein C (activated)	bovine plasma	sequence of active-site peptide in heavy chain, 18 residues	253
Thrombin	human	complete sequence of A chain, 36 residues and *N*-terminal sequence of B chain, 50 residues	254
Complement proteins			
Subcomponent $C1_q$, C chain	human	*N*-terminal, 42 residues	255
Subcomponents of C1, activated forms $C\bar{1}r$ $C\bar{1}s$	human	two peptide chains a and b *N*-terminal, 20 residues, *N*-terminal, 29 residues	256
C4	human serum	*N*-terminal sequence, 12 residues of α, 8 residues of β-, and 19 residues of γ-chains	257
C4	human	*N*-terminal sequence, 21 residues of α-, 9 residues of β-, and 21 residues of γ-chains	258
C5	human	*N*-terminal sequence of $C5_a$ which has been derived from the *N*-terminus of the α-chain of C5, 25 residues	259
HLA antigens and antibodies			
DNP-Antibody, L-chain	rabbit	*N*-terminal, 20 residues	260

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
DNP-*p*-Aminobenzoyl-glutamate-antibody, L-chain	rabbit	*N*-terminal, 20 residues	260
Antibody K16-167 (anti-streptococcal group A variant polysaccharide) L-chain	rabbit	sequence of variable region, 109 residues	261
Anti-*p*-azobenzoate antibody	rabbit	sequence of CNBr fragment of heavy chain, 19 residues	262
Anti-*p*-azophenyl-arsonate antibody L-chain	A/J mice	complete sequence of variable region, 108 residues	263
Anti-pneumococcal antibody, H-chain	rabbit	complete sequence of variable region	264
Bence–Jones cryoprotein	human	complete sequence of variable region peptides; ability to crystallize at 4 °C	265
HLA-B system antigen	chicken lymphocytes	*N*-terminal, 27 residues	266
HLA-linked B-cell alloantigen	human lymphoblastoid cells	*N*-terminal sequence of two non-covalently associated subunits p29 and p34, 11 and 15 residues	267
HLA-antigens HLA-A2 HLA-B7	human human	sequence around the site of glycosylation, 15 and 17 residues	268
Immunoglobulin (k type) L-chain	mouse myeloma MPC 11	*N*-terminal sequence of *in vitro* synthesized protein, 18 residues	269
Immunoglobulin (k type) L chains	rabbits hyper-immunized with streptococcal vaccines	*N*-terminal sequence of six homogeneous k-chains, 4 residues; two different *N*-terminal sequences were found	270
Immunoglobulin L-chain	MOPC-321 mouse myeloma cells	*N*-terminal, 15 residues	271
Immunoglobulin λ-chains	water buffalo	partial sequence of *C*-terminal tryptic peptide, 9 residues	272
Immunoglobulin λ-chains	MOPC-104Eλ_1 RPC-20λ_1	*N*-terminal sequence of *in vitro* synthesized protein, 19 residues;	273

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
	MOPC-315$_2$ mouse myeloma	λ_1L chain precursors identical, λ_2L chain precursor differs in at least 3 out of 19 positions	
Immunoglobulin λ chains	porcine	complete sequence of micro heterogeneous proteins, 214 residues	274
Immunoglobulin A allotypes a1 a3	rabbit	*N*-terminal sequence of Aa1 fragment, 34 residues, and residues 67 to 96, *N*-terminal sequence of Aa_3 fragment, 27 residues	275
Immunoglobulin H chains, I_gA_k Gom I_gM_λ Moo	canine Airdale terrier Scottish terrier	complete sequences of the variable regions, 117 and 112 residues	276
Immunoglobulin new H chains	human myeloma	complete sequence, 249 residues	277
Immunoglobulin H chains $I_gG2(k)$ I_gM (k)	human	complete sequences of the variable regions, 135 and 137 residues	278
Immunoglobulin H chains, I_gG3	human	sequence of 'hinge' region, 62 residues	279
Immunoglobulins I_gA1 I_gA2	human	complete sequence of *C* region of heavy chains of α_2 and location of *N*-acetyl glucosamine residues	280
Immunoglobulin 'a' negative	rabbit immunized with group A *Streptococcus*	partial sequence of H chain	281
Immunoglobulin I_gG3 m(g)	human	sequence of pFc' fragment obtained by pepsin digestion of I_gG3, 112 residues	282
J Chain from I_gM	human	partial sequences of four fragments, 59 residues of a total of 129	283
J Chain	human with Waldenstrom's macroglobulinemia	complete sequence, 129 residues; pairing of 5 disulphide bonds still needs to be clarified	284
Myeloma protein, H chain	mouse	complete sequence of variable region, 106 residues	285

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Miscellaneous			
Amyloid A protein (AA)	duck liver	complete sequence, 86 residues	286
Amyloid P component	human liver (amyloid deposits)	*N*-terminal, 27 residues	287
Anthopleurin A	*Anthopleura xanthogrammica*	complete sequence, 49 residues	288
Antifreeze peptide 3	*Pseudopleuronectus americanus* (Walbaum)	complete sequence of smallest of three peptides, 37 residues	289
Antiplasmin	human	*N*-terminal, 4 residues	290
α-1-Antitrypsin (Z-variant)	human plasma	*N*-terminal, 8 residues of tryptic peptide	291
Apolipoprotein C-II	human	complete sequence, 78 residues; lacking in Cys and His	292
Apo mucin	Ovine submaxillary gland	partial sequence of three tryptic peptides representing 106 residues of a total of 650 amino-acids; one-third of the residues are either Thr or Ser	293
Apovitellenin I	duck's egg yolk	complete sequence, 82 residues; high degree of homology between hen, duck, and emu apovitellenin I	294
L-Arabinose binding protein	*E. coli* B/r	complete sequence, 306 residues	295
Bacillomycin L	*Bacillus subtilis*	complete sequence of cyclic peptide, 7 residues; contains D-Asp, D-Ser, and D-Tyr	296
Biotin carboxyl carrier protein	*E. coli*	complete sequence of fragment containing the single biotin residue, 82 residues	297
Casein	human	*N*-terminal, 28 residues	298
α_{s2}-Casein	bovine milk	complete sequence, 207 residues, and preliminary data on location of phosphate groups	299

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Casein precursors	ovine mammary gland	*N*-terminal, 29 residues	300
Ceruloplasmin	human serum	sequence around the single cysteine residue in the L-chain, 32 residues	301
Elongation factor T_u	*E. coli* B	partial sequence, 180 amino-acids out of a total of 400 accounted for, three cysteines located	302
	E. coli	partial sequence, alignment of tryptic peptides and location of −SH groups	303
	E. coli B	partial sequence, alignment of 11 CNBr fragments	304
α-Fetoprotein	human serum	*N*-terminal, 17 residues	305
Fimbrial protein	*Moraxella nonliquefaciens*	*N*-terminal, 49 residues; presence of an uncommon amino-acid at the *N*-terminus	306
Gut GLI-1 protein	porcine gut	partial sequence, 100 residues; *C*-terminal 10 residues includes the sequence of glucagon	307
Hepatitis B surface antigen, subtype adw	human	*N*-terminal, 9 residues, and *C*-terminal, 3 residues of two major polypeptide components	308
Initiation factor IF-3	*E. coli*	complete sequence of two forms, IF-31 and IF-3s, 181 and 175 residues; the first 6 residues of IF-1 are missing in IF-3s	309
Lactogen	ovine placenta	partial sequence of CNBr fragments	310
Lactogen precursor	human placenta	*N*-terminal sequence of *in vitro* synthesized protein, 19 residues	311

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Leghaemoglobin C_2	Soybean (*Glycine max* 'Lincoln')	complete sequence, 141 residues; the two major components 'a' and 'c' differ in only 10 positions	312
Lipoprotein (high density)	baboon plasma	*N*-terminal sequence of apolipoprotein I, 30 residues, *C*-terminal, 4 residues	313
LIV-Protein (Leu, Ile, Val-binding protein)	*E. coli*	complete sequence, 344 residues	314
Lysozyme precursor	egg-white	*N*-terminal sequence of *in vitro* synthesized protein, 19 residues	315
Major apolipoprotein (apo VLDL-II)	white leghorn plasma	complete sequence, 82 residues of two identical polypeptide chains held together by a disulphide bond at position 76	316
Mating factor	*Saccharomyces cerevisiae*	complete sequence, 13 residues	317
Metallothionein-I	mouse liver	complete sequence, 61 residues	318
Metallothioneins	horse kidney human liver rabbit liver	complete sequence of all three proteins, 60 residues; all have the same 4 residues at the *N*-terminus and the same *C*-terminus	319
Zn-Metallothionein	human liver	complete sequence, 61 residues	320
Mucin	bovine cervical mucus	partial sequence of tryptic peptides, 22 and 19 residues	321
Non-histone protein, HMG-T	*Salmo gairdnerii* testis	*N*-terminal, 29 residues; shows considerable similarity to HMG-1 and HMG-2 chromosomal proteins	322
Non-histone protein, HMG-17	calf thymus	complete sequence, 89 residues	323
Phosphocarrier, protein HPr	*Staphylococcus aureus*	complete sequence, 70 residues	324
Phosphorylated polypeptide (E_4)	bovine embryonic dental enamel	*N*-terminal, 43 residues	325

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
Prealbumin	rat plasma	*N*-terminal, 30 residues	326
Preproalbumin	rat liver	*N*-terminal sequence of *in vitro* synthesized protein, 24 residues	327
Proalbumin	bovine liver microsomes	*N*-terminal, 10 residues	328
Proalbumin	chicken liver microsomes	*N*-terminal, 12 residues	329
Prolipoprotein	*E. coli* (toluene-treated)	*N*-terminal, 19 residues	330
Prolipoprotein (outer membrane)	*E. coli*	*N*-terminal sequence of *in vitro* synthesized protein, 20 residues	331
Protein A	*Staphylococcus aureus*	*N*-terminal sequence of five tryptic peptides still bound to the cell wall	332
	Staphylococcus aureus	complete sequence of I_gG-F_c binding region, 235 residues out of 395 residues	333
Protein C	human parotid saliva	*C*-terminal, 4 residues	334
Protein S	human plasma	*N*-terminal, 8 residues	335
Protease inhibitors II and V	Tracy soybean	*N*-terminal, 20 residues and *N*-terminal, 16 residues respectively	336
Proteinase inhibitor II_b	potato	complete sequence of active fragment prepared by incubating inhibitor with trypsin, 40 residues	337
Protein phosphatase inhibitor-1	rabbit skeletal muscle	sequence of a peptide at the phosphorylation site, 10 residues	338
Proteolipid P7 apoprotein	rat brain myelin	*N*-terminal sequence extended to 31 residues	339
Purothionin A	wheat flour (*Triticum vulgare* 'Manibota 3')	complete sequence of two polypeptide chains, 45 residues differing in 5 positions	340

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Table 1 (*cont.*)

Protein	*Source*	*Comments*	*Ref.*
C-Reactive protein (CRP)	human	complete sequence, 187 residues	341
Rhodopsin	bovine eye lens	*N*-terminal, 16 residues	342
Silk fibroin peptide C_p	*Bombyx mori*	complete sequence, 59 residues made up of repeated sequences	343
Statherin	human parotid saliva	complete sequence, 43 residues	344
Thymic factor (FTS: facteur thymique serique)	porcine serum	complete sequence, 9 residues	345
Thyroxine-binding globulin	human plasma	*N*-terminal, 20 residues	346
Trypsin inhibitor	human pancreas	complete sequence, 56 residues	347
Trypsin and trypsin inhibitors P-3 and P-4	chick peas (*Cicer arietinum* L.)	*N*-terminal sequences, 6 residues and 4 residues from each fragment after oxidation	348
Trypsin-chymotrypsin elastase inhibitor CII	Soybean	complete sequence, 76 residues	349

Novel *N*-Terminal Amino-acids.—*N*-Methyl alanine has been shown to be at the *N*-terminus of *E. coli* ribosomal proteins S11 and L33, and protein L16 has *N*-methyl methionine at its *N*-terminus.[350] The fimbrial protein of *Moraxella nonliquefaciens* was shown to have an uncommon amino-acid at its *N*-terminus.[351] The structure of this amino-acid is being studied, but high-resolution mass spectrometry indicates that it could be either *o*(*m* or *p*)-methyl-L-phenylalanine or L-α-amino-γ-phenyl butyric acid. Dimethylproline was found at the *N*-terminus of *Crithidia oncopelti* cytochrome *c*557 by 220 MHz proton magnetic resonance spectroscopy.[352]

Isopeptide Linkages in Proteins.—Chromosomal protein A24 contains a non-histone polypeptide and histone 2A, and has two *N*-terminal amino-acids and a single *C*-terminal residue. This suggested the presence of a branched structure and indicated that the polypeptide was linked to the histone 2A molecule in such a way as to prevent the detection of its *C*-terminal amino-acid. A search was made for an altered histone 2A peptide in a tryptic digest of the A24 protein.[353] The peptide was isolated and its amino-acid composition and *C*-terminal amino-acid were determined. The peptide was subjected to sequential Edman degradation. The first cycle released two glycines and a single lysine on hydrolysis of the PTH-derivatives with hydriodic acid. These data are consistent with a branched

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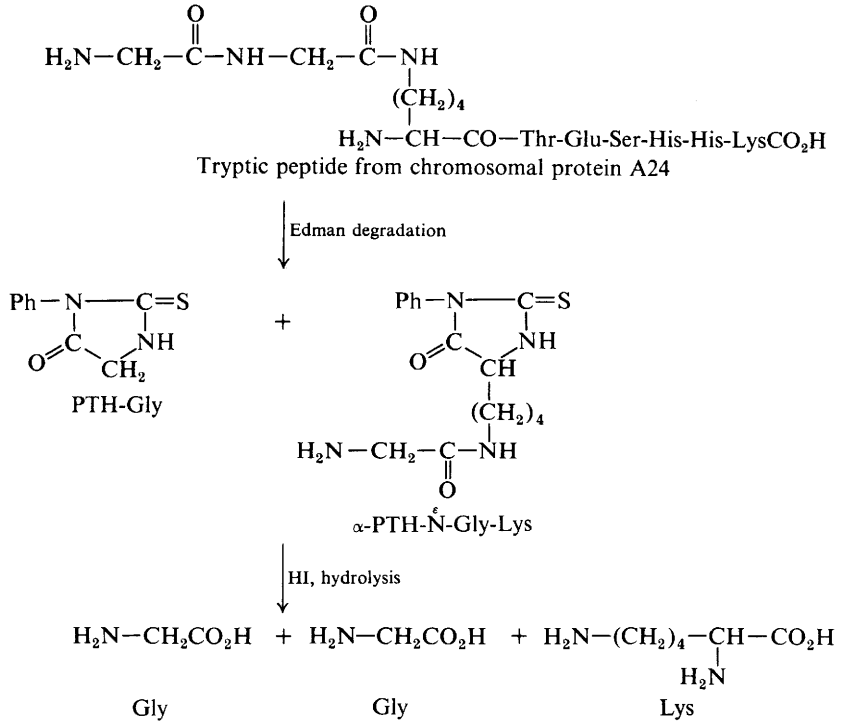

Scheme 4

structure arising from the two glycines at the *N*-terminus being attached to the rest of the peptide in an isopeptide linkage as seen in Scheme 4. The lysine residue was at position 119 of histone 2A in the chromosomal protein. This is the first occurrence of an isopeptide linkage among chromosomal proteins.

This type of linkage was also observed in *E. coli* ribosomal protein S11.[354] Determination of the *N*-terminus of protein S11 by the dansylation technique gave one spot corresponding to DNS-*N*-methylalanine and a second spot in the position of α- or ε-DNS-Lys on thin layer chromatography on polyamide plates. This is due to the reaction of the amino group of Lys with dansyl chloride. Bis-DNS-Lys is seen when Lys is the *N*-terminal amino-acid. It was therefore concluded that protein S11 contained *N*-methylalanine at its *N*-terminus. However, when S11 was subjected to automatic Edman degradation in the sequenator, two amino-acids were released in the first step. Two amino-acids were seen in all the subsequent steps, indicating that two polypeptide chains were present in the sample. The data also suggested that the two chains were homologous because an amino-acid seen in one step of the analysis also occurred in the next step. It seemed that the difference between the two chains was an additional amino-acid at the *N*-terminus of one of them that induced a shift in the entire sequence. In order to resolve this discrepancy, the *N*-terminal tryptic peptide was

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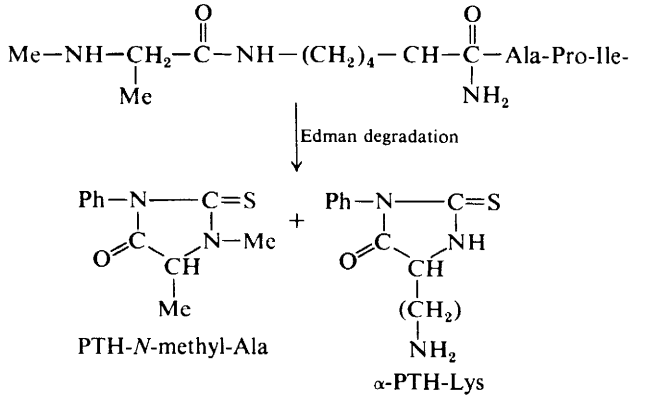
isolated and subjected to the micro-dansyl-Edman sequencing technique. This gave DNS-*N*-methylalanine and α- or ε-DNS-Lys in the first cycle. Bis-DNS-Lys was not seen, nor was Lys identified in any further step of the sequence. The peptide was then subjected to the subtractive micro-dansyl-Edman sequencing technique. Before each degradation step, a fraction of the peptide was hydrolysed with 6M-HCl, the liberated amino-acids were dansylated and identified on micro-polyamide thin-layer plates. In these experiments, bis-DNS-Lys was found before the first residue had been cleaved from the peptide, but not in any succeeding step. This result confirmed that Lys occurred at the *N*-terminus of the peptide, and that the second amino group became accessible to dansyl chloride on hydrolysis prior to reaction with the reagent. Automated sequence analysis of S11 revealed two PTH-amino acids in the first cycle. One of them migrated in the position of α-PTH-N^{ϵ}-PTC-Lys on silica gel thin-layer plates. Results with synthetic isopeptides showed that α-PTH-Lys and α-PTH-N^{ϵ}-PTC-Lys migrated to the same position on silica gel. Scheme 5 shows the behaviour of the *N*-terminal peptide of S11 during Edman degradation.



Scheme 5

N-methylalanine was quantitatively cleaved from S11 in the sequenator whereas Lys strongly overlapped in the second cycle. When the protein was subjected to a double cleavage, the overlap almost disappeared. This is the first occurrence of an isopeptide linkage among ribosomal proteins.

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

1 Introduction

A list of proteins which have been modified is given in Table 1. Space limitations have necessitated a certain amount of selectivity in compiling the Table and, apart from isolated cases where chemical modification is an integral part of the work, the following procedures have not been covered: (i) phosphorylation, (ii) radioactive iodination, and (iii) chemical modification for sequencing purposes.

Table 1 *Chemical modification of proteins*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Acetylcholinesterase	electric eel	isopropylmethylfluorophosphate	Ser	study of spontaneous reactivation	1
Acetylcholinesterase	electric eel	methyl(acetoxymethyl) nitrosamine–photoirradiation		inactivation by photolysis of reaction product	2
Acetylcholine receptor	rat muscle	4-(*N*-maleimido)benzyltri[^{3}H]-methylammonium iodide	Cys	2 subunits labelled	3
Acetylcholine receptor	*Torpedo californica*	[^{3}H]bis(3-azidopyridinium)-1,10-decane di-iodide–photo-irradiation		40 000 and 60 000 polypeptides labelled	4
Acetylcholine receptor	*Torpedo californica*	dithiothreitol	cystine	S—S dimer	5
Acetylcholine receptor	*Torpedo californica*	DTNB	Cys	S—S bridge links δ-subunits	6
Acetylcholine receptor	*Torpedo californica*	*N*-ethylmaleimide	Cys	prevents formation of L oligomer	7
Acetylcholine receptor	*Torpedo californica*	^{125}I–lactoperoxidase	Tyr	preferential labelling of 40 000, 50 000, and 60 000	8
Acetylcholine receptor	*Torpedo californica*	iodoacetamide	Cys	immunological characterization	9
Acetyl-CoA transferase	*E. coli*	DTNB, *N*-ethylmaleimide	Cys	thiol reacts faster in CoA complex	10
Acetyl-CoA transferase	*E. coli*	pyridoxal 5′-phosphate–sodium borohydride	Lys	essential Lys	11
α-*N*-Acetylglucos-aminidase	human	*N*-ethylmaleimide, iodoacetamide	Cys	no effect on activity	12

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
N-Acetylglutamate synthase	*E. coli*	various thiol reagents	Cys	essential thiols	13
N-Acetyl-β-D-hexosaminidase	human	2-acetamido-2-deoxy-β-D-glucopyranosyl isothiocyanate		specific irreversible inhibitor	14
N-Acetyl-β-D-hexosaminidase	human	poly(*N*-vinylpyrrolidone)	Lys	active stabilized enzyme	15
N-Acetylmuramyl-L-alanine amidase	bacteriophage	*p*-chloromercuribenzoate	Cys	essential thiol	16
Acid phosphatase	*Aspergillus niger*	*N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide	Trp	essential Trp	17
Acid phosphatase	human prostrate	CNBr-activated Sepharose		stabilized enzyme	18
Aconitase	porcine	butanedione	Arg	single essential Arg	19
Aconitase	porcine	phenacyl bromide	Cys	essential Cys	20
Acyl-CoA : glycine *N*-acyltransferase	bovine liver	*p*-azido[^{14}C]benzoyl-CoA–photoirradiation		one active site per molecule	21
Adenine nucleotide carrier	bovine heart	8-azido-[^{14}C]ADP–photo-irradiation		8-azido-AMP is not a photolabel	22
Adenosylhomo-cysteinase	lupin	*N*-ethylmaleimide	Cys	inhibits enzyme	23
Adenylate cyclase	horse thyroid	iodide		inhibits enzyme	24
Adenylate cyclase	mouse	*N*-ethylmaleimide	Cys	at least 2 proteins necessary for activity	25
Agglutinin	wheat germ	dimethyl(2-methoxy)-5-nitrobenzyl-sulphonium bromide	Trp	Trp at monosaccharide binding site	26
D-Alanine carboxy-peptidase	*Bacillus subtilis*	[^{14}C]- or [^{35}S]penicillin iodoacetamide	Ser Cys	labelled pronase and tryptic peptides isolated	27
Alanine dehydrogenase	*Bacillus natto*	*p*-chloromercuribenzoate	Cys	inhibits enzyme	28
Albumin	bovine serum	*N*-dansylaziridine		nucleophiles other than thiol react	29
Albumin	bovine serum	DTNB	Cys	kinetics of cleavage of S—S	30
Albumin	bovine serum	methoxypolyethylene glycols–[^{14}C]cyanuric chloride	Lys	loss of immunogenicity	31
Albumin	chicken egg white	*N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide	Cys	0.6 mol per molecule	32

Albumin	human serum	1,2-cyclohexanedione *N*-acetylimidazole	Arg, Tyr, Lys	bilirubin and diazepam bind to separate sites	33
Albumin	human serum	diethylenetriaminepenta-acetic acid–isobutylchloroformate–triethylamine		simple procedure for covalent coupling of metal binding ligand	34
Albumin	human serum	photoirradiation–sensitizer	His	stoicheiometry and kinetics	35
Alcohol dehydrogenase	*Drosophila melanogaster*	DTNB	Cys	single thiol per subunit	36
Alcohol dehydrogenase	horse liver	α-bromo-β-(5-imidazolyl)-[^{14}C]-propionic acid, 1,3-dibromo-acetone	Cys	potential use of L and D isomers	37

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Alcohol dehydrogenase	horse liver	ethylacetimidate, 4-bromobutyramide, various ketones–sodium borohydride	Lys	mechanism of catalysis studied	38
Alcohol dehydrogenase	horse liver	iodo[^{14}C]acetate	Cys	study of subunit interactions	39
Alcohol dehydrogenase	horse liver	[^{14}C]phenylglyoxal	Arg	2 Arg modified; one is Arg-84	40
Alcohol dehydrogenase	horse liver	styrene oxide	Cys-43, Cys-152	modified tryptic peptides identified	41
Alcohol dehydrogenase	human liver	ethyl acetimidate	Lys	altered mechanism	42
Alcohol dehydrogenase	yeast	arylazido-[3-^{3}H]β-alanine-NAD^{+}–photoirradiation		photoaffinity label, synthesis detailed	43
Alcohol dehydrogenase	yeast	*N*-(4-chloromercuriphenyl)-4-chloro-3,5-dinitrobenzamide	Cys, Lys	new bifunctional reagent	44
Alcohol dehydrogenase	yeast	diethyl pyrocarbonate	His	essential His in NADH site ?	45
Alcohol dehydrogenase	yeast	[4-^{3}H]-NADH–acid	Trp	electrophilic attack at C-3	46
Aldehyde dehydrogenase	sheep liver	DTNB, disulphiram, iodoacetamide	Cys	4 thiols react with DTNB, 2 thiols with other reagents	47
Aldehyde reductase	human liver	*p*-mercuribenzoate	Cys	most active Cys not essential	48
Aldolase	rabbit heart	dihydroxy-[^{14}C]acetone-phosphate–sodium borohydride	Lys	80% inactivation	49
Aldolase	rabbit muscle	di-*N*-(2-nitro-4-azidophenyl)-cystamine-*SS*-dioxide–photoirradiation	Cys	lipid soluble, cleavable	50
Aldolase	rabbit muscle	sodium borohydride	Lys	N^{6}(2-deoxy-2-glucitol)L-lysine isolated; stereospecific reaction	51
Alkaline phosphatase	*E. coli*	butane-2,3-dione	Arg	fewer Arg modified in Zn^{2+} enzyme than Hg^{2+} enzyme	52
D-Amino-acid transaminase	*Bacillus sphaericus*	β-chloro-D-alanine		inactivates enzyme	53
4-Aminobutyric acid aminotransferase	rat brain	4-amino-hex-5-enoic acid	Lys	irreversible inhibitor; mechanism proposed	54
6-Aminohexanoic acid cyclic dimer hydrolase	*Acromobacter guttatus*	*p*-chloromercuribenzoate di-isopropylfluorophosphate	Cys Ser	essential Cys	55

δ-Aminolevulinic acid synthase	*Rhodopseudomonas spheroides*	dimethylsuberimidate	Lys	dimer	56
c-AMP receptors	*E. coli*	[^{14}C]DTNB	Cys	cAMP induces DTNB mediated S—S cross-linking	57
c-AMP receptors	sarcoma 37	8-azido-[^{32}P]-AMP–photo-irradiation		photolabelling of whole freeze-fractured cells is feasible	58
c-AMP receptors	porcine kidney	[^{3}H]-c-AMP–photoirradiation		evidence of >2 receptors	59
c-AMP receptors	rat	8-azido-[^{32}P]-AMP–photo-irradiation		improved synthesis of reagent	60
α-Amylase	*Bacillus amylo-liquefaciens*	CNBr-activated dextran		extends circulatory life	61

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Antigen E	ragweed	2-chloro-4-hydroxy-6-methoxy-polyethyleneglycoltriazine, glyoxylic acid–cyanoborohydride	Lys	reduced allergenic activity	62
α_1-Antitrypsin	human plasma	[^{14}C]formaldehyde–sodium borohydride, formaldehyde–sodium borotriti-ide	Lys	study of properties of labelled proteins	63
α_1-Antitrypsin–trypsin	porcine	$^{18}OH^-$		new carboxy-terminal Lys labelled; evidence for acyl ester	64
Apolipoprotein A-II	monkey	iodoacetic acid	Cys	properties different from human	65
Arginase	human liver	nylon–glutaraldehyde		reactivation of subunits	66
Arom multienzyme complex	*Neurospora crassa*	dimethylsuberimidate	Lys	2 subunits each 165 000	67
L-Asparaginases	*Citrobacter freundii*	*p*-chloromercuribenzoate	Cys	2 out of 3 enzymes have essential Cys	68
L-Asparaginase	*E. coli*	5-diazo-4-oxo-L-[5-^{14}C]norvaline	Ser-9	radioactive peptide sequenced	69
Aspartase	*E. coli*	CNBr-activated Sepharose		immobilized enzyme stabilized	70
Aspartate aminotransferase	porcine	3-bromo-1,1,1-trifluoropropanone	Cys	selectively modify Cys-45,-82 and 390	71
Aspartate aminotransferase	porcine heart	dibromohydroxymercurifluorescein, other thiol reagents	Cys	evidence for reaction of mercurochrome with Cys	72
Aspartate aminotransferase	porcine heart	pentane-2,4-dione	Lys	1 Lys reacts 200× faster than remainder	73
Aspartate aminotransferase	porcine heart	[1-^{14}C]vinylglycine	Lys-258	mechanism of inactivation	74
Aspartate transcarbamylase	*E. coli*	phenylglyoxal	Arg	2 Arg per regulatory chain	75
Aspartate transcarbamylase	*E. coli*	pyridoxal 5′-phosphate–sodium borotriti-ide	Lys	inactivation reversed by photolysis	76
Aspartokinase II -homoserine dehydrogenase II	*E. coli*	iodo[2-^{14}C]acetic acid	Cys	identical subunits	77
Aspartokinase II -homoserine dehydrogenase II	*E. coli*	DTNB	Cys	protected by Thr	78
		N-acetylimidazole	Tyr	inhibits kinase activity	
		methylacetimidate	Lys	no effect on catalysis	

ATPase	bovine heart	*N*-4-azido-2-nitrophenyl-amino[^{3}H]butyryl-ADP–photoirradiation		α and β subunits labelled	79
ATPase	bovine heart	ATP-γ-4-(*N*-2-chloroethyl-*N*-methylamino)benzylamidate		binds to β-subunit(s) of factor F_1	80
ATPase (Na^+, K^+-dependent)	canine kidney	ethylacetimidate	Lys	unexpected cross-linking	81
ATPase (Na^+, K^+-dependent)	*Electrophorous electricus*	*S*-mercuri-*N*-dansyl cysteine	Cys	fluorescent probe	82
ATPase	*E. coli*	[^{14}C]dicyclohexylcarbodi-imide	carboxyl	reactive protein purified	83
ATPase (Na^+, K^+-dependent)	human erythrocytes	ozone	Cys	oxidation prevented by DTNB, semicarbazide stops cross-links	84

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
ATPase	*Neurospora crassa*	*p*-chloromercuri-benzoate and phenyl sulphonate, *N*-ethyl-maleimide	Cys	mercurials inhibit, *N*-ethylmaleimide does not	85
ATPase	rabbit	pyridoxal 5′-phosphate–sodium borotriti-ide	Lys	single essential Lys in 30 000 fragment	86
ATPase (Ca^{2+}-dependent)	rabbit	DTNB	Cys	2 reactive groups protected by ATP	87
ATPase	*Saccharomyces cerevisiae*	methyl-4-mercaptobutyrimidate	Lys	structural model for ATPase proposed	88
ATPase (Na^+, K^+-dependent)	rabbit	*N*-ethylmaleimide	Cys	inactivates	89
ATPase (Na^+, K^+-dependent)	rabbit	butane-2,3-dione DTNB	Arg Cys	Arg in nucleotide site protects Arg from reaction	90
ATPase	rabbit	*N*-ethyl[^{14}C]maleimide, DTNB	Cys	3 S—S, 20 SH	91
ATPase	spinach chloroplasts	butane-2,3-dione	Arg	1 essential Arg	92
ATPase	yeast	oligomycin–sodium borotriti-ide		binds to low mol. wt. component	93
ATP synthetase	bovine heart	butane-2,3-dione, phenylglyoxal	Arg	most reactive Arg involved in phosphate exchange	94
Bacteriorhodopsin	*Halobacterium*	*N*-bromosuccinimide	Trp	essential for proton pump	95
Beta adrenergic receptor	turkey erythrocytes	*N*-(2-hydroxy-3-naphthoxypropyl)-*N*′-(2-nitro-5-azidophenyl) ethylenediamine–photoirradiation		synthesis and characterization of photoaffinity label	96
β-Bungarotoxin	*Bungarus multicinctus*	various reagents		evidence for toxicity being due to phospholipase A activity	97
γ-Butyrobetaine hydroxylase	*Pseudomonas*	DTNB	Cys	13 thiols, no S—S	98
Calcitonin	bovine, porcine	succinic anhydride, citraconic anhydride	α-NH_2, Tyr-12	acylated amino enhances activity	99
Carbonic anhydrase B	bovine	bromoacetazolamide	His	second equivalent of reagent reacts with N-3 of first	100
Carbonic anhydrase B	human	bromo[^{13}C]acetic acid	His-200	^{13}C-n.m.r.	101
Carboxylesterase	human	diethyl-*p*-nitrophenyl phosphate, di-isopropylfluorophosphate	Ser	essential Ser	102
		various thiol reagents	Cys	Cys not in active site ?	

Carboxypeptidase A	bovine	diazotized *p*-arsanilic acid	Tyr-248	resonance Raman study	103
Casein	bovine	iodoacetamide	Cys	SDS binds to alkylated casein in co-operative manner	104
Catalase	human erythrocyte	various di-imidates	Lys	role of quaternary structure	105
Catalase	porcine erythrocyte	*p*-chloromercuribenzoate, iodoacetate	Cys	specific association caused by air oxidation of thiols	106
Cathepsin B	bovine spleen	benzyloxycarbonyl-Phe-diazomethyl ketone	Cys	inactivates	107
CDP-diglyceride : inositol transferase	rat liver	*N*-ethylmaleimide, *p*-chloro-mercuribenzene sulphonate	Cys	inhibits enzyme	108

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Cellulase	*Aspergillus niger*	various reagents	Trp, His, Tyr, carboxyl	1 essential Trp, essential carboxyl	109
Chlorocruorin	*Spirographis*	iodoacetic acid, performic acid	Cys	2 types of subunits	110
Choriomammotropin	human	performic acid, hydrogen peroxide, iodoacetic acid	Met	under non-denaturing conditions 5/6 Met oxidized by performic acid	111
Chorionic gonadotropin receptor	human	^{131}I–lactoperoxidase	Tyr	method for labelling receptor specifically	112
Chorismate mutase–prephenate dehydratase	*E. coli*	DTNB	Cys	one Cys in dehydratase active site	113
Chromatin non-histone proteins	rat liver	formaldehyde, glutaraldehyde, 4-methylmercaptobutyrimidate	Lys	a single high mol. wt. aggregate stabilized	114
Chymotrypsin	bovine	di-isopropylfluorophosphate	Ser-195	^{31}P-n.m.r. study	115
Chymotrypsin	bovine	1-(2-chloroethyl)-3-([1-^{14}C] cyclohexyl)-1-nitrosourea	Ser-195	cyclohexylisocyanate is the reactive species	116
Chymotrypsin	bovine	methyl *p*-nitrobenzenesulphonate	His-57	rate of deacylation studied	117
Chymotrypsin	bovine	*p*-nitrophenyl[^{14}C]acetate	Lys	kinetics of deacylation	118
Chymotrypsin	bovine	*p*-nitrophenyl-N^2-acetyl-N^1-arylmethylcarbazates	Ser-195		119
		2-hydroxy-5-nitrobenzyl bromide	Trp	Trp less accessible in Ser-modified enzyme	
Chymotrypsin	bovine	phenylmethylsulphonylfluoride–NaOH	Ser-195	thermodynamics of binding	120
		methyl *p*-nitrobenzenesulphonate	His-57		
Chymotrypsin	bovine	phenylmethylsulphonylfluoride–NaOH	Ser	anhydroprotein characterized which retains Ser-195	121
Chymotrypsin and trypsin	bovine	tetranitromethane	Tyr	pure monomeric nitrated trypsin isolated	122
Chymotrypsin, trypsin, elastase		various *p*-nitrophenyl carbamates		simple preparation outlined	123
Citrate synthetase	*E. coli*	DTNB	Cys	4 accessible thiols	124
Coat protein	tobacco mosaic virus	*N*-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidin-3-yl)methylmaleimide	Cys	oligomers dissociate as pH increases	125

Collagen	human (osteogenesis imperfecta)	sodium borotriti-ide		clinical symptoms may result from labile cross-links	126
Collagen	rat	sodium cyanoborohydride		dihydrohydroxymerodesmosine identified	127
Complement C4	human	iodo[^{14}C]acetamide	Cys	three chain structure	128
Complex III	bovine heart	^{125}I–lactoperoxidase *p*-diazoniumbenzene [^{35}S]-sulphonate	Tyr	peptides I, II, IV, V deeper than III and IV; VII very deeply buried	129
Concanavalin A		methyl 4-azidobenzoimidate–photoirradiation in presence of membrane	Lys	Con A-binding site identified	130

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Coupling factor 1	bovine heart	dimethyl-3,3′-dithiobispropionimidate	Lys	minimum stoicheiometry is $\alpha_2 \beta_2 \gamma_2 \delta \varepsilon$	131
		Cu^{2+}/*o*-phenanthroline	Cys		
Coupling factor 1	spinach chloroplasts	5-iodoacetamidofluorescein	Cys	attaches remote from active site	132
Coupling factor 1	spinach chloroplasts	*N*-ethylmaleimide, *N*-phenylmaleimide, *o*-[^{14}C]phenylenedimaleimide	Cys	*o*-phenylenedimaleimide is potent inhibitor of photophosphorylation	133
Coupling factor 1	spinach chloroplasts	phenylglyoxal	Arg	essential Arg	134
Coupling factor 1	spinach chloroplasts	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Tyr	model of coupling factor proposed	135
		various maleimides	Cys		
Creatine kinase	chicken	2-mercuri-4-nitrophenol	Cys	reactive thiol not in active site	136
Creatine kinase	rabbit muscle	iodoacetamide, methylmethanethiosulphonate	Cys	n.m.r., e.p.r., and kinetic studies	137
Creatine kinase	rabbit muscle	2-mercaptoethanol	Cys	restores activity to inactive enzyme	138
Creatine kinase	rabbit muscle	mixed disulphide of 2-thiopyridine and 2-thiobenzyl[^{14}C]diazoacetate–photoirradiation	Thr, Ser	new bifunctional reagent for active site of thiol enzymes	139
γ-Cystathionase	rat liver	2-amino-4-[^{14}C] and [^{2}H] and [^{3}H] pentynoic acid		Cys or Tyr modified ? mechanism proposed	140
γ-Cystathionase	rat liver	butane-2,3-dione	Arg	essential Arg	141
γ-Cystathionase	rat liver	β,β,β-trifluoro[1-^{14}C]alanine	Lys	mechanistic study	142
Cytochrome *c* oxidase	bovine heart	*N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)maleimide	Cys	spin label used to study denaturation	143
Cytochrome *c*-552	*Euglena gracilis*	acetic anhydride, maleic anhydride	Lys	alteration of crevice structure	144
Cytochrome *c*	horse heart	ethylthioltrifluoroacetate followed by hydrolysis	Lys	cytochrome oxidase binding site involves Lys around haeme crevice	145
Cytochrome *c*	horse heart	ethylthioltrifluoroacetate	Lys	kinetics of oxidation studied	146
Cytochrome *c*	horse heart	^{13}C-enriched *O*-methylisourea	Lys	^{13}C-n.m.r. study	147
Cytochrome *c*	horse heart	methyl 4-mercaptobutyrimidate *p*-azidophenacyl bromide–photoirradiation in presence of cytochrome *c* oxidase	Lys	1 : 1 complex of 2 proteins in high yield	148

Cytochrome *c*	horse heart	^{125}I–lactoperoxidase	Tyr-74	active monoiodo-protein	149
Cytochrome *c*	horse heart	trifluoromethylphenyl isocyanate	Lys	6 mono-substituted proteins isolated	150
Cytochrome b_5	rabbit liver	acetylimidazole	Tyr	2 Tyr in membrane-protein region	151
		N-bromosuccinimide	Trp	not shielded; 1 Trp in same region, buried	
Cytochrome P448	rat and rabbit	various thiol reagents	Cys	thiols necessary for hydroxylation of benzo[*a*]pyrene	152
Cytochrome *c*–chrochrome oxidase	horse, bovine	4-fluoro-3-nitro-phenylazide–photoirradiation	Lys	covalent complex if Lys-13, but not Lys-22, labelled	153
Cytochrome *c* oxidase	bovine heart	dithiobissuccinimidylpropionate, dimethyl-3,3′-dithiobispropionimidate	Lys	low levels of reagent gives II + V, III + V, V + VII, IV + VI; high levels gives 'unit complex'	154

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Cytochrome *c* reductase	porcine liver	DTNB	Cys	single thiol protected by NADP	155
Deoxyhaemoglobin	human	pyridoxal 5′-sulphate and phosphate, 2-nor-2-formyl-pyridoxal 5′-phosphate	α-NH_2	*X*-ray diffraction study	156
Dihydrofolate reductase	*E. coli*	various thiol reagents	Cys	Cys-85 protected by substrate	157
Dihydrofolate reductase	*Lactobacillus casei*	(i) *N*-bromosuccinimide, (ii) dimethyl(2-hydroxy-5-nitrobenzyl)-sulphonium bromide	Trp	critical Trp-21 oxidized, Trp-5 and 129 alkylated	158
DNA photolyase	yeast	*N*-bromosuccinimide, diketene, potassium ferricyanide	Cys	diketene probably reacts with Lys and Arg	159
DNA polymerase	calf thymus	*N*-ethylmaleimide	Cys	enzymes A and C have very different reactivities	160
Dopa decarboxylase	porcine kidney	sodium borohydride	Lys	phosphopyridoxyl peptide sequenced	161
Elastase	porcine	various peptide chloromethyl-ketones	Ser	can construct selective inhibitors	162
Elongation factor G	*E. coli*	(2-nitro,4-azidobenzoyl)hydrazone of periodate oxidized GTP, γ-(4-azidobenzyl)amide of GTP–photoirradiation		can form specific complex with GTP without ribosomes	163
Elongation factor G	*E. coli*	GTP or GDP–photoirradiation	Cys	nucleotide linked to binding site	164
Elongation factor G	*E. coli*	butane-2,3-dione	Arg	1 essential Arg	165
Estrogen receptor	rat uterus	various photoreactive estrogen analogues–photoirradiation		first example of photoaffinity labelling of estrogen receptor	166
Ethanolamine ammonialyase	*Clostridium*	DTNB	Cys	further evidence for thiols being involved in adenosylcobalamin-dependent rearrangements	167
Factor H_1	*E. coli*	dimethylsuberimidate	Lys	dimeric species mainly	168
		4-chloro-7-nitrobenzofurazan	Cys	under oxidizing conditions Lys not Cys labelled	
Factor VIII	human	dimethylsuberimidate, ethyl acetimidate	Lys	most reactive Lys not essential	169
Fatty acid synthetase	yeast	iodoacetamide	Cys	labelled peptide sequenced	170
Fatty acid synthetase	yeast	iodo[^{14}C]acetamide	Cys	rapid high resolution gel system for separating subunits	171

Fatty acid synthetase	yeast	[^{14}C]palmityl-CoA	Ser	labelled peptides isolated	172
Ferredoxin	*C. pasteurianum*	glass beads		immobilized enzyme stabilized	173
Ferredoxin	spinach	trinitrobenzene sulphonic acid	Lys	loss of NADP photoreduction activity	174
Ferredoxin : NADP oxido–reductase	spinach	*N*-ethylmaleimide	Cys	inhibits high activity form more than low	175
Ferroxidase II	human serum	iodoacetic acid	Cys	no dissociation into subunits	176
Fibrinogen	human	iodoacetate, iodoacetamide	His	disulphide bonds are stable	177
Flavocytochrome b_2	yeast	bromopyruvate		proposed as affinity label	178
Formate–nitrate reductase	*E. coli*	*N*-ethylmaleimide, *p*-chloromercuribenzoate	Cys	inhibitors of reconstitution	179

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Formlyglycinamide ribonucleotide amidotransferase	chicken liver	(i) iodoacetate–glutamine, (ii) iodo-[^{14}C]acetate at 4 °C	Cys	method for specifically labelling active site Cys	180
Fructose bisphosphate aldolase	bovine liver	methyl[1-^{14}C]acetimidate	Lys	Lys-27 unreactive	181
Fructose bisphosphate aldolase	bovine liver	[*U*-^{14}C]fructose bisphosphate–sodium borohydride	Lys	absolute configuration of carbinolamine determined	182
Fructose bisphosphate aldolase	rabbit muscle	ethyl phosphoglycollate–sodium borohydride	Lys	imidate ester intermediate proposed	183
β-Fructosidase	yeast	[^{3}H]conduritol-B-epoxide	carboxyl	only L-form reacts	184
Fucosyltransferase	human	*N*-ethylmaleimide	Cys	selectivity inhibits one enzyme	185
Fumarase	porcine	3,4-bis(bromomethylbenzoate)–$^{3}H_2O$, pH 8	His	^{3}H incorporated at C-2	186
Galactose oxidase	*Dactylium dendroides*	iodo[^{14}C]acetamide	His	essential His	187
		N-bromosuccinimide	Trp	inhibits His alkylation	
Galactose oxidase	*D. dendroides*	*N*-bromosuccinimide	Trp	reduces enzymic activity to 2%	188
Galactose-1-phosphate uridyltransferase	*E. coli*	diethylpyrocarbonate	His	inactivates	189
		pyridoxal 5′-phosphate, 5-nitrosalicylaldehyde	Lys	activity not affected	
		p-hydroxymercuribenzoate	Cys	inactivates	
Galactose receptor	*Salmonella typhimurium*	5-iodoacetamidofluorescein	Met	distant reporter group	190
β-Galactosidase	*E. coli*	*N*-bromoacetyl-β-D-glucosylamine	Met	reactivated with β-mercaptoethanol	191
Globulin (corticosteroid binding)	human	6β-bromo[^{3}H]progesterone	Cys	affinity label	192
Glucagon	porcine	^{125}I–lactoperoxidase	Tyr	preparation of pure monoiodo-derivative	193
Glucagon	porcine	methyl iodide	Met	reversible; method for isotopic labelling	194
4-α-Glucanotransferase amylo-1,6-glucosidase	dogfish	DTNB	Cys	8/32 react rapidly in native protein	195
Glucose dehydrogenase		nylon–glutaraldehyde		useful for glucose determination in body fluids	196

Glucose oxidase	*Aspergillus niger*	periodate	Arg	10 000 peptide split off	197
Glucose-6-phosphate dehydrogenase	*Leuconostoc mesenteroides*	butane-2,3-dione	Arg	2 essential Arg modified	198
Glucosephosphate isomerase	human	*N*-bromo-[^{14}C]acetylethanolamine phosphate	His	affinity label	199
Glutamate dehydrogenase	bovine liver	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Cys	protected by NADH, α-ketoglutarate	200
Glutamate dehydrogenase	bovine liver	[^{14}C]dimethyladipimidate	Lys	labelled peptides sequenced	201
Glutamate dehydrogenase	bovine liver	nitroxide derivative of *p*-chloromercuribenzoate	Cys	0.6 mole per mole of protomer	202

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Glutamate dehydrogenase	bovine liver	pyridoxal 5′-phosphate–sodium borohydride	Lys-126, 333	Lys-333 modification deploymerizes enzyme into hexamers no	203
Glutamate dehydrogenase	bovine liver	various thiol reagents iodo[^{14}C]-acetic acid	Cys Cys, Met	no inactivation inactivates, 50% of label in Met-169	204
Glutamate dehydrogenase	*Neurospora crassa*	pyridoxal 5′-phosphate *N*-ethyl[^{14}C]maleimide	Lys Cys	labelled Lys identified	205
Glutaminase	rat	L-2-amino-4-oxo-5-chloro-pentanoic acid, 6-diazo-5-oxo-L-norleucine		inhibition protected by glutamine	206
Glutamine synthetase	*Bacillus subtilis*	iodoacetic acid, DTNB	Cys	one S—S, 2 thiols	207
γ-Glutamylcysteine synthetase	ovine brain	cystamine	Cys	inhibits	208
γ-Glutamylcysteine synthetase	rat liver	L-2-amino-4-oxo-5-chloro-[^{14}C]-penanoate		reacts at glutamate binding site	209
γ-Glutamylcysteine synthetase	rat liver	dimethylsuberimidate	Lys	gives 100 000 cross-linked species	210
γ-Glutamyl transpeptidase	human kidney	6-diazo-5-oxo-L-[^{14}C]norleucine, L-azaserine		γ-glutamyl binding site is on light subunit	211
γ-Glutamyl transpeptidase	rat kidney	6-diazo-5-oxo-L-[^{14}C]norleucine		mechanism of reaction proposed	212
γ-Glutamyl transpeptidase	tumour AH-130	6-diazo-5-oxo-L-norleucine		study of cellular uptake of amino-acids and glutathione	213
Glutathione peroxidase	ovine erythrocytes	cyanide		inactivates, releases Se	214
Glyceraldehyde 3-phosphate dehydrogenase	rabbit muscle	various chloromethylakanediones	Cys	reagents for cross-linking thio groups	215
Glyceraldehyde 3-phosphate dehydrogenase	rabbit muscle	methyl[^{14}C]acetimidate	Lys	Lys-306 unavailable	216
Glyceraldehyde 3-phosphate dehydrogenase	*Scenedesmus obliquus*	dithiothreitol	Cystine	converts to NADPH specific form	217

Glyceraldehyde 3-phosphate dehydrogenase	sturgeon muscle	bromotrifluoroacetone	Cys	essential thiol plays role in subunit communication	218
Glycine reductase	*Clostridium sticklandii*	potassium borohydride–DTNB	Cys, Se-Cys	3 titratable groups	219
Glycogen	rabbit liver	iodoacetamide	Cys	tendency to aggregate	220
Glycogen synthetase	rabbit muscle	[γ-^{32}P]ATP	Ser	labelled peptide sequenced	221
Glycoprotein	monkey	iodoacetic acid	Cys	2 identical subunits	222
GMP synthetase	*E. coli*	L-2-amino-4-oxo-5-chloropentanoic acid, 6-diazo-5-oxonorleucine, iodo[^{14}C]acetamide	Cys	84% of radioactivity in one tryptic peptide	223
Growth hormone	horse	trinitrobenzene sulphonic acid	Lys	inactivates	224
Growth hormone	human	iodoacetamide	Cys	S—S dimer isolated	225

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Guanylate cyclase	rat liver	various thiol reagents	Cys	inhibits	226
Haeme oxygenase	rat liver	various thiol reagents	Cys	essential Cys	227
Haemoglobin	cat, rat	diethylpyrocarbonate	His	loss of triethyltin-binding site	228
Haemoglobin	canine	dilithium carbamyl phosphate	α-NH_2	clinical study	229
Haemoglobin	human	acetyl-3,5-dibromosalicylic acid	Lys	anti-sickling agent	230
Haemoglobin	human	*N*-bromosuccinimide	Trp	2 Trp become inaccessible on complex formation	231
Haemoglobin	human	5′-deoxypyridoxal	α-NH_2	anti-sickling agent	232
Haemoglobin	human	*N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide	Cys	ligand induced conformational changes occur in isolated subunits	233
Haemoglobin	human	preincubated carbamyl phosphate	α-NH_2	cyanate is active species	234
Haemoglobin	human	31 different carbonyl compounds	Lys	effect on sickling evaluated	235
Haemoglobin	human	cystamine, cystine-dimethyl ester	Cys β-93	resistance to haemolysis not impaired	236
Haemoglobin	human	hydrazine–trypsin	Arg-141	α-carboxyl converted to hydrazide	237
Haemoglobin	human	*N*-(2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide	Cys-93	oxygen binding studied	238
Haemoglobin	human	[^{3}H]- and [^{14}C]acetic anhydride	Lys	competitive labelling	239
Haemoglobin	human	iodoacetamide, *N*-ethylmaleimide	Cys	blocks Cu^{II} oxidation	240
Haemoglobin	human	1-fluoro-2,4-dinitrobenzene, dimethyladipimidate	Lys	circular dichroism studies	241
Haemoglobin	human	*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide and -maleimide	Cys	labelled molecules form radical pairs	242
Haemoglobin	rat	diazenedicarboxylic acid bis-*NN*′-dimethylamide, diazenedicarboxylic acid bis-*NN*′-ethylpiperazinide	Cys	mapping exposed thiols	243
Haemoglobin	trout	CNBr-activated Sepharose or Sephadex		bound enzyme has altered properties	244
Haemopexin	human	tetranitromethane, *N*-acetylimidazole	Tyr	react only when denatured; low reactivity of Trp, Tyr indicates	245

		N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide	Trp	compact core	
Hexokinase	yeast	cyanuryl fluoride, tetranitromethane, *N*-acetylimidazole	Tyr	2 Tyr modified by first 2 reagents, 1 Tyr by last	246
Hexokinase	yeast	substituted carbodi-imide–nitrotyrosyl ethyl ester	Glu	essential Glu, labelled peptide isolated	247
Hexokinase	yeast	1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide	Tyr	single Tyr reacts; inhibits	248
Hexokinase	yeast	various alkylating reagents	Cys	single essential Cys	249
Hexosaminidases A and B	human kidney	iodoacetic acid	Cys	one major subunit	250

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Histamine-*N*-methyl transferase	guinea-pig brain	2′-*O*-[(*R*)-formyl(adenin-9-yl) methyl]3′-*S*-homocysteinyl-3′-deoxy-(*R*)-glyceraldehyde		affinity label	251
Histidinol dehydrogenase	*Salmonella typhimurium, E. coli*	*N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide	Cys	amino-acid sequence around modified Cys determined	252
Histones	*Arbacia lixula*	various spin labels	Cys	study of histone–histone interactions	253
Histones	calf thymus	[^{14}C]- and [^{3}H]acetic anhydride	Lys	competitive labelling	254
Histones	calf thymus	ethylacetimidate	Lys	suitable for mapping	255
Histones	calf thymus	fluorescamine	Lys	used in assays for protamine-displaced histones	256
Histones	calf thymus	*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide	Cys	some non-specific labelling of Lys	257
Histones	calf thymus	glycyl azide agarose		study of complexes with non-histone proteins	258
Histone H3	Chinese hamster	iodoacetamide, hydrogen peroxide	Cys	study of S—S links	259
Histone H1	fruit fly	iodoacetate	Cys	Cys confers aggregating ability	260
Histones	rat liver	acetic anhydride	Lys	affects nuclease sensitivity	261
HLA antigens	human	iodo[^{3}H]acetate, dithiobis-(succinimidyl propionate)	Cys	non S—S bonded form contains one heavy and one light chain	262
Homoserine dehydrogenase	*Rhodospirillum rubrum*	*p*-mercuri[^{14}C]benzoate	Cys	spontaneously reactivates	263
4-Hydroxyiso-phthalate hydroxylase	*Pseudomonas putida*	various thiol reagents	Cys	inhibit	264
Hydroxylamine oxido–reductase	*Nitrosomonas europaea*	hydrogen peroxide	His, Tyr?	irreversible inactivation	265
4-Hydroxyphenyl-pyruvate dioxygenase	human liver	various thiol reagents	Cys	5 thiols and one S—S bridge per 87 000 dalton	266
3(17)β-Hydroxysteroid dehydrogenase	*Pseudomonas testosteroni*	dimethylsuberimidate	Lys	monomer, dimer, trimer, and tetramer found	267
Hypocalcemic protein	bovine parotid	various reagents		Trp, Tyr, amino S—S, His play a role in hypocalcemic activity?	268

Hypoxanthine–guanine phosphoribosyltransferase	human	iodoacetic acid maleic anhydride	Cys Lys	subunit mol. wt. 26 000	269
Hypoxanthine–guanine phosphoribosyltransferase	rat liver	DTNB, *p*-chloromercuribenzoate 2,4,6-trinitrobenzenesulphonic acid	Cys Lys	protected by substrate protected by substrate and product	270
Immunoglobulin G	human	iodo[^{14}C]acetamide	Cys	complement-activating capacity enhanced	271
Immunoglobulins	mouse	carbodi-imide–various amines formaldehyde–sodium borohydride	carboxyl Lys	study of circulatory survival of modified antibodies	272

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Immunoglobulins	rabbit	^{125}I	Tyr	tryptic mapping of iodinated light chains is rapid assay for relatedness	273
Initiation factor IF3	*E. coli*	[^{14}C]formaldehyde–sodium borohydride followed by photoirradiation	Lys	cross-linking between IF3 and both 30S proteins and 16S RNA	274
Initiation factor IF3	*E. coli*	1. (i) [^{14}C]formaldehyde–sodium borotriti-ide, (ii) [^{3}H]*N*-ethyl-maleimide; 2. photoirradiation or formaldehyde	(*i*) Lys (*ii*) Cys	protein–RNA cross-linking	275
Insulin	bovine pancreas	formaldehyde–sodium borohydride	Lys	n.m.r. studies	276
Isocitrate dehydrogenase	bovine heart	bromoacetate, diethyl pyro-carbonate, photo-oxidation	His	essential His	277
Isocitrate dehydrogenase	porcine heart	[^{14}C]methylmethanethiol-sulphonate	Cys	essential Cys	278
Isocitrate dehydrogenase	porcine heart	butane-2,3-dione	Arg	10/24 Arg modified	279
Isocitrate dehydrogenase	porcine heart	substituted carbodi-imide–glycinamide or [^{14}C]glycine ethyl ester	Asp, Glu	1 Asp and 1 Glu essential	280
Isocitrate dehydrogenase	porcine heart	2,4-[^{14}C]pentanedione	Lys	1 to 3 Lys in active site	281
Isocitrate lyase	flax	sodium borohydride–DTNB	Cys	3 S—S, 8 SH	282
L-Isoleucyl tRNA synthetase	*E. coli*	L-isoleucylbromomethylketone	Cys	reactive Cys not essential	283
L-Isoleucyl tRNA synthetase	*E. coli*	[α-^{32}P]ATP-Ile-tRNA^{Ile} synthetase complex–photoirradiation		labelled peptide sequenced	284
L-Isoleucyl tRNA synthetase	*E. coli*	pyridoxal 5′-phosphate	Lys	protected by tRNA	285
Δ^5-3-Ketosteroid isomerase	*Pseudomonas testosteroni*	3-oxo-4-estren-17β-yl acetate–photoirradiation	Asp-38	Asp converted to Ala	286
Kinase (AMP dependent)	bovine heart	DTNB	Cys	3 reactive Cys; most reactive not essential	287

Kinase (AMP dependent)	porcine brain	[γ-^{32}P]ATP [^{14}C]2′,3′-dialdehyde derivative of ATP–sodium borohydride	His Lys	tryptic peptide isolated model of ATP binding area proposed	288
Kinase (AMP dependent)	rabbit muscle	various thiol reagents	Cys	inhibit protein	289
Kinase	pigeon liver	activated nylon–glutaraldehyde		activity and stability of different preparations compared	290
Kinase inhibitor	rabbit muscle	cyclohexane-1,2-dione	Arg	4/6 Arg modified; inactivates	291
Lac repressor	*E. coli*	*N*-bromosuccinimide	Trp-209, Met Cys	protected by inducer	292
Lac repressor	*E. coli*	2-chloromercuri-4-nitrophenol, 2-bromoacetamido-4-nitrophenol	Cys	first reagent modifies Cys-107, -140, and -268; second reagent modifies Cys-107 and -140	293

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Lac repressor	*E. coli*	tetranitromethane	Tyr	Tyr-7,-17 most reactive	294
		N-acetylimidazole	Lys, Tyr	operator, but not inducer, binding activity lost	
Lac repressor	*E. coli*	photoirradiation	Trp	1 Trp per protomer modified	295
α-Lactalbumin	human	2-hydroxy-5-nitrobenzyl bromide	Trp	3 accessible Trp	296
Lactate dehydrogenase	bovine heart	N^6-[(6-aminohexyl)carbamoyl-methyl]-NAD–glutaraldehyde		number of bound cofactor molecules varied from 0 to 8	297
Lactate dehydrogenases	lactic acid bacteria	*p*-hydroxymercuribenzoate, iodoacetamide	His, Lys	no essential thiol	298
Lactate dehydrogenase	porcine heart	methyl acetimidate	Lys	17/24 Lys modified	299
Lactate dehydrogenase	porcine heart	methyl-ε-(*N*-2,4-dinitrophenyl)-aminocaproimidate	Lys	no essential Lys with extraordinary activity modified	300
Lactate dehydrogenase	porcine heart	methylmethanethiosulphonate	Cys	residual catalytic capacity	301
L-Lactate	*Mycobacterium smegmatis*	dimethylsuberimidate	Lys	octameric structure	302
L-Lactate oxidase	*Mycobacterium smegmatis*	diethyl[^{14}C]pyrocarbonate	His	essential His	303
Lipase	porcine pancreas	citraconic anhydride	Lys	necessary Lys for reactivating effect of colipase	304
		cyclohexane-1,2-dione	Arg	no effect on activity	
Lipoamide dehydrogenase	porcine heart	*N*-acetylhomocysteine thiolactone	Lys	introduces 5 to 6 extra thiol groups for immobilization	305
Lipoamide dehydrogenase	porcine heart	activated glass beads		co-immobilization of NAD^+	306
Lipoamide dehydrogenase	porcine heart	iodoacetamide	Cys	pH profile of alkylation is analogous with papain	307
Lipoamide dehydrogenase	porcine heart	DTNB	Cys	study of immobilized enzyme	308
Lipoproteins (low density)	human, canine	cyclohexane-1,2-dione	Arg	modifies half of Arg residues	309
Lipoproteins	human	(i) ^{125}I (ii) 1,5-difluoro-2,4-dinitrobenzene	(i) Tyr (ii) Lys	exchange of proteins between subclasses inhibited by cross-linking	310
Lipotropin	porcine pituitary	citraconic anhydride	Lys	opiate binding studies	311

Lutropin	ovine	various acylating reagents	Lys	receptor binding study	312
Lutropin	ovine	tetranitromethane–sodium hydrosulphite	Tyr	circular dichroism studies	313
Lysate	*Limulus polyphemus*	di-isopropylfluorophosphate, phenylmethylsulphonylfluoride	Ser	essential Ser	314
		iodoacetic acid	Cys	single chain	
Lysosyme	chicken egg white	4-chloro-7-nitrobenz-2-oxa-1,3-diazole	Tyr-23	pH-dependent absorption spectrum	315
Lysosyme	chicken egg white	iodo[^{14}C]acetic acid	Cys	random labelling of single Cys	316
Lysosyme	chicken egg white	potassium tri-iodide	Trp-108	n.m.r. study of lanthanide binding	317
Lysosyme	chicken egg white	glutaraldehyde	Lys	2 cross-links identified	318

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Table 1 *(cont.)*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Malate dehydrogenase	porcine heart	*N*-ethylmaleimide	Cys	NADH binding altered	319
Malic enzyme	pigeon liver	bromo[^{14}C]pyruvate	Cys	affinity label	320
Malic enzyme	pigeon liver	ethoxyformic anhydride	His	His at nucleotide binding site	321
Membrane proteins	*Ascaris suum*	β-mercaptoethanol	Cystine	reduced membrane contains 17 polypeptides	322
Membrane proteins	bovine heart	[^{14}C]*N'*-(*N''*-n-nonyl-4-sulphamoylphenyl)maleimide	Cys	labelled proteins isolated	323
Membrane proteins	bovine heart	^{125}I–lactoperoxidase	Tyr	29 500 protein is carboxyatractylate binding protein	324
Membrane proteins	chick synaptosomes	^{125}I–lactoperoxidase	Tyr	11 proteins labelled	325
Membrane proteins	*E. coli*	1-azidopyrene–photoirradiation		de-energized cells increase labelling	326
Membrane proteins	*E. coli*	dithiobis(succinimidylpropionate), glutaraldehyde	Lys	specific protein–protein interactions	327
Membrane proteins	*E. coli*	*N*-ethylmaleimide	Cys	study of amino-acid transport	328
Membrane proteins	hamster	Cu^{2+}–*o*-phenanthroline	Cys	more S—S bonding in surface than cellular proteins	329
Membrane proteins	human erythrocyte	acetaldehyde		reacts with spectrin, actin	330
Membrane proteins	human erythrocyte	Cu^{2+}–*o*-phenanthroline	Cys	band 3 cross-linked	331
Membrane proteins	human erythrocyte	diamide, tetrathionate	Cys	intact cells cross-link spectrin only; ghosts band 3 as well	332
Membrane proteins	human erythrocyte	diazo[^{125}I]di-iodosulphanilic acid	Tyr, His, Lys	surface labelling, different from lactoperoxidase	333
Membrane proteins	human erythrocyte	ethyl(4-azidophenyl)-1,4-dithiobutyrimidate, 4,4'-dithiobisphenylazide–photoirradiation		cross-linking in milliseconds	334
Membrane proteins	human erythrocyte	fluorescamine	Lys	not specific for cell surface	335
Membrane proteins	human erythrocyte	^{125}I–lactoperoxidase	Tyr	decreased iodination after phospholipase *c* treatment	336
Membrane proteins	human fibroblast	*N*-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide	Lys	3 polypeptides labelled after incubation with factor XIIIa	337
Membrane proteins	human platelets	^{125}I–lactoperoxidase *N*-ethylmaleimide	Tyr Cys	evidence for S—S links being integral part of structure	338

Membrane proteins	mouse 3T3 cells	[^{125}I]epidermal growth factor derivatized with methyl 3-[(*p*-azidophenyl)dithio]propionimidate–photoirradiation		labelling epidermal growth factor receptor	339
Membrane proteins	rabbit erythrocyte	^{125}I–lactoperoxidase	Tyr	study of phenylhydrazine-treated rabbits	340
Membrane proteins	rabbit kidney	various thiol reagents	Cys	SH involved in organic acid transport?	341
Membrane proteins	rabbit muscle	glutaraldehyde, dimethylsuberimidate	Lys	aggregates >400 000 dalton	342
		Cu^{2+}–*o*-phenanthroline	Cys		

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Membrane proteins	rabbit muscle	cyclohepta-amylose–fluorescamine complex	Lys	calsequestrin internally located; ATPase is transmembrane protein	343
		diazotized [^{35}S]sulphanilic acid	His		
Membrane proteins	rabbit muscle	dimethyl-3,3′-dithiobispropionimidate	Lys	series of polypeptides up to 900 000 dalton formed	344
		Cu^{2+}–*o*-phenanthroline	Cys		
Membrane proteins	rabbit muscle	*N*-[^{14}C]ethylmaleimide, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide	Cys	2 proteins labelled	345
Membrane proteins	rat adipocytes	8-azido-[2-^{3}H]adenosine–photoirradiation		labelled proteins involved in adenosine transport?	346
Membrane proteins	rat adipocytes	*N*-(4-azido-2-nitrophenyl)-2-amino-2-deoxy-D-[6-^{3}H]glucose–photoirradiation		4 membrane components labelled	347
Membrane proteins	rat intestine	[^{125}I]iodonaphthylazide–photoirradiation		hydrophobic photoactive label	348
Membrane proteins	rat liver	*p*-diazobenzenesulphonate		effect on redox events studied	349
Membrane proteins	rat liver	[^{125}I]N^{ϵ}-4-azido-2-nitrophenyl-glucagon–photoirradiation		23 000—25 000 protein labelled	350
Membrane proteins	rat myocardium	various reagents		Ca^{2+}-binding study	351
Membrane proteins	rabbit muscle	*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide	Cys	3 Cys modified; no loss of activity	352
Membrane proteins	rat muscle	*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide	Cys	SH protected by ATP are less mobile than rest	353
Membrane proteins	*Salmonella typhimurium*	CNBr-activated dextran		labelling exposed proteins	354
Membrane proteins	various	pyridoxal[^{32}P]phosphate–sodium borohydride	Lys	preparation of labelled phosphate detailed	355
Methaemoglobin	horse	*O*-methylisourea	Lys	all Lys reactive	356
L-Methionine γ-lyase	*Pseudomonas ovalis*	various thiol reagents	Cys	80—100% inhibition	357
N-Methylglutamate dehydrogenase	*Pseudomonas aminovorans*	iodoacetamide, *p*-chloromercuribenzoate	Cys	inhibits enzyme	358

Methylglyoxal synthetase	*Proteus vulgaris*	3-bromo and -iodoacetol phosphates		affinity label	359
Mitochondrial subparticles	porcine heart	[^{14}C]mersalyl, [^{3}H]-*N*-ethyl-maleimide	Cys	labelled 30 000—32 000 dalton protein partially purified	360
Modulator of cyclic nucleotide phosphodiesterase	bovine heart	various reagents	His, Tyr, Met, Lys, carboxyl	activity lost when Met and carboxyl modified	361
M-protein	Sendai virus	dimethylsuberimidate	Lys	2 × 35 000 dimer	362
Myelin protein P2	rabbit	performic acid, iodo[^{14}C]acetamide	Cys	2 Cys present	363
Myeloperoxidase	canine	iodoacetate	Cys	2 subunits: 57 500 and 10 500	364
Myoglobin	horse heart	mesohaeme monosulphuric anhydride	Lys-45	oxygen binding studied	365

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Myosin	bovine heart	6,6′-dithiobis(inosinyl imidodiphosphate)–^{14}CN	Cys	critical Cys on light chains	366
Myosin	bovine heart	iodo[^{14}C]acetamide	Cys	CNBr peptide containing essential thiol characterized	367
Myosin	*Physarum polycephalum*	6,6′-dithiobis(inosinyl imidodiphosphate)	Cys	inhibited at very low rate	368
Myosin	rabbit heart	*N*-ethylamaleimide	Cys	depresses ATPase activity	369
Myosin	rabbit heart	iodo[^{14}C or ^{3}H]acetate	Cys	CNBr peptides mapped	370
Myosin heads	rabbit muscle	iodo[^{14}C]acetate, [^{14}C]*N*-ethylmaleimide	Cys	specifically labelled essential thiols	371
Myosin	rabbit muscle	Co^{III} complexes		labels active site	372
Myosin	rabbit muscle	dimethyl-3,3′-dithiobispropionimidate, methyl 4-mercaptobutyrimidate	Lys	myosin heads in close contact on filament surface	373
Myosin	rabbit muscle	DTNB	Cys	study of Mn^{II} binding	374
Myosin	rabbit muscle	*N*-ethylmaleimide, fluorodinitrobenzene	Cys	investigation of SH involvement in hydrolysis	375
Myosin	rabbit muscle	[^{14}C]fluorodinitrobenzene, [^{14}C]-*N*-ethylamaleimide	Cys	reactivity of essential thiols in presence of Mg and MgADP	376
Myosin	rabbit muscle	2′,3′-isopropylidene adenosine 5′-dibromo-hydrogen succinate		improved synthesis	377
Myosin	rabbit muscle	β-naphthoquinone-4-sulphonate	Lys	Cys reacts unless protected	378
Myosin	rabbit muscle	*p*-*NN*′-phenylenedimaleimide, 2,4-dinitro-1,5-difluorobenzene, 4,4′-difluoro-3,3′dinitrodiphenyl sulphone	Cys	cross-linking reagents used to probe conformational changes on binding of nucleotides	379
Myosin	rabbit muscle	trinitrobenzenesulphonate	Lys	blue shift in absorption spectrum when ATP added	380
Myotoxin a	*Crotalus viridis*	iodoacetamide	Cys	essential S–S	381
Neocarzinostatin	*Streptomyces carzinostaticus*	^{3}H- or ^{125}I-labelled 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester ammonium chloride–1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide	Ala-1, Lys-20	no inhibition	382

Nerve growth factor	mouse	succinic anhydride	Lys	dissociation studies	383
Neuraminidase	*Streptomyces griseus*	various reagents		essential Arg	384
Nitrate reductase	*Neurospora crassa*	diazotized 3-aminopyridine adenine dinucleotide	Cys	binds at NADPH site	385
Nucleoside triphosphatase	avian myeloblastosis virus	azido-ATP–photoirradiation		inhibition prevented by ATP	386
Ornithine transcarbamylase	bovine	pyridoxal 5′-phosphate	Lys	essential Lys in unique environment	387
Ovoinhibitor	chicken	*N*-chlorosuccinimide	Met	selective effects on protease inhibition	388
		2-nitrophenylsulphenylchloride	Trp	no effect on protease inhibition	
2-Oxo-4-hydroxyglutarate aldolase	bovine liver	DTNB, *p*-mercuribenzoate	Cys	inactivation; Cys not essential	389

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Papain	papaya latex	*N*-acyl-aminoacetaldehydes	Cys	n.m.r. evidence for hemithioacetal formation	390
Papain	papaya latex	benzamidoacetaldehyde	Cys	essential Cys; hemithioacetal	391
Papain	papaya latex	8α-bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin, 7α-bromoacetyl-10-methylisoalloxazine	Cys-25	semi-synthetic enzyme synthesis	392
Papain	papaya latex	diazomethyl ketones of Z-Phe and Z-Phe-Phe	Cys	inhibitors	393
Papain	papaya latex	*meso*-1,4-bis(acetatomercuri)-2,3-diethoxybutane–mercuric chloride	Cys	dimerizes protein	394
Papain	papaya latex	Ti^{IV} oxide and activated cellulose		active water-insoluble derivative	395
Papain, thiolsubtilisin		iodoacetic acid, iodoacetamide 3-iodopropionate	Cys	reactivity of 2 enzymes compared	396
PZ-peptidase	chick embryo	*N*-ethylmaleimide, *p*-hydroxymercuribenzoate	Cys	inhibit enzyme	397
Peroxidase	horseradish	photoirradiation–sensitizer		several amino-acids including His, Tyr destroyed with eosin Y as sensitizer	398
Phenol-sulphotransferase	rat liver	butane-2,3-dione, phenylglyoxal	Arg	essential Arg	399
Phosphate translocator	spinach	pyridoxal 5′-phosphate–sodium borotriti-ide	Lys	inhibits phosphate transport	400
Phosphate transporter	pea chloroplasts	*p*-chloromercuriphenyl sulphonic acid	Cys	inhibits phosphate transporter but not adenine nucleotide transporter	401
Phosphodiesterase II	rat intestine	iodoacetic acid	Cys	essential Cys	402
Phosphoenolpyruvate carboxylase	*E. coli*	butane-2,3-dione	Arg	essential Arg	403
Phosphofructokinase	rabbit muscle	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Cys	1 Cys per subunit	404
Phosphofructokinase	rabbit muscle	iodo[^{14}C]acetate followed by bromo[^{3}H]acetate	Cys	single highly reactive thiol identified	405

Phosphofructokinase	rabbit muscle	pyridoxal 5′-phosphate–sodium borohydride	Lys	citrate site Lys specifically labelled	406
		7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, 4-dimethylamino-4′-maleimidostilbene	Cys	fluorescence energy transfer studies	
6-Phosphogluconate dehydrogenase	yeast	*N*-alkylmaleimides	Cys	inactivates; chain length of alkyl not important	407
Phosphoglycerate kinase	yeast	butane-2,3-dione, cyclohexane-1,2-dione	Arg	essential Arg	408
Phosphoglycerate kinase	yeast	tetranitromethane	Tyr	active site-labelled peptide sequenced	409
		[^{14}C]methoxyamine	carboxyl	modified active site peptide isolated	
Phospholipase A_2	*Bitis gabonica*	*p*-bromophenacyl bromide	His-45		410
		pyridoxal 5′-phosphate–sodium borohydride	Lys	Lys-11, 33, 58, 111 modified with nett one Lys per molecule	

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Phospholipase A_2	*Naja naja naja*	*p*-bromophen[^{14}C]acyl bromide	His	half site reactivity; evidence for asymmetric dimer	411
		ethoxyformic anhydride	Lys, Tyr, His		
		N-bromosuccinimide	Trp		
Phospholipase C	*Bacillus cereus*	diethylpyrocarbonate	His	1 essential His; 4 His bind Zn	412
Phospholipase C	*Bacillus cereus*	ethyl acetimidate, 2,4,6-trinitro benzene sulphonic acid, pyridoxal 5′-phosphate–sodium borohydride	Lys	2 essential Lys	413
Phospholipase C	*Bacillus cereus*	*N*-ethyl-5-phenylisoxazolium-3′-sulphonate, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodi-imide–DNP-ethylene diamine or glycine ethyl ester	carboxyl	single essential carboxyl	414
Phosphorylase b	rabbit muscle	tetranitromethane	Tyr	2/34 Tyr modified	415
Phosphotransferase	*E. coli*	*N*-ethylmaleimide	Cys	essential Cys unmasked by phosphate-accepting sugar	416
Phycobili proteins	*Cyanidium caldarium*	iodo[^{14}C]acetamide	Cys	Cys involved in some bilin–apoprotein linkages	417
Polynucleotide phosphorylase	*E. coli*	DTNB	Cys	1/3 Cys react in native protein	418
Porphobilinogen oxygenase	wheat germ, rat liver	succinic anhydride, acetic anhydride	Lys	subunit association prevented	419
Porphobilinogen synthase	bovine liver	iodo[^{14}C]acetate, iodo[^{14}C]-acetamide	Cys	each reagent alkylates a different essential Cys	420
Porphobilinogen synthase	bovine liver	CNBr-activated Sepharose		immobilized enzyme for hybridization studies	421
Postproline protease	lamb kidney	various reagents		evidence for Asp-His-Ser triad	422
Prealbumin	human serum	*N*-bromo[2-^{14}C]-acetyl-L-thyroxine	Gly-1, Lys-9, Lys-15	labelled in ratio 29 : 63 : 9	423
Prolactin	ovine	tetranitromethane, potassium tri-iodide	Tyr	Tyr-44 most reactive	424

Prostaglandin synthetase	sheep vesicular	[*acetyl*-^{3}H]aspirin	amino	labelled protein purified	425
Protease	*Bacillus cereus*	*N*-hydroxysuccinimide esters of amino-acids and peptides	Tyr	increase in proteolytic activity	426
Protease VI	*Mucor miehei*	diazoacetylnorleucine methyl ester–Cu^{2+}, 1,2-epoxy-3-(*p*-nitrophenoxy)propane	Asp	kinetics study	427
Proteinase B inhibitors	yeast	tetranitromethane–sodium dithionite	Tyr-41	nitrated inhibitor more suitable for chromatography	428
Proteoglycan 'link proteins'	bovine	CNBr	Met	one prominent peptide obtained	429
Pyridine dinucleotide transhydrogenase	bovine heart	DTNB	Cys	50% reactivation with cyanide	430
Pyrophosphate phosphohydrolase	yeast	hydroxylamine, *N*- and *O*-methylhydroxylamine, glycine methyl ester	Asp	essential activated Asp	431

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Pyruvate carboxylase	chicken liver	trinitrobenzenesulphonate	Lys	active Lys not involved in activator binding	432
Pyruvate dehydrogenase	*Azobacter vinelandii*, *E. coli*	*N*-ethyl[2,3-^{14}C[maleimide	Cys	determination of chain stoicheiometries	433
Pyruvate dehydrogenase	*E. coli*	4-dimethylamino-4′-maleimidostilbene, *N*-[*p*-(2-benzoxazolyl)-phenyl]maleimide, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide	Cys	one thiol per chain modified	434
Pyruvate kinase	rabbit muscle	5′-*p*-[^{14}C]fluorosulphonylbenzoyladenosine	Lys, Tyr	affinity label for adenine nucleotide sites in proteins	435
Rhodanese	bovine liver	*N*-bromosuccinimide	Cys	oxidation to S—S	436
Rhodanese	bovine liver	DTNB	Cys	additional thiol reacts in sulphur-free enzyme	437
Rhodopsin	bovine retina	five spin labelled maleimides	Cys	2 thiols per molecule labelled	438
Rhodopsin	bovine retina	11-*cis*-[15-^{3}H]retinal–sodium borohydride	Lys	labelled active site	439
Ribonuclease A	bovine pancreas	*N*-(4-azido-2-nitrophenyl)-2-aminoethyl sulphonate–photoirradiation		non-specific surface labelling	440
Ribonuclease A	bovine pancreas	dimethylsuberimidate–spermine	Lys	most active product has 8 spermines per molecule	441
Ribonuclease A	bovine pancreas	β-mercaptoethanol	cystine	mild conditions; kinetics	442
Ribonuclease A	bovine pancreas	tetranitromethane	Tyr Arg	Tyr-76, 115 and Arg-39, 85 do not belong to antigenically relevant regions	443
		cyclohexane-1,2-dione			
Ribonuclease inhibitor	human placenta	iodoacetic acid	Cys	8 free thiols	444
Ribonucleoside triphosphate reductase	*Lactobacillus leichmannii*	[^{14}C]-*N*-ethylmaleimide	Cys	alkylation modulated by effectors	445
Ribulosebisphosphate carboxylase	*Rhodospirillum rubrum*	3-bromo-1,4-dihydroxy-2-butanone, 1,4-bisphosphate	Lys	essential Lys	446

Ribulosebisphosphate carboxylase–oxygenase	spinach	*N*-bromoacetylethanolamine phosphate		Mg^{2+} changes specificity from Cys to Lys	447
Ribulosebisphosphate carboxylase–oxygenase	spinach	pyridoxal 5′-phosphate–sodium borohydride	Lys	essential Lys	448
Ribosomal protein L12	*E. coli*	[^{14}C]- and [^{3}H]acetic anhydride	Lys	competitive labelling	449
Ribosomes 50S	*E. coli*	fluorescein isothiocyanate	Lys	modified proteins used to reassemble ribosome	450
Ribosomes 30S	*E. coli*	maleic anhydride	Lys, Cys	prevents polyuridylic acid binding	451
Ribosomes 30S	*E. coli*	2-methoxy-5-nitrotropone	Lys	identification of proteins in mRNA binding site	452
Ribosomes	*E. coli*	formaldehyde	Lys	specific cross-linking	453
Ribosomes	*E. coli*	photoirradiation–methylene blue–air		inactivation *via* singlet oxygen	454

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ribosomes	*E. coli*	photoirradiation		sequences of RNA linked to S4 and S20 determined	455
Ribosomes	*E. coli*	poly(4-thiouridylic acid)–photo-irradiation		photoaffinity label for mRNA binding site	456
Ribosomes	rat liver	formaldehyde–sodium borotriti-ide	Lys	reactivity as a function of tempera-ture studied	457
Ribosomes	rat liver	^{125}I-lactoperoxidase	Tyr	free and membrane bound ribosomes studied	458
RNase	bovine pancreas	3-ethoxy-2-ketobutanal	Arg	Arg-39, 85 react in presence of ligand, Arg-10 as well in absence of ligand	459
RNase		[^{14}C]uridine 2′(3′),5′diphosphate–photoirradiation		specific cross-linking	460
tRNA ligases	yeast, *E. coli*	DTNB	Cys	sensitivity to reagent varies with different ligases	461
RNA polymerase	*E. coli*	*NN′*-bis(2-carboximidoethyl) tartaramide dimethyl ester		model for subunit arrangement proposed	462
RNA polymerase	*E. coli*	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	Cys	rapid reaction of unique Cys	463
RNA polymerase	*E. coli*	fluorescamine	Lys	inactivation at level of initiation	464
RNA polymerase	*E. coli*	methyl 4-mercaptobutyrimidate, dimethylsuberimidate	Lys	spatial orientation for subunits proposed	465
		NN′-(1,4-phenylene)bismaleimide	Cys		
Rubredoxin	*Pseudomonas oleovorans*	CNBr-activated Sepharose		first example of water-insoluble derivative of enzyme of this type	466
Salivary proteins	human	^{125}I–lactoperoxidase	Tyr	2 major iodine binding fractions	467
Serine protease	*Bacillus subtilis*	phenylmethylsulphonylfluoride	Ser	active site Ser	468
Somatotropin	human	iodo[^{14}C]acetamide	Cys	circular dichroism studies	369
Somatotropin	human	hydrogen peroxide	Met	modification of Met-170 affects physical parameters	470
Spectrin	human erythrocytes	inorganic phosphate	Ser, Thr	one labelled tryptic peptide	471
Spectrin	human erythrocytes	various dithiobisalkylimidate homologues	Lys	chain length of reagent used to discriminate dimer, multimer	472

Subtilisin BPN′		various peptide chloromethyl-ketones	His, Ser	substrate hydrolysis rates can be used to design inhibitors	473
Subtilisins		di-isopropylfluorophosphate, phenylmethylsulphonylfluoride	Ser	study of I^- quenching of fluorescence of inactivated enzymes	474
Sucrase–isomaltase	rabbit	various reagents		carboxyl groups at the 2 active centres	475
Sulphating enzyme	rat liver	*p*-chloromercuribenzoate	Cys	essential Cys	476
Superoxide dismutase	bovine erythrocytes	diethylpyrocarbonate	His	Zn protects all except His-19	477
Superoxide dismutase	*Photobacterium leiognathi*	various reagents		essential His, Trp; 1 Tyr and 1 Trp per subunit metal ligands?	478

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Thrombin	bovine	di-isopropylfluorophosphate	Ser	evidence for at least 2 types of binding sites on platelet surface	479
		1-chloro-3-tosylamido-7-amino-L-2-heptanone	His		
		N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide	Trp	essential Trp	
Thrombin	bovine	tetranitromethane	Tyr	abolishes high affinity binding	480
Thrombins	human	[^{3}H]-*m*-[*o*-(2-chloro-5-fluorosulphonylphenylureido)phenoxybutoxy]benzamidine, *o*-(2-chloro-5-fluorosulphonylphenylureido) methoxybenzene		second reagent lacks active site-directed benzamidine and only slowly inactivates enzyme	481
Thrombin	human	[^{3}H]di-isopropylfluorophosphate	Ser-254	serine protease	482
Thymidine kinase	*E. coli*	various 5′-derivatives of thymidine and analogues		assessment as affinity labels	483
Thymidylate synthetase	*Lactobacillus casei*	*N*-ethylmaleimide, iodoacetamide *p*-chloromercuribenzoate	Cys	1,4—1,8 modified thiols give inactivation	484
Thymidylate synthetase	*L. casei*	*cis*- and *trans*-$Pt(NH_3)_2Cl_2$	Cys	*trans* not *cis* reacts with essential Cys	485
Thymidylate synthetase	*L. casei*	various thiol reagents	Cys	1 Cys involved in deoxyuridylate binding	486
Thyroglobulin	rat	^{125}I and ^{131}I–thyroid peroxidase	Tyr	iodination takes place in rigid sequence	487
Thyroxine-binding globulin	human	iodo[^{14}C]acetic acid	Cys	single polypeptide chain	488
Toxin (fragment A)	*Corynebacterium diphtheriae*	2-hydroxy-5-nitrobenzyl bromide	Trp-153	essential Trp	489
Toxin	*C. diphtheriae*	tetranitromethane	Tyr	essential Tyr in fragment A	490
		formaldehyde–sodium borohydride	Lys	decrease in toxicity after 20 Lys modified	
Toxin	scorpion	^{125}I–lactoperoxidase	Tyr	mono- and di-iodo toxins active	491
Transcortin	human	bromo[^{14}C]acetyltestosterone and bromo[^{14}C]acetylated derivatives of monohydroxylated progesterone		1 Met and 1 His in active site	492

Transferrin	chicken egg white, human serum	ethoxyformic anhydride, photoirradiation–methylene blue	His	2 essential His in each iron-binding site	493
Transferrin	rabbit	(i) bismethylsuberimidate, 4-methyl-mercaptobutyrimidate	(i) Lys	2 transferrin per membrane protein; complexes identified	494
		(ii) Cu^{2+}–*o*-phenanthroline, all in presence of membranes	(ii) Cys		
Transferrin binding components	rabbit reticulocytes	*N*-[^{14}C]ethylmaleimide	Cys	disrupts association with transferrin	459
Tropoelastin	chicken	iodoacetate	Cys	evidence for proforms	496
Tropomyosin	rabbit muscle	[^{14}C]acetic anhydride	Lys	Lys-7 most accessible	497
		iodoacetamide	Met	Met-8,281 in overlap core	
Troponin C	rabbit muscle	bromotrifluoroacetone	Cys	^{19}F-n.m.r. study	498
Trypsin	bovine	formaldehyde–sodium borohydride	Lys	increased stability	499

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Table 1 (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Trypsin	bovine	phenylmethylsulphonylfluoride–alkali	Ser-195	Ser-195 plays different roles in stabilizing various enzyme-inhibitor complexes	500
Trypsin, chymotrypsin	bovine	activated poly(*N*-vinylpyrrolidone)		immobilized enzymes stabilized	501
Trypsin inhibitor	*Alocasia macrorhiza*	trinitrobenzene sulphonate	Lys	antichymotryptic activity lost faster than antitryptic	502
Trypsin inhibitor	bovine	tetranitromethane	Tyr-10, 21	lanthanides used as shift probes	503
Tryptophanase	*E. coli*	*N*-ethylmaleimide	Cys	reactivity used as conformation probe	504
Tryptophanase	*E. coli*	phenylglyoxal	Arg	essential Arg	505
Tryptophanyl tRNA synthetase	bovine pancreas	γ-(*p*-azidoanilide)ATP–photo-irradiation, *N*-chlorambucilyl-Trp-tRNA^{Trp}		sequential affinity labelling	506
Tubulin	chicken brain	dimethyl-3,3′-(tetramethylene-dioxy)dipropionimidate	Lys	cross-linked Lys better than Cys for gel electrophoresis	507
Tubulin	ovine brain	8-azidoguanosine[β,γ-^{32}P]-triphosphate–photoirradiation		incorporated in β-monomer	508
Tyrosyl tRNA synthetase	yeast	*p*-chloromercuribenzoate, *N*-ethylmaleimide	Cys	2 rapidly reacting thiols per subunit	509
Ubiquinone cytochrome *c* reductase	bovine heart	dithiobissuccinimidylpropionate, dimethyl-3,3′-dithiobispropion-imidate	Lys	major pairs I and II, II and VI, I and V, VI and VII	510
UDP-galactose-4-epimerase	bovine liver	*N*-ethylmaleimide, showdomycin	Cys	>3 thiol groups modified before inactivation	511
		2-hydroxy-5-nitrobenzyl bromide	Trp	partial inactivation	
Urea amidolyase	*Candida utilis*	DTNB, *N*-ethylmaleimide	Cys	inhibits enzyme	512
Urease	bovine	*p*-chloromercuribenzene sulphonate, *N*-ethylmaleimide	Cys	inhibit enzyme	513
Uridine diphosphoglucose pyrophosphorylase	*Dictyostelium discoideum*	tosyllysylchloromethylketone		specific activity increases with increased exposure to reagent	514
Uteroglobin	rabbit	iodoacetate	Cys	2 similar subunits	515

[500] N. Levilliers, M. Péron-Renner, and J. Pudles, *J. Mol. Biol.*, 1977, **111**, 279.
[501] B.-U. Von Specht and W. Brendel, *Biochim. Biophys. Acta*, 1977, **484**, 109.
[502] S. Sumathi and T. N. Pattabiraman, *Biochim. Biophys. Acta*, 1977, **485**, 167.
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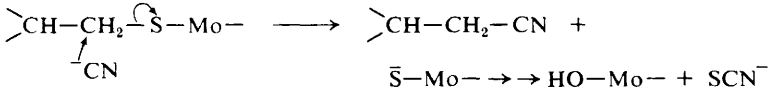
[504] O. Raibaud and M. E. Goldberg, *European J. Biochem.*, 1977, **73**, 591.
[505] M. N. Kazarinoff and E. E. Snell, *J. Biol. Chem.*, 1977, **252**, 7598.
[506] V. Z. Akhverdyan, L. L. Kisselev, D. G. Knorre, O. I. Lavrik, and G. A. Nevinsky, *J. Mol. Biol.*, 1977, **113**, 475; V. V. Zinoviev, N. G. Rubtsova, O. I. Lavrik, E. G. Malygin, V. Z. Akhverdyan, O. O. Favorova, and L. L. Kisselev, *F.E.B.S. Letters*, 1977, **82**, 130.
[507] R. F. Ludueña, E. M. Shooter, and L. Wilson, *J. Biol. Chem.*, 1977, **252**, 7006.
[508] R. L. Geahlen and B. E. Haley, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4375.
[509] H. G. Faulhammer and F. Cramer, *European J. Biochem.*, 1977, **75**, 561.
[510] R. J. Smith and R. A. Capaldi, *Biochemistry*, 1977, **16**, 2629.
[511] C. R. Geren, L. M. Geren, and K. E. Ebner, *J. Biol. Chem.*, 1977, **252**, 2089.
[512] A. Waheed and P. A. Castric, *J. Biol. Chem.*, 1977, **252**, 1628.
[513] S. Mahadevan, F. D. Sauer, and J. D. Erfle, *Biochem. J.*, 1977, **163**, 495.
[514] F. J. De Toma, K. E. Kindwall, and C. A. Reardon, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 350.
[515] A. Nieto, H. Ponstingl, and M. Beato, *Arch. Biochem. Biophys.*, 1977, **180**, 82.

Table 2 *Preliminary X-ray data on proteins*

Protein	*Source*	*Space group*	*Cell dimensions*		
			a/Å	*b*/Å	*c*/Å
L-Asparaginase	*E. coli* KY3598	$I222$ [a]	119.4	62.3	87.3
		$P22_12_1$ [a]	119.7	62.7	87.2
Plastocyanin	Poplar leaves	$P2_12_12_1$	29.6	46.9	57.6
Fibrinogen fragments[d]	Bovine blood	$P2_1$	134	97.3	174
γ-Crystallin fraction II	Calf lens	$P4_12_12$ or $P4_32_12$	57.8 (3)	—	98.7 (4)
γ-Crystallin fraction II	Calf lens	$P4_12_12$ or $P4_32_12$	58.0	—	98.6
γ-Crystallin fraction IIIb	Calf lens	$P2_12_12_1$	58.6	69.8	117.6
Actin/pancreatic DNase inhibitor	Rabbit/calf spleen	$P2_12_12$	42	230	77
Elongation factor EF-Tu in complex with GDP[j]	*E. coli* MRE600	$P2_12_12_1$	144	93	69
Trypsin inhibitor	Soybean (var. Tracy)	$P2_1$	25.919 (7)	43.23 (1)	19.905 (5)
Lactoferrin	Human milk	$P2_12_12_1$	155.5	97.3	55.5
C-Phycocyanin	*Agmenellum quadruplicatum* [n]	$P321$	184.5	184.5	60.5
cis-Benzene glycol dehydrogenase	*Pseudomonas putida*	$I422$	133.1	133.1	273.8
Antiviral protein	*Phytolacca americana*	$P1$	49.2	49.0	65.6
Ribulose bisphosphate carboxylase	Tobacco	$P42_12$	230	230	315
Leucine amino-peptidase	Bovine lens	$P6_322$	132	—	122
Haemagglutinin membrane glycoprotein	Hong Kong influenza virus	$P4_1$ or $P4_3$	164	—	178
Superoxide dismutase	*Bacillus stearothermophilus*	$P2_12_12$	111.8	74.6	51.3
		$P2_12_12$	111.2	72.8	51.3
		$P2_12_12_1$	112.4	78.0	50.5
Aspartate: 2-oxoglutarate aminotransferase	Pig heart	$P2_12_12_1$	124.7	130.9	55.7
L-Glutaminase-asparaginase	*Acinetobacter glutaminosificans*	$I222$	96.7	112.4	70.9
	Pseudomonas 7A	$P2_12_12_1$	118.0	131.2	85.1
Aconitase	Pig heart	$P2_12_12$	174.1 (9)	72.0 (4)	72.8 (4)
Basic copper-containing protein	Cucumber seedlings	$P2_12_12_1$	30.8	45.6	66.6

2 Re-investigation of Known Reagents and Reactions

Cyanide Treatment of Molybdenum Iron–Sulphur Flavin Hydroxylases.—It is now well known that cyanide removes sulphur from these enzymes in the form of free thiocyanate but the origin of the labile sulphur remains to be established. Coughlan [516] presents a well reasoned argument, compatible with the experimental results, which invokes nucleophilic attack by cyanide on a molybdenum-bonded active site cysteine (Scheme 1). This mechanism explains the production of one mole of dehydroalanine after hydrolysis of the cyanide-treated enzyme.



Scheme 1

Carbodi-imide.—This reagent is frequently used to catalyse reactions of acidic functions and is a convenient means of modifying carboxy groups in proteins. It has generally been assumed that by-products resulting from carbodi-imide catalysed polymerization of proteins only occur at elevated pH. However, in a study [517] of four typical proteins it has been shown that polymerization side-reactions can be a serious problem even in dilute acid.

***N*-Bromosuccinimide.**—Inactivation of the enzyme rhodanese by *N*-bromosuccinimide, previously attributed to the destruction of an essential tryptophan, has been shown [518] to be a result of oxidation of an essential thiol. The enzyme can be reactivated by incubation with thiosulphate and it is postulated that a disulphide bond is generated between the active site thiol and a second thiol which can be brought close to the active site in the flexible native structure.

Photolysis of Tryptophan.—Small peptides have been used [519] to investigate the photodecomposition products of tryptophan. Irradiation of Ala-Gly-Trp-Leu yielded the following identifiable photoproducts, Ala-Gly-Asp-Leu, Ala-Gly-(*N'*-formylkynurenine)-Leu, Ala-Gly-(β-(3-oxindolyl)alanine)-Leu and ammonia.

Δ^5-3-Ketosteroid Isomerase.—The previously reported [520] inactivation of this enzyme by 3-oxo-4-estren-17β-yl acetate-dependent photolysis has been investigated [521] and a reaction new to protein chemistry revealed. The chemical change, which was identified by amino-acid analysis and sequencing, was shown to be a conversion of Asp to Ala, *i.e.* a reductive decarboxylation.

Iodination.—A number of studies of the iodination of proteins have been reported. A cautionary paper [522] presents results demonstrating that transfer of radio-iodine from iodinated proteins to unlabelled carrier takes place during performic

[516] M. P. Coughlan, *F.E.B.S. Letters*, 1977, **81**, 1.
[517] R. Timkovich, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 1463.
[518] K. Guido and P. Horowitz, *Biochim. Biophys. Acta*, 1977, **485**, 95.
[519] L. A. Holt, B. Milligan, D. E. Rivett, and F. H. C. Stewart, *Biochim. Biophys. Acta*, 1977, **499**, 131.
[520] R. J. Martyr and W. F. Benisek, *Biochemistry*, 1973, **12**, 2172.
[521] J. R. Ogez, W. F. Tivol, and W. F. Benisek, *J. Biol. Chem.*, 1977, **252**, 6151.
[522] R. C. Montelaro, A. C. Herman, and D. P. Bolognesi, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 38.

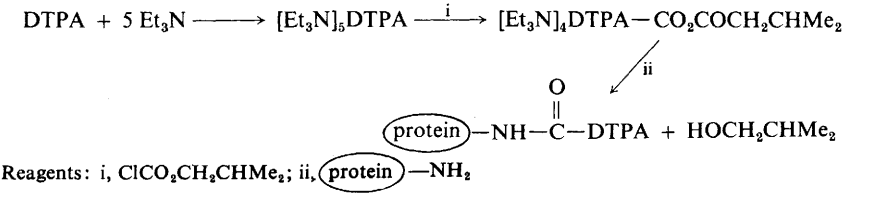
acid oxidation (a routine procedure prior to peptide mapping). Up to 80% of the iodine label was lost from the original protein. Radioiodine release and transfer were not observed when proteins were denatured by reduction and carboxymethylation and the authors recommend this procedure for mapping. The results suggest that previous mapping studies should be carefully evaluated. Differences in the sites of iodination following four methods of radioiodination have been studied [523] using fibrinogen and small peptides. The chloramine-T procedure as a method for surface specific iodination has been evaluated [524] for virus particles and it is concluded that internal labelling of the viral envelope at high iodide concentration is due to a new reaction pathway rather than alteration of the viral envelope. It is suggested that two iodinating species exist: (i) a membrane impermeable 'iodamine-T' complex at low iodide concentration and (ii) membrane permeable nascent iodine at high iodide concentration. The latter labels the inner proteins.

Peroxidase–Iodide Oxidation.—Peroxidases catalyse the peroxide-dependent oxidation of thiols in the presence of iodide ions. This reaction has been investigated [525] using bovine serum albumin and β-lactoglobulin and it is shown to be mediated by oxidation of iodide to iodine. Protein thiols react with iodine to yield a sulphenyl iodide derivative.

3 New Reagents and Techniques

Transglutaminase catalyses the formation of an amide bond between the γ-carboxy group of peptide-bound glutamine and a variety of amines. An assessment has been made [526] of the feasibility of using transglutaminase for labelling glutamine in isolated proteins, intact cells, membranes, and virus particles. Previous workers [527] have advocated the method for cell labelling. The author [526] concludes that the technique is useful when specific, limited labelling is required. Steric and specificity constraints limit the reaction to highly exposed residues. It should be noted that transglutaminase becomes significantly self-labelled in experiments employing amines of high specific activity and, in addition, it strongly adsorbs to membranes. Hence precautions must be taken to prevent spurious results.

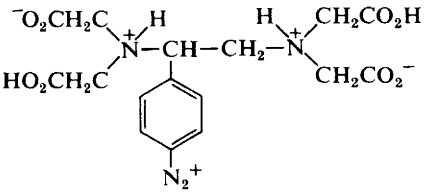
The covalent attachment of chelating groups to proteins provides a convenient method for specifically locating metal ions within the macromolecule. The



Scheme 2

[523] K. A. Krohn, L. C. Knight, J. F. Harwig, and M. J. Welch, *Biochim. Biophys. Acta*, 1977, **490**, 497.
[524] R. C. Montelaro and R. R. Rueckert, *Arch. Biochem. Biophys.*, 1977, **178**, 555.
[525] E. L. Thomas and T. M. Aune, *Biochemistry*, 1977, **16**, 3581.
[526] V. Iwanij, *European J. Biochem.*, 1977, **80**, 359.
[527] A. Dutton and S. J. Singer, *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 2568.

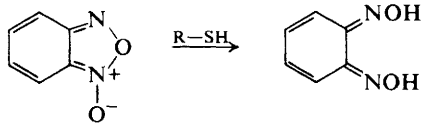
resulting metal–protein complexes can be useful as probes of biological systems. A simple procedure has been reported [528] for covalently coupling diethylenetriaminepenta-acetic acid (DTPA) to proteins (Scheme 2). An alternative method [529] of chelate introduction is to use a 'bifunctional' chelating agent such as 1-(*p*-benzenediazonium)ethylenedinitrilotetra-acetic acid (1) which is attached mainly



(1)

to histidine and lysine *via* nucleophilic displacement of nitrogen. This type of reagent offers versatility in the choice of the reactive group on the aromatic ring and therefore the selective labelling of amino-acids.

The use of benzofuroxan as a chromophoric oxidizing agent for thiol groups has been evaluated.[530] The concomitant reduction of the reagent (Scheme 3)



Scheme 3

yields a product which strongly absorbs at 416 nm (far removed from protein absorptions) and the reaction can be readily monitored photometrically. The cysteine-reacting compound, α-bromo-β(5-imidazolyl)propionic acid, has been shown [531] to be the most specific label for liver alcohol dehydrogenase known and may be a useful reagent for other metalloenzymes. The resolution of the reagent into its L- and D-isomers and the separate use of these for labelling has yet to be exploited. Lactoperoxidase [532] has been used to catalyse the peroxide-dependent incorporation of thiocyanate into proteins. At low peroxide concentrations cysteine is modified, while at high peroxide levels thiocyanogen is formed which reacts with tyrosine, tryptophan, or histidine in a similar manner to halogenation. A study [533] of the oxidation of glutathione in rat red blood cells has led to the discovery of the unique behaviour of rat haemoglobin towards diazenes, *e.g.* diazenedicarboxylic acid bis-*N'*-methylpiperazinide. These reagents may be of use in mapping exposed, reactive, thiol groups in proteins.

The observation that reduced pyridoxal 5'-phosphate–enzyme complexes slowly dissociated under fluorescent lights prompted a study [534] of the reactivation

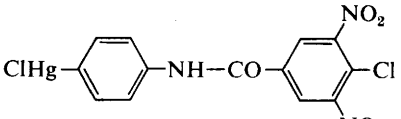
[528] G. E. Krejcarek and K. L. Tucker, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 581.
[529] C. S. H. Leung and C. F. Meares, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 149.
[530] M. Shipton, T. Stuchbury, K. Brocklehurst, J. A. L. Herbert, and H. Suschitzky, *Biochem. J.*, 1977, **161**, 627; M. Shipton and K. Brocklehurst, *ibid.*, 1977, **167**, 799.
[531] K. H. Dahl and J. S. McKinley-McKee, *European J. Biochem.*, 1977, **81**, 223.
[532] T. M. Aune, E. L. Thomas, and M. Morrison, *Biochemistry*, 1977, **16**, 4611.
[533] N. S. Kosower, E. M. Kosower, and R. L. Koppel, *European J. Biochem.*, 1977, **77**, 529.
[534] J. M. Ritchey, I. Gibbons, and H. K. Schachman, *Biochemistry*, 1977, **16**, 4584.

of a variety of enzymes pre-treated with pyridoxal 5′-phosphate and borohydride. Aspartate transcarbamoylase was quantitatively regenerated after irradiation with two 15 W fluorescent bulbs. The general occurrence of this photoreaction illustrates the care that needs to be taken when using pyridoxylated proteins. On the positive side, the reaction is potentially useful, *e.g.* in difference labelling or as a temporary handle.

The topography of the external surface of human erythrocytes has been studied [535] with the non-penetrating label, [^{125}I]diazodi-iodosulphanilic acid, whose parent compound, diazosulphanilic acid, reacts primarily with tyrosine, histidine, and lysine. Its pattern of reactivity differs from other commonly used reagents and it is a useful additional vectorial probe for membrane surfaces. The reagent provides further evidence for the complexity of the red cell membrane and the result emphasizes the necessity for taking into account the differences resulting from the properties of the probes when interpreting information from topographical experiments.

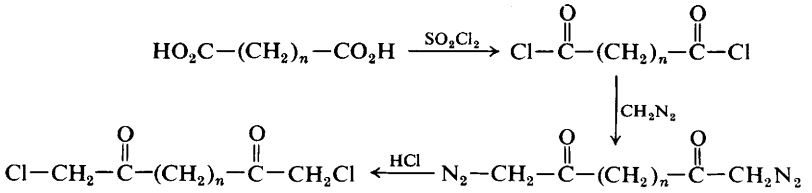
4 Chemical Cross-linking

A comprehensive review of chemical cross-linking in the study of membrane structure has appeared [536] which contains a useful summary of the problems still to be solved before the method can merit general application. Two new kinds of bifunctional reagents have been described.[537, 538] The heterobifunctional compound, *N*-(4-chloromercuriphenyl)-4-chloro-3,5-dinitrobenzamide (2), which



(2)

links lysine and cysteine, has been used to probe the quaternary structure of yeast alcohol dehydrogenase.[537] Only dimers were formed and therefore the tetrameric enzyme probably has D_2 symmetry. A series of chloromethylalkanedione derivatives of variable chain length which can be used to cross-link thiol groups has been synthesized [538] (Scheme 4). The reagents were used to cross-link glyceraldehyde 3-phosphate dehydrogenase. Maximum cross-linking was obtained



Scheme 4

[535] D. A. Sears, J. M. Friedman, and J. N. George, *J. Biol. Chem.*, 1977, **252**, 712.
[536] K. Peters and F. M. Richards, in 'Annual Review of Biochemistry', ed. E. E. Snell, 1977, vol. 46, p. 523.
[537] J. Diopoh and M. Olomucki, *European J. Biochem.*, 1977, **75**, 441.
[538] D. P. Bloxham, *Biochem. J.*, 1977, **167**, 201.

with bischloromethylhexanedione and the optimal thiol separation is therefore calculated to be 1.21—1.45 nm.

An interesting method for preventing the formation of aggregates during intramolecular cross-linking experiments has been proposed.[539] The protein is attached to a solid support *via* a cleavable reagent, the cross-linking is performed, and then the protein is released from the matrix. The method was demonstrated using trypsin and papain attached to CL-Sepharose—$NH-(CH_2)_6-NHCO-(CH_2)_2-S-S-(CH_2)_2-CO-$(*N*-hydroxysuccinimide) and CL-Sepharose—(glutathione-2,2′-dipyridyl disulphide) respectively. Protein recovery is over 80% but the procedure suffers from the very serious limitation that proteins containing an essential disulphide may be inactivated when the protein is released from the resin.

X-Ray diffraction studies of protein denaturation and renaturation have been successfully carried out with lysosyme cross-linked using glutaraldehyde.[540] Electron density difference maps indicated the locations of intermolecular cross-links but showed no appreciable differences in the protein conformation.

The technique of reductive cross-linking has not found general acceptance for analysing nucleic acid–protein interactions, mainly because of low yields. Recently it has been shown [541] that the use of sodium cyanoborohydride as the reducing agent markedly increases the yield of covalent nucleic acid–protein complexes from reductive cross-linking of viral mRNA to initiation factors. The method involves sodium periodate oxidation of the 5′-terminal 'cap' to convert the 2′,3′-*cis*-diol to a reactive dialdehyde, incubation of this species in cell-free protein synthesizing systems, and subsequent reduction with cyanoborohydride. In contrast to borohydrides, this reducing agent does not react with aldehydes at neutral pH and hence there is no competing conversion of the dialdehydes in oxidized mRNA back to non-reactive *cis*-diols. The improved yield of cross-linked products should make the procedure more generally attractive.

It has been shown [542] that formaldehyde, under very mild conditions, can successfully produce RNA–protein cross-linking in ribosomal subunits. The adducts formed are rather unstable and the reaction is readily reversed. Despite this, the authors have identified regions of RNA to which the proteins are joined thereby establishing some new topographical groupings of proteins and RNA in both ribosomal subunits. Although formaldehyde is by no means an ideal cross-linking reagent the experiments have demonstrated that even a non-specific reagent displays a high degree of selectivity in the proteins it links to RNA. Because cross-linking was made with intact subunits it was possible to demonstrate contacts between a single protein and widely separated regions of the RNA.

5 Photocross-linking

A review [543] on protein–nucleic acid photointeraction covers the literature for 1976 and the first half of 1977. The authors emphasize the important point that

[539] G. P. Royer, S. Ikeda, and K. Aso, *F.E.B.S. Letters*, 1977, **80**, 89.
[540] A. Yonath, A. Sielecki, J. Moult, A. Podjarny, and W. Traub, *Biochemistry*, 1977, **16**, 1413.
[541] N. Sonenberg and A. J. Shatkin, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4288.
[542] K. Möller, J. Kinke, A. Ross, G. Buddle, and R. Brimacombe, *European J. Biochem.*, 1977, **76**, 175.
[543] J. Sperling and A. Havron, *Photochem. and Photobiol.*, 1977, **26**, 661.

the reliability of the method depends on (i) the ability of both purines and pyrimidines to form covalent adducts with a major number of amino-acids and (ii) specific covalent bonds must be formed only between neighbouring residues in the native structure. The second requirement has been shown to be true for photocross-linking of ribonuclease with its competitive inhibitor uridine-2′(3′), 5′-diphosphate.[544] The amino-acid residues which took part in the photochemical reaction were identified as Ser-80, Ile-81, and Thr-82. The results suggest that Ile-81 is modified subsequent to initial attack on Ser-80 and that a different pathway operates for Thr-82 modification. Specificity has also been observed [545] in the photocross-linking of guanosine-5′-triphosphate to the nucleotide-binding site of elongation factor G. Raney nickel treatment liberates the nucleotide from the complex. Hence the nucleotide is cross-linked through a sulphur atom. There is some experimental evidence for bonding to the essential cysteine but linkage to methionine is not ruled out. *In vivo* experiments have been carried out to probe the structural association between protein and DNA as they occur in cells.[546] U.v. irradiation of bacteriophage M13-infected *E. coli* induces the formation of a covalent cross-link between progeny single-stranded DNA and the M13 DNA-binding protein, the product of gene *5*. The cross-linked product was isolated and the protein component cleaved with cyanogen bromide or trypsin. The cross-link was shown to be located within the peptide spanning residues 70—77.

In studies designed to elucidate the role that initiation factor IF-3 plays in protein synthesis two photolytic methods have been used to cross-link IF-3 with the 16S RNA of the 30S ribosomal unit. In the first [547] the irradiation was performed in the presence of a sensitizing system using light of wavelength 364—366 nm. Under these conditions singlet oxygen is the active species and the reaction resembles cross-linking with bifunctional electrophiles except that no bridging molecule is involved and the specificity is lower. In the second method [548] u.v. light without a sensitizer is used. The resulting cross-linked complexes have been isolated and subjected to limited hydrolysis with a ribonuclease yielding two subparticles each containing a characteristic set of proteins.

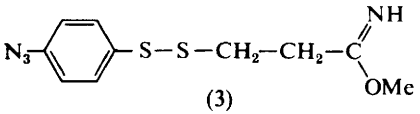
The regions of *E. coli* 16S RNA which are covalently linked to the ribosomal proteins S4 and S20 after u.v. irradiation have been determined.[549] This work complements earlier studies of the protein components of the cross-linked complexes.

The ATP-binding domain of three different aminoacyl-tRNA synthetases from *E. coli* has been probed [550] by direct, specific photochemical cross-linking of ATP using 254 nm light. Detailed studies on isoleucine-tRNA synthetase have shown that the entire nucleotide is incorporated. Proteolytic digestion of the [α-^{32}P]-

[544] A. Havron and J. Sperling, *Biochemistry*, 1977, **16**, 5631.
[545] M. S. Rohrbach and J. W. Bodley, *Arch. Biochem. Biophys.*, 1977, **183**, 340.
[546] L. Lica and D. S. Ray, *J. Mol. Biol.*, 1977, **115**, 45.
[547] B. S. Cooperman, J. Dondon, J. Finelli, M. Grunberg-Manago, and A. M. Michelson, *F.E.B.S. Letters*, 1977, **76**, 59.
[548] C. L. Pon, R. Brimacombe, and C. Gualerzi, *Biochemistry*, 1977, **16**, 5681.
[549] B. Ehresmann, C. Backendorf, C. Ehresmann, and J. P. Ebel, *F.E.B.S. Letters*, 1977, **78**, 261.
[550] V. T. Yue and P. R. Schimmel, *Biochemistry*, 1977, **16**, 4678.

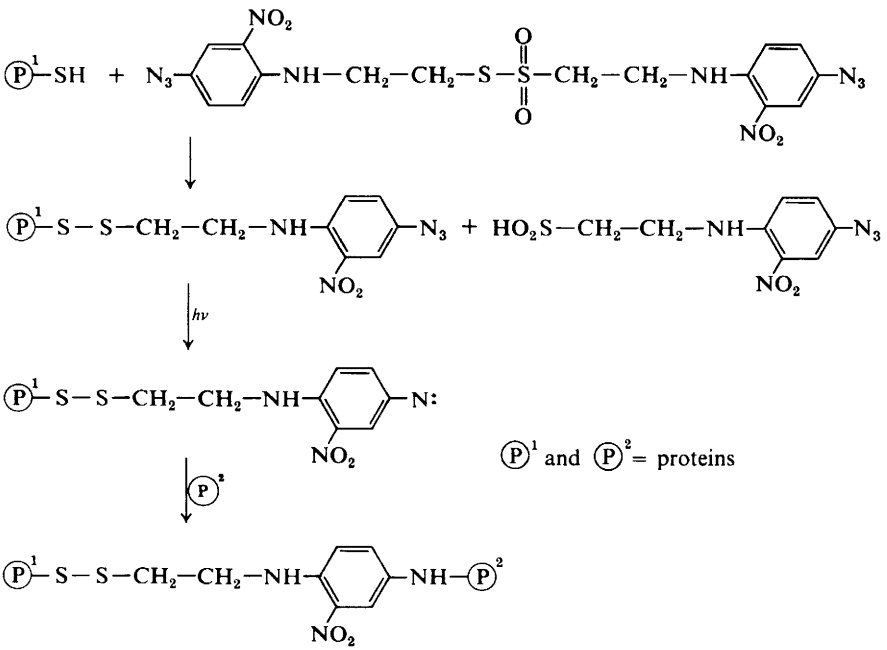
ATP-Ile-tRNA synthetase complex gives rise to the labelled peptide Lys-Val-Ala-Gly-Asx-X where X does not correspond to a natural amino-acid.

Photosensitive heterobifunctional cross-linking reagents are being exploited in the study of membrane receptors. Methyl 4-azidobenzoimidate has been used[551] to attach concanavalin A to the surface of erythrocyte ghosts. Only high molecular weight material was produced which indicated that multimer complexes were formed. A more useful result was obtained[552] from experiments designed to label the cell surface receptor for epidermal growth factor (EGF).



(3)

The photoactive, cleavable reagent, methyl-3[(*p*-azidophenyl)dithio]propionimidate (3), was used to link ^{125}I-labelled EGF with mouse 3T3 cells. A single radioactive band of molecular weight 190 000 was observed. This band was absent if a non-binding variant clone of mouse cells was used. A new photosensitive, lipid soluble, cleavable, cross-linking reagent has been synthesized for membrane studies.[553] The reagent di-*N*-(2-nitro-4-azidophenyl) cystamine-*SS*-dioxide cross-links proteins through thiol groups (Scheme 5), which are expected



Scheme 5

[551] T. H. Ji, *J. Biol. Chem.*, 1977, **252**, 1566.
[552] M. Das, T. Miyakawa, C. F. Fox, R. M. Pruss, A. Aharonov, and H. R. Herschman, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2790.
[553] C.-K. Huang and F. M. Richards, *J. Biol. Chem.*, 1977, **252**, 5514.

to be among the few reactive groups to be within the lipid bilayer. In an attempt to eliminate non-specific cross-linking in fluid membranes resulting from random collisions, flash photolysis has been used together with various azidoimidates to cross-link proteins in human ghosts.[554] The flash discharges within milliseconds and the time period of cross-linking is estimated to be of the same order. An improved gel electrophoresis system was used to analyse the cross-linked products.

Cytochrome *c* labelled at lysyl-13 with a 4-nitrophenylazide group forms a covalent complex with the polypeptide of molecular weight 23 700 of cytochrome oxidase after photoirradiation.[555] When the label is located at lysyl-22 no covalent protein binding is observed.

6 Photolabelling

A method for *non-specificially* labelling all exposed residues on a protein has been applied to ribonuclease A.[556] A reactive aryl nitrene is generated by flash photolysis of *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulphonate. The reactive nitrene is inserted within about two milliseconds into those carbon–hydrogen bonds exposed to solvent. Labelling of the intestinal microvillus membrane with the hydrophobic photoactive reagent [5-^{125}I]iodonaphthyl-1-azide, which unspecifically labels those portions of membrane proteins which are inserted into the lipid bilayer matrix, yields radioactive products of molecular weights 99 000, 86 000, 65 000, 54 000, and 30 000.[557] The mixed disulphide of 2-thiopyridine and 2-thiobenzyl[^{14}C]diazoacetate has been used [558] to photolabel thiol enzymes. After irradiation at 254 nm labelled products were identified. About 30% of the carbene produced on photoysis was shown to react with serine and threonine with *O*-[^{14}C]carboxymethylthreonine as the major product.

7 Distant Reporter Group

A highly sensitive method for demonstrating ligand-induced conformational changes in protein molecules in solution has been described.[559] The method utilizes an environmentally sensitive reporter group known to be distant from the active site. This is achieved by placing two reporter groups sufficiently far apart that bound ligand cannot interact directly with both simultaneously. By monitoring both reporters it is possible to distinguish between a direct effect (in the vicinity of binding) and an indirect effect (the change in the microenvironment of a residue some distance from the ligand-binding site). The method has been applied to the galactose receptor of *Salmonella typhimurium* using the fluorophores 5-iodoacetamidofluorescein, attached to a single methionine, and tryptophan. The distance between the two dyes was established by fluorescence energy transfer

[554] D. J. Kiehm and T. H. Ji, *J. Biol. Chem.*, 1977, **252**, 8524.
[555] R. Bisson, H. Gutweniger, C. Montecucco, R. Colonna, A. Zanotti, and A. Azzi, *F.E.B.S. Letters*, 1977, **81**, 147.
[556] R. R. Matheson, jun., H. E. Van Wart, A. W. Burgess, L. I. Weinstein, and H. A. Scheraga, *Biochemistry*, 1977, **16**, 396.
[557] K. Sigrist-Nelson, H. Sigrist, T. Bercovici, and C. Gitler, *Biochim. Biophys. Acta*, 1977, **468**, 163.
[558] J. Henkin, *J. Biol. Chem.*, 1977, **252**, 4293.
[559] R. S. Zukin, P. R. Hartig, and D. E. Koshland, jun., *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1932.

methods to be 41 ± 10 Å. Binding of galactose perturbs both fluorophores and it was concluded that a ligand-induced conformational change is propagated a minimum of 30 Å through the receptor molecule.

8 Competitive Labelling

The technique of competitive labelling has been used to probe the topography of the *E. coli* ribosomal protein L12 in the native 50S subunit.[560] All the lysines are exposed and have pK_a values varying from 9.7 to 11. Lysines-29, 51, and 59 exhibit an interesting pK_a minimum between pH 7.5 and 8 which indicates that the amino terminal region from residues 1 to 59 undergoes a pH-dependent conformational change. This region may be near the site of interaction of L12 with the 50S subunit. The reactivity of the amino groups of free histones and histones in chromatin as a function of ionic strength has been studied.[561] No unusual reactivity was observed. Competitive labelling experiments [562] on deoxy- and liganded-haemoglobin have revealed that only those amino groups known to form salt bridges in deoxy- but not in liganded-haemoglobin have different reactivities in the two structures.

9 Affinity Labelling

The technique of affinity labelling is now well established and Volume 46 of 'Methods in Enzymology'[563] is devoted entirely to this subject. Affinity labels for a variety of proteins have been reported in 1977. Space limitations preclude a separate discussion of each example and the reader is referred to Table 1 for a comprehensive coverage.

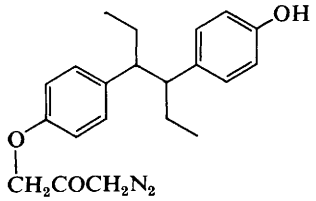
The reagent A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD^+ has been synthesized and shown to be a substrate for yeast alcohol dehydrogenase.[564] It has potential use for other NAD^+- or NADH-dependent enzymes.

Affinity labelling of human corticosteroid-binding globulin (transcortin) is rendered difficult by its rapid inactivation in the absence of steroid substrates. A method has been developed [565] for labelling transcortin which is initially saturated with cortisol. Labelling with 6β-bromo[^{3}H]progesterone was achieved after prior absorption of transcortin onto DEAE filter discs. The reagent reacted with active site cysteine and the product, progesterone-6-*S*-L-cysteine, was identified after acid hydrolysis of the labelled protein. Displacement kinetic studies have been used to assess the utility of bromoacetylated derivatives of progesterone and testosterone as affinity labels for transcortin.[566] Specific labelling was obtained with 11α-bromoacetoxyprogesterone, 16α-bromoacetoxyprogesterone, and 17β-bromoacetyltestosterone.

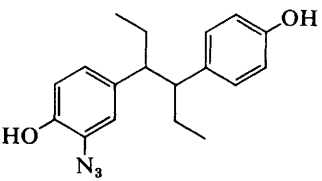
Detailed studies [567] have been made of estrogen photoaffinity labels and

[560] S. Hasnain, L. P. Visentin, and H. Kaplan, *European J. Biochem.*, 1977, **80**, 35.
[561] B. L. Malchy, *Biochemistry*, 1977, **16**, 3922.
[562] D. Bresciani, *Biochem. J.*, 1977, **163**, 393.
[563] '*Methods in Enzymology*', ed. W. B. Jakoby and M. Wilchek, Academic Press, 1977, vol. 46.
[564] S. Chen and R. G. Guillory, *J. Biol. Chem.*, 1977, **252**, 8990.
[565] M. S. Khan and W. Rosner, *J. Biol. Chem.*, 1977, **252**, 1895.
[566] F. Le Gaillard and M. Dautrevaux, *Biochim. Biophys. Acta*, 1977, **495**, 312.
[567] J. A. Katzenellenbogen, K. E. Carlson, H. J. Johnson, jun., and H. N. Myers, *Biochemistry*, 1977, **16**, 1970; J. A. Katzenellenbogen, H. N. Myers, H. J. Johnson, jun., R. J. Kempton, and K. E. Carlson, *ibid.*, 1977, **16**, 1964.

their capacity for labelling the estrogen receptor. Simple chemical and radiochemical synthesis of hexestrol diazoketopropyl ether (4) and hexestrol azide (5) are reported. This is the first example of the labelling of a steroid hormone receptor by a photoaffinity procedure. It should be noted that the reagents are more lipophilic than estradiol and therefore may bind strongly to non-receptor proteins.



(4)



(5)

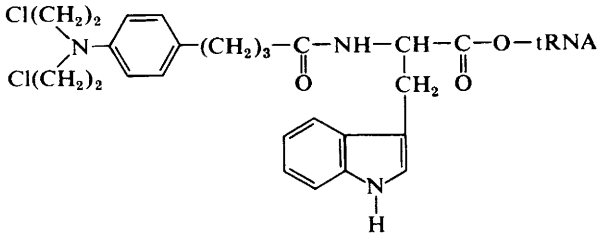
An important step towards isolation of the beta adrenergic receptor has been achieved by the synthesis of the photoaffinity probe, *N*-(2-hydroxy-3-naphthoxypropyl)-*N'*-(2-nitro-5-azidophenyl)ethylenediamine.[568] The acetylcholine receptor, both free and membrane bound, has been labelled with [^{3}H]bis(3-azidopyridinium)-1,10-decane di-iodide.[569] The 40 000 and 60 000 molecular weight subunits were labelled in the purified receptor while the membrane-bound receptor was labelled on the 40 000 and 50 000 subunits suggesting that specific ligand binding sites are located on the 40 000 subunit.

Poly(4-thiouridylic acid) which acts as a mRNA for the synthesis of polyphenylalanine has been used to label the ribosomal mRNA binding site.[570]

A new approach to affinity labelling, the photoactivation of one of the reaction products, has been achieved using acetylcholinesterase and the substrate methyl-(acetoxymethyl)nitrosamine.[571] Difficulties will certainly be encountered in achieving specificity in a procedure such as this, since there is no guarantee that the product will still be in the active site when photolysis occurs.

Affinity labelling of tRNA synthetases continues to be actively pursued and the interesting new technique of 'double affinity labelling' has been used to convert the two site bifunctional enzyme, tryptophanyl-tRNA synthetase, into a single site bifunctional enzyme.[572] This is performed using the affinity labels γ-(*p*-azidoanilide)ATP and *N*-chloroambucilyl-Trp-tRNA (6) which label the ATP and tRNA binding sites respectively. The tRNA analogue labels only one active site in the dimer and the kinetics of the resulting one site monofunctional enzyme have been studied.[573] The double affinity labelling approach was used to produce tRNA synthetase labelled at only one of the ATP sites.[572] This was achieved as follows: (i) alkylation with the tRNA analogue (ii) modification with γ-(*p*-azido-

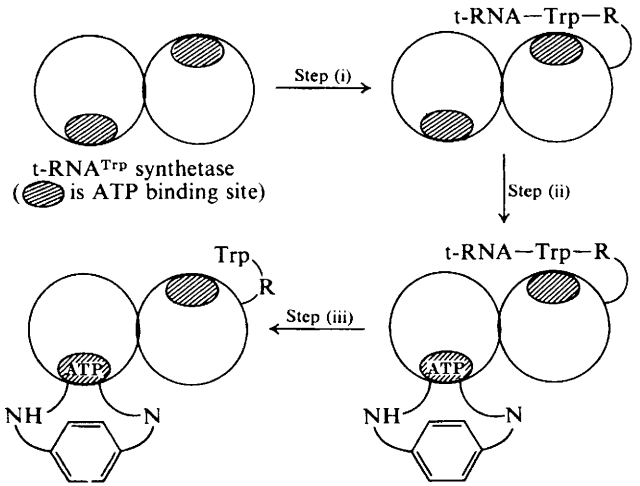
[568] F. J. Darfler and G. V. Marinetti, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 1.
[569] V. Witzemann and M. A. Raftery, *Biochemistry*, 1977, **16**, 5862.
[570] I. Fiser, K. H. Scheit, and E. Kuechler, *European J. Biochem.*, 1977, **74**, 447.
[571] M. P. Goeldner and C. G. Hirth, *F.E.B.S. Letters*, 1977, **82**, 151.
[572] V. Z. Akhverdyan, L. L. Kisselev, D. G. Knorre, O. I. Lavrik, and G. A. Nevinsky, *J. Mol. Biol.*, 1977, **113**, 475.
[573] V. V. Zinoviev, N. G. Rubtsova, O. I. Lavrik, E. G. Malygin, V. Z. Akhverdyan, O. O. Favorova. and L. L. Kisselev, *F.E.B.S. Letters*, 1977, **82**, 130.



(6)

anilide)ATP, and (iii) alkaline hydrolysis to split off the tRNA. Since one ATP binding site is protected from reaction with γ-(*p*-azidoanilide)ATP after alkylation the procedure yields a bifunctional enzyme which has one blocked ATP binding site, *i.e.* only one active centre (Scheme 6).

Three analogues of methionyl-tRNA synthetase substrates which contain photolabile groups have been synthesized.[574] These are (i) the product from alkylation of the 4-thiouridine at the 8-position of $tRNA^{fMet}$ with [^{14}C]-*p*-azidobromoacetanilide, (ii) the product from condensation of [^{14}C]-*p*-azidobenzoic acid hydrazide



Scheme 6

with periodate oxidized $tRNA^{fMet}$, and (iii) [^{3}H]methioninyl-8-azido-adenosine 5′-phosphate which is an analogue of the methionyl adenylate intermediate in the aminoacylation reaction. The most efficient cross-linking occurs at pH 5.5—6.0 which is evidence for cross-linking being associated with normal enzyme–substrate interactions. High ionic strength, which destabilizes enzyme–tRNA complexes, completely inhibits cross-linking.

[574] R. Wetzel and D. Söll, *Nucleic Acid Res.*, 1977, **4**, 1681.

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

1 Introduction

The year 1977 has seen many important advances in the methods and instrumentation of crystallography as well as a wide variety of interesting structures which have been determined.

With conventional *X*-ray sources, a new diffractometer has been described and there have been several reports on the equipment and methodology of data collection on film. The interest in the use of synchrotron radiation has continued and there have been several papers describing suitable instruments for data collection and methods of processing the data obtained using them. Several groups have reported the use of computer-controlled graphic systems for the interpretation of electron-density maps and for the subsequent examination of the molecular topology. Together with the use of very fast refinement techniques, these graphic systems allow the examination of a structure without a model ever having to be built.

Two immunoglobulin structures have been reported, one being an intact human IgE molecule. The structures of deoxy- and met-myoglobin have been redetermined and the structures of several haemoglobin variants and complexes have been reported. The work reported on lysozyme includes an investigation of the effects of denaturants on the molecule in structural terms. Two protease structures, actinidin and penicillinopepsin, have been reported together with refinements and substrate-binding studies for several previously determined proteolytic enzymes. Of the enzymes of the glycolytic pathway, the structures of glucose-6-phosphate isomerase, D-glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase have been described. Other structures reported include dihydrofolate reductase (the first folate-binding enzyme to have its structure determined), wheat-germ agglutinin, ferritin, and arabinose binding protein.

The analysis of the folding patterns for the known protein structures has continued apace and there have been several papers reporting classifications of 'super-secondary structures' together with methods for automatically identifying these structures. The topology of β-sheet structures and improved methods of predicting these structures have also been studied in detail.

Many large biological structures and even whole organs can be studied by fibre diffraction or low-angle *X*-ray scattering techniques. Results have been reported from whole muscles and muscle components, chromatin, and microtubules. The structures of collagen and keratin have been examined and *X*-ray diffraction experiments with membranes, lipoproteins, and synthetic polypeptides have been reported.

Much useful information on the preferred conformations and hydrogen-bonding patterns of amino-acids and peptides can be obtained by crystallographic structure determinations and can give an idea of the structures likely to be seen in proteins. Investigation of the structures of complexes of amino-acids with metal ions and nucleic acids can give an insight into the possible interactions of the amino-acids in proteins with these proteins. The structures reported in 1977 are listed in Table 1 (see p. 166).

Preliminary crystallographic data on a wide range of proteins are presented in

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

Compound	*Space group*	*Cell dimensions* *a*/Å	*b*/Å	*c*/Å	α/°	β/°	γ/°	*Z*	*R*	*Ref.*
NN-Bis(2-hydroxyethyl)Gly [a]	$P2_1/n$	9.963 (2)	11.045 (3)	7.667 (1)	—	111.78 (2)	—	4	0.047	*b*
Gly hydrochloride	$P2_1/n$	7.126 (3)	5.238 (2)	13.767 (6)	—	97.30 (2)	—	4	0.066	*c*
L-Ala hydrochloride	$P2_12_12_1$	7.148 (3)	17.590 (8)	5.202 (2)	—	—	—	4	0.090	*c*
DL-Asp hydrochloride	$P2_1/a$	11.14 (4)	6.31 (4)	11.24 (8)	—	120.20 (2)	—	4	—	*d*
Calcium L-Glu chloride hydrate [e]	$P2_12_12_1$	5.0731 (5)	8.2168 (5)	23.067 (2)	—	—	—	4	0.045	*f*
L-Glu/L-pyroGlu	$P2_1$	18.473 (5)	7.190 (1)	5.113 (1)	—	96.77 (2)	—	2	0.0685	*g*
L-pyroGlu	$P2_1/a$	9.194 (2)	8.744 (1)	7.976 (2)	—	116.29 (2)	—	4	0.042	*g*
N-Pivalyl-*N'*-methyl-L-Gln-methylamide	$P2_12_12$	6.62 (2)	16.42 (3)	13.16 (3)	—	—	—	4	0.059	*h*
L-Arg-L-Glu [i]	$P2_12_12_1$	5.047 (8)	9.849 (8)	32.500 (12)	—	—	—	4	0.083	*j*
N-Acetyl-L-His-*N*-methylamide [k]	$P2_12_12_1$	13.951 (2)	15.705 (2)	4.898 (1)	—	—	—	4	0.066	*l*
L-His hydrochloride hydrate	$P2_12_12_1$	15.36	8.92	6.88	—	—	—	4	0.036	*m*
									0.121 [n]	*o*
L-Met hydrochloride	$P2_12_12_1$	7.087 (4)	24.643 (8)	5.297 (3)	—	—	—	4	0.044	*p*
5-Methyl-L-Met hydrochloride [q]	$P2_1$	9.500 (8)	7.223 (6)	8.884 (7)	—	114.9 (2)	—	2	0.022	*r*
N-Formyl-L-Met	$P2_12_12_1$	7.507 (2)	14.875 (1)	7.561 (1)	—	—	—	4	0.053	*s*
N-Acetyl-L-Phe-*N*-methylamide [k]	$P2_1$	11.695 (1)	4.966 (1)	11.531 (1)	—	116.6 (1)	—	2	0.039	*t*
D-2-Hydroxy-Phe hydrochloride	$Pca2_1$	16.667 (5)	7.438 (2)	16.659 (5)	—	—	—	8	0.035	*u*
N-Acetyl-DL-Trp-*N*-methylamide [k]	$P2_1/a$	14.023 (7)	11.896 (6)	9.105 (5)	—	107.0 (1)	—	4	0.049	*v*
N-Acetyl-L-Trp	$P2_12_12_1$	7.470 (1)	25.912 (4)	6.247 (1)	—	—	—	4	0.060	*w*
DL-Val hydrochloride	$P2_1/c$	11.722 (4)	7.007 (3)	11.143 (4)	—	120.53 (3)	—	4	0.063	*x*
LL-Diaminopimelic acid hydrochloride	$I2$	11.252 (2)	5.088 (1)	18.047 (3)	—	94.22 (1)	—	4	0.0295	*y*
meso-Diaminopimelic acid hydrochloride	$P2_1/n$	11.024 (3)	5.108 (1)	18.320 (3)	—	97.11 (2)	—	4	0.0391	*y*
N^6-(*N*-Gly-carbonyl)adenosine	$P2_1$	7.572 (1)	21.015 (4)	4.909 (1)	—	106.32 (2)	—	2	0.046	*z*
N^6-(*N*-Thr-carbonyl)adenosine	$P2_1$	10.477 (1)	16.894 (2)	5.001 (1)	—	94.69 (5)	—	2	0.041	*z*
5-{*N*-(L-Leu)amino}uridine [aa]	$I222$	10.883 (3)	23.774 (5)	17.038 (7)	—	—	—	8	0.080	*bb*
α-Gly-Gly [n]	$P2_1/c$	8.025 (2)	9.543 (4)	7.788 (2)	—	106.50 (2)	—	4	0.030	*cc*
t-Butyloxycarbonyl-Gly-L-Ala	$P2_1$	5.15 (9)	20.93 (2)	7.62 (1)	—	128.55	—	2	0.047	*dd*
β-Ala-L-His (carnosine) [ee]	$C2$	24.740 (10)	5.428 (10)	7.992 (10)	—	100.12 (2)	—	4	0.036	*ff*

L-Cys-L-Cys [gg]	$P2_12_12_1$	8.629 (7)	23.500 (9)	5.145 (2)	—	—	—	4	0.042	*hh*
t-Butyloxycarbonyl-L-Cys-L-Cys disulphide methylester [ii]	$P2_12_12_1$	17.404 (1)	10.565 (2)	9.137 (5)	—	—	—	4	0.078[n]	*jj*
Benzyloxycarbonyl-Gly-L-Pro	$P4_32_12$	9.455 (1)	—	34.889 (5)	—	—	—	8	0.051	*kk*
cyclo-L-Leu-L-His monohydrate	$P2_12_12_1$	29.074 (1)	6.017 (1)	8.306 (1)	—	—	—	4	0.039	*ll*
L-Pro-L-hydroxyPro monohydrate	$P2_1$	6.264 (3)	8.940 (3)	10.336 (5)	—	101.5	—	2	0.059	*mm*
DL-Leu-Gly-Gly [nn]	$P2_1/c$	11.52 (2)	12.44 (2)	9.70 (1)	—	102.6 (2)	—	4	0.092	*oo*
t-Butyloxycarbonyl-L-Pro-L-Leu-Gly hydrate [pp]	$P2_1$	10.036 (1)	18.142 (2)	6.191 (1)	—	101.77 (2)	—	2	0.037 0.086[n]	*qq*
N-Benzyloxycarbonyl-α-aminoisobutyryl-Pro-α-aminoisobutyryl-Ala methyl ester [rr]	$P2_12_12_1$	9.694	15.034	18.595	—	—	—	4	0.107	*ss*
Tyr-Gly-Gly-Phe [tt]	$P2_12_12$	12.912	21.73	10.76	—	—	—	4	0.089	*uu*
Gly-Gly-Phe-Leu [tt]	$P2_1$	9.11	6.20	19.76	—	102.6	—	2	0.073	*uu*
[Phe,[4]Val[6]]Antamanide [vv]	$P2_12_12$	15.084 (4)	21.920 (6)	11.015 (3)	—	—	—	2	0.090	*ww*
Aqua(L-glutamato)cadmium(II) hydrate	$P2_12_12_1$	11.61 (1)	10.79 (1)	7.286 (7)	—	—	—	4	0.038	*xx*
Bis(*N*-acetylglycinato)-1,10-phenanthrolinecopper(II)	$C2/c$	8.85 (1)	16.45 (1)	14.74 (3)	—	102.3 (1)	—	4	0.036 —	*yy*
(Glycylglycinato)(cytidine)copper-(II)dihydrate	$P2_1$	4.710 (4)	26.99 (3)	14.99 (2)	—	90.1 (1)	—	2	0.127	*zz*
Dichlorobis(δ-*N*-hydro-L-ornithinato)copper(II) dihydrate	$P2_1$	5.218 (3)	15.636 (2)	12.058 (4)	—	93.37 (2)	—	2	0.050	*aaa*
Diaqua(glycyl-L-tyrosinato)copper(II) dihydrate	$P\bar{2}_12_12_1$	12.498 (3)	10.429 (2)	11.978 (2)	—	—	—	4	0.046	*bbb*
Glycyl-L-histidyl-glycinatocopper(II) dihemihydrate	$C2$	19.040 (6)	6.943 (4)	12.201 (4)	—	103.89 (2)	—	4	0.035	*ccc*
Bis(DL-proline)manganese(II) dibromide dihydrate	$P2_1/c$	9.375 (1)	9.195 (2)	10.122 (2)	—	106.38 (2)	—	2	0.038	*ddd*
Bis-η-cyclopentadienyl-L-prolinatomolybdenum hexafluorophosphate	$P2_1$	9.842 (3)	9.939 (2)	8.921 (2)	—	100.22 (4)	—	2	0.044	*eee*
Bis-η-cyclopentadienyl-L-leucinato-molybdenum hexafluorophosphate	$P2_1/b$	6.44 (1)	10.04 (1)	30.05 (3)	—	—	94.1 (1)	4	0.069	*eee*
Bis(L-isoleucinato)nickel(II) dihydrate	$P1$	5.98 (3)	13.80 (9)	5.86 (3)	96.8 (3)	112.6 (3)	87.5 (9)	1	—	*fff*

Table 1 *cont.*

Compound	Space group	Cell dimensions a/Å	b/Å	c/Å	α/°	β/°	γ/°	Z	R	Ref.
Dichloro{(2*S*,*SR*)-methionine sulphoxide}platinum(II) hydrate [ggg]	$P2_12_12_1$	13.420 (4)	10.888 (2)	8.066 (1)	—	—	—	4	0.0296	*hhh*
DL-Cysteinatothallium(I) [iii]	$P2_1/a$	10.841 (6)	7.672 (2)	8.391 (2)	—	114.58 (3)	—	4	0.063	*jjj*
Bis(L-asparaginato)zinc(II)	$P2_1$	12.323 (1)	5.027 (2)	9.702 (2)	—	99.12 (4)	—	2	0.045	*kkk*

(*a*) Bicine buffer. (*b*) V. Cody, J. Hazel, and D. Langs, *Acta Cryst.*, 1977, **B33**, 905. (*c*) B. Di Blasio, V. Pavone, and C. Pedone, *Cryst. Struct. Comm.*, 1977, **6**, 745. (*d*) B. Dawson, *Acta Cryst.*, 1977, **B33**, 882. (*e*) Calcium ion is co-ordinated by oxygens from five different glutamates. (*f*) H. Einspahr, G. L. Gartland, and C. E. Bugg, *Acta Cryst.*, 1977, **B33**, 3385. (*g*) Z. Taira and W. H. Watson, *ibid.*, 1977, **B33**, 3823. (*h*) A. Aubry and J. Protas, *ibid.*, p. 2534. (*i*) Salt bridge between γ-carboxy-group of the glutamate and the guanidyl group of the arginine. (*j*) T. N. Bhat and M. Vijayan, *Acta Cryst.*, 1977, **B33**, 1754. (*k*) β-Sheet torsion angles. (*l*) Y. Harada and Y. Iitaka, *Acta Cryst.*, 1977, **B33**, 250. (*m*) D. Hohlwein, *ibid.*, p. 649. (*n*) Neutron-diffraction study. (*o*) H. Fuess, D. Hohlwein, and S. A. Mason, *Acta Cryst.*, 1977, *ibid.*, p. 654. (*p*) B. Di Blasio, V. Pavone, and C. Pedone, *Cryst. Struct. Comm.*, 1977, **6**, 845. (*q*) Vitamin U, hydrochloride. (*r*) G. Del Re, E. Gavuzzo, E. Giglio, F. Lelj, F. Mazza, and V. Zappia, *Acta Cryst.*, 1977, **B33**, 3289. (*s*) Cheng Cheng-San and R. Parthasarathy, *ibid.*, p. 3332. (*t*) Y. Harada and Y. Iitaka, *ibid.*, p. 247. (*u*) A. Mostad, C. Rømming, and L. Tressum, *Acta Chem. Scand.*, 1977, **B31**, 119. (*v*) Y. Harada and Y. Iitaka, *Acta Cryst.*, 1977, **B33**, 244. (*w*) T. Yamane, T. Andou, and T. Ashida, *ibid.*, p. 1650. (*x*) B. Di Blasio, G. Napolitano, and C. Pedone, *ibid.*, p. 542. (*y*) S. E. Hull, O. Kennard, H. J. Rogers, and M. V. Kelemen, *ibid.*, p. 3832. (*z*) R. Parthasarathy, J. M. Ohrt, and G. B. Chheda, *Biochemistry*, 1977, **16**, 4999. (*aa*) Has antiviral properties. (*bb*) P. Narayanan and H. M. Berman, *Acta Cryst.*, 1977, **B33**, 2047. (*cc*) Å. Kvick, A. R. Al-Karaghouli, and T. F. Koetzle, *ibid.*, p. 3796. (*dd*) M. Gadret, J. M. Leger, and A. Carpy, *ibid.*, p. 1067. (*ee*) Extended chain conformation. (*ff*) H. Itoh, T. Yamane, T. Ashida, and M. Kakudo, *Acta Cryst.*, 1977, **B33**, 2959. (*gg*) *cis*-Peptide bond and a right-handed disulphide bond (torsion angle 94°). (*hh*) S. Capasso, C. Mattia, and L. Mazzarella, *Acta Cryst.*, 1977, **B33**, 2080. (*ii*) Cyclo-cystine compound with *cis* peptide bond. (*jj*) Y. Hata, Y. Matsuura, N. Tanaka, T. Ashida, and M. Kakudo, *Acta Cryst.*, 1977, **B33**, 3561. (*kk*) I. Tanaka, T. Kozima, T. Ashida, N. Tanaka, and M. Kakudo, *ibid.*, p. 116. (*ll*) I. Tanaka, T. Iwata, N. Takahashi, T. Ashida, and M. Tanihara, *ibid.*, p. 3902. (*mm*) B. Arnoux, T. Prangé, and C. Pascard, *Cryst. Struct. Comm.*, 1977, **6**, 29. (*nn*) *trans*-Peptide in extended conformation. (*oo*) K. N. Goswami, V. S. Yadava, and V. M. Padmanabhan, *Acta Cryst.*, 1977, **B33**, 1280. (*pp*) Unique *trans*-butyloxycarbonyl–proline bond. Conformation is a β-I turn with an opening of the proline NCαC′ angle. (*qq*) T. Ashida, I. Tanaka, Y. Shimonishi, and M. Kakudo, *Acta Cryst.*, 1977, **B33**, 3054. (*rr*) Two β-turns, giving an incipient 3_{10} helix. The compound is the *N*-terminal tetrapeptide of alamethicin. (*ss*) N. Shamala, R. Nagaraj, and P. Balaram, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 292. (*tt*) Enkephalin related fragments showing 3_{10} helical conformation. (*uu*) M.-C. Fournie, Zaluski, T. Prangé, C. Pascard, and B. P. Roques, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 1199. (*vv*) Molecule has two-fold symmetry. (*ww*) I. L. Karle and E. Duesler, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2602. (*xx*) R. J. Flook, H. C. Freeman, and M. L. Scudder, *Acta Cryst.*, 1977, **B33**, 801. (*yy*) L. P. Battaglia, A. Bonamartini-Corradi, G. Marcotrigiano, and G. C. Pellacani, *ibid.*, p. 3886. (*zz*) D. J. Szalda and T. J. Kistenmacher, *ibid.*, p. 865. (*aaa*) F. S. Stephens, R. S. Vagg, and P. A. Williams, *ibid.*, p. 438. (*bbb*) A. Mosset and J.-J. Bonnet, *ibid.*, p. 2807. (*ccc*) P. De Meester and D. J. Hodgson, *ibid.*, p. 3505. (*ddd*) T. Glowiak and Z. Ciunik, *ibid.*, p. 3237. (*eee*) K. Prout, S. R. Critchley, E. Cannillo, and V. Tazzoli, *ibid.*, p. 456. (*fff*) J. A. Muir and A. Ortiz, *J. Appl. Cryst.*, 1977, **10**, 489. (*ggg*) Formed by the reaction of potassium platinum tetrachloride with methionine sulphoxide. (*hhh*) W. A. Freemau, *Acta Cryst.*, 1977, **B33**, 191. (*iii*) In the crystal it forms Tl_2(D-cysteinate)(L-cysteinate) complexes. (*jjj*) H. C. Freeman and C. J. Moore, *Acta Cryst.*, 1977, **B33**, 2690. (*kkk*) F. S. Stephens, R. S. Vagg, and P. A. Williams, *ibid.*, p. 433.

Table 2 (see p. 170). An increase in the number of plant proteins being studied is apparent. The conditions used for producing crystals are presented and show that salting out with ammonium sulphate is still widely used, although poly(ethylene glycol) is being used successfully more often.

Structures which have been determined at low resolution are listed in Table 3 (p. 176). It is anticipated that several of these proteins will be solved at high resolution in the near future.

Low-angle *X*-ray scattering data can be very useful in determining the overall shape and dimensions of biological macromolecules, and the results reported in 1977 are listed in Table 4 (p. 178).

2 Equipment and Methods

A combination of speed with accuracy is needed for the collection of diffraction data from biological macromolecules, and the four-circle diffractometer gives just this combination. A diffractometer which collects a number of reflections quasi-simultaneously has been reported [1] and increases the rate of data acquisition significantly. It carries a bank of five proportional counters and can be used with crystals of axial lengths from 50 Å up to 130 Å, measuring 200—250 reflections per hour. The authors discuss in detail the geometry and data-collecting modes of the machine. The minimization of crystal damage by cooling is now in fairly general use and the construction of a new cooling device has been reported.[2] Crystal damage can be further minimized using monochromatic radiation, and Bauspiess and co-workers have described [3] a bicrystal monochromator which allows not only for very clean radiation but also for optimal intensity of the beam.

A computer-controlled microdensitometer suitable for scanning diffraction patterns from biological macromolecules has been built and evaluated.[4] The authors conclude that its overall performance is limited mainly by the properties of the *X*-ray film. The performance of a commercially available scanner, the Syntex AD–1 flat-bed autodensitometer, has also been examined and compared with that of its competitors.[5] With the recent advances in electron-diffraction methods, the report of a digital scanning and recording system for electron-diffraction patterns is of interest.[6]

Wood and Abola [7] report a data-collection operating system written in FORTRAN IV and operating on an 8K computer, while Hunt and Schwalbe [8] have determined equations for allowing crystals to be oriented even when the calculated adjustment exceeds the allowed movement on one arc of the goniometer. Methods of determining the degree of overlapping in twinned or grown-together crystals have been reported.[9] Rayment and co-workers [10] have devised an ingenious

[1] D. W. Banner, P. R. Evans, D. J. Marsh, and D. C. Phillips, *J. Appl. Cryst.*, 1977, **10**, 45.
[2] C. Thaxton and E. Lattman, *J. Appl. Cryst.*, 1977, **10**, 60.
[3] W. Bauspiess, U. Bonse, W. Graeff, and H. Rauch, *J. Appl. Cryst.*, 1977, **10**, 338.
[4] J. F. W. Mallett, J. N. Champness, A. R. Faruqi, and T. H. Gossling, *J. Phys.* (*E*), 1977, **10**, 351.
[5] M. J. Ross and R. M. Stroud, *Acta Cryst.*, 1977, **A33**, 500.
[6] A. M. MacLeod and J. N. Chapman, *J. Phys.* (*E*), 1977, **10**, 37.
[7] M. K. Wood and E. E. Abola, *J. Appl. Cryst.*, 1977, **10**, 206.
[8] W. E. Hunt and C. H. Schwalbe, *J. Appl. Cryst.*, 1977, **10**, 502.
[9] W. Denner, H. D'Amour, H. Schulz, and W. Stoeger, *J. Appl. Cryst.*, 1977, **10**, 177.
[10] I. Rayment, J. E. Johnson, and D. Suck, *J. Appl. Cryst.*, 1977, **10**, 365.

Cell angles			*Mol. wt. & No. of subunits*	*Mol. wt. of Asym. unit*	V_m*	*Precipitant*	pH	*Ref.*
α	β	γ						
—	—	—	136 000 (4)	34 000	2.39	n-Propanol	5.0	*b*
—	—	—	136 000 (4)	68 000	2.41	n-Propanol	5.0	*b*
—	—	—	10 500 (1)	10 500	1.9	Ammonium sulphate	6.0	*c*
—	92.56°	—	340 000 (6)	330 000	3.43	Dialysis to low ionic strength	6.2	*e*
—	—	—	19 870 (1)	19 870	2.07	Phosphate buffer	7.0	*f*
—	—	—	20 000 (1)	20 000	2.09	Phosphate buffer [g]	7.0	*h*
—	—	—	20 000 (1)	40 000	3.01	Phosphate buffer [g]	7.0	*h*
—	—	—	74 000 (1)	74 000	2.55	PEG6000 [ccc]	6.6	*i*
—	—	—	40 000 (1)	80 000	2.98	PEG6000	7.0	*k*
—	103.63 (2)°	dgh	6800 (1)	6800	1.59	Ammonium sulphate	4.5	*l*
—	—	—	85 000 (1)	85 000	2.47	Ethanol	7.8	*m*
—	—	120°	29 000 (1α, 1β)	174 000 [$(\alpha\beta)_6$]	1.7	Ammonium sulphate	7.0	*o*
—	—	—	440 000 (4)	110 000	2.75	Ammonium sulphate	7.4	*p*
80.6°	112.9°	114.7°	31 000 (1)	62 000	2.15	PEG6000	7.4	*q*
—	—	—	560 000 (8 large + 8 small)	560 000	3.72	Phosphate buffer	6.0	*r*
—	—	—	326 000 (6)	54 000	2.84	2-Methyl-2,4-pentanediol	8.0	*s*
—	—	—	197 000 (3)	394 000	3.04	Sodium citrate	7.0	*t*
—	—	—	40 000 (2)	40 000	2.67	2-Methyl-2,4-pentanediol	6.3, 7.8	*u*
—	—	—	40 000 (2)	40 000	2.60		6.3	
—	—	—	40 000 (2)	40 000	2.77		6.3, 7.8	
—	—	—	92 700 (2)	92 700	2.45	PEG6000	5.4	*v*
—	—	—	140 000 (4)	35 000	2.75	PEG400	7.2	*w*
—	—	—	140 000 (4)	140 000	2.35	2-Methyl-2,4-pentanediol	7.2	*w*
—	—	—	66 000 (1)	66 000	3.46	Ammonium sulphate	7.8	*x*
—	—	—	10 100 (1)	10 100	2.31	Ammonium sulphate	6.0	*y*

* Units are $Å^3$ $dalton^{-1}$.

Table 2 (*cont.*)

Protein	*Source*	*Space group*	*Cell dimensions*		
			a/Å	*b*/Å	*c*/Å
D-Glyceraldehyde-3-phosphate dehydrogenase	Sturgeon	$P6_122$	82.2	82.2	458
Phosphofructokinase	*Bacillus stearothermophilus*	$I222$	122	84	61
B-Phycoerythrin	*Porphyridium cruentum*	$R3$ or	111.0 189.1	— 189.1	— 60.1
α-Amylase	Pig pancreas	$P2_12_12_1$	70.6	114.7	118.5
		$P2_12_12_1$	56.0	88.3	104.1
Cow-pea chlorotic mottle virus		$P2_12_12_1$	522	383	308
Pancreatic polypeptide	Chicken or turkey pancreas	$C2$	34.18	32.91	28.45
Plastocyanin	Pea leaves	$P2_12_12_1$	49.0	53.3	82.6
Plastocyanin	Corn leaves	$P1$	24.8	30.0	58.5
Prothrombin fragment I [hh]	Bovine blood	$P4_12_12$ or $P4_32_12$	79.5	79.5	84.9
t-RNA-Asp	Yeast	$C222_1$	61	68	148
		$C222_1$	171	98	150
		$P6_222$	98	98	300
		$P6_322$	98	98	150
Aspartate aminotransferase	Chicken heart mitochondria	$P1$	55.6	58.7	76.0
Trypsinized elongation factor	*E coli*	$P4_12_12$	70.4	70.4	161.4
Lactollin	Bovine milk	$P2_12_12_1$	77.4	47.9	34.3
Fructose bisphosphatase	Chicken liver	$R3$	304	304	80.4
[des 16—20]Ribonuclease S′ [oo]	—	$P3_121$	44.64	44.64	97.3
[*p*-F-Phe 8, des 16—20]Ribonuclease S′	—	$P3_121$	44.64	44.64	97.1
Cholera toxin	*Vibrio cholerae*	$P2_1$	79.9	92.0	60.7
Fructose bisphosphatase	Turkey liver	$R3$	303	303	75
Haemocyanin subunit	Horse-shoe crab	$R32$	115	—	285

Cell angles			*Mol. wt. & No. of subunits*	*Mol. wt. of Asym. unit*	V_m*	*Precipitant*	pH	*Ref.*
α	β	γ						
—	—	—	145 000 (4)	72 500	3.2	Ammonium sulphate	7.0	*z*
—	—	—	130 000 (4)	32 500	2.4	Sodium phosphate	6.9	*aa*
116.8°	—	—	265 000	88 000 [bb]	2.26	Sodium	7.0	*cc*
—	—	120°	$[(\alpha\beta)_6\gamma]$			phosphate		*cc*
—	—	—	53 000 (1)	106 000	2.26	Calcium	?4 °C	*dd*
—	—	—	53 000 (1)	53 000	2.42	chloride	?25 °C	*dd*
—	—	—	4 600 000 (180)	4 600 000	3.3	Sodium phosphate	6.0	*ee*
—	105.26°	—	4240 (1)	4240	1.82	Tris/HCl, by cooling	7.0	*ff*
—	—	—	10 500 (1)	21 000	2.57	Ammonium sulphate	7.0	*gg*
96.17°	87.13°	78.67°	10 500 (1)	21 000	2.02	Ammonium sulphate	7.0	*gg*
—	—	—	22 000 (1)	22 000	3.05	Ammonium sulphate, or phosphate buffer	7.5	*ii*
—	—	—	25 000	25 000	3.07	Spermine/ ammonium sulphate	6.8	*jj*
—	—	—	25 000	100 000	3.14	Spermine/iso-	6.8	*jj*
—	—	—	25 000	50 000	4.16	propanol	6.8	*jj*
—	—	—	25 000	25 000	4.16	Spermine	6.8	*jj*
85.3°	109.2°	115.6°	90 000 (2)	90 000	2.34	PEG2000	7.5	*kk*
—	—	—	36 000 (1)	36 000	2.32	PEG4000	7.5	*ll*
—	—	—	12 000 (1)	12 000	2.69	—	—	*mm*
—	—	—	143 000 (4)	143 000	5.0	PEG4000	7.2	*nn*
—	—	—	—	—	—	Ammonium sulphate	5.3	*pp*
—	—	—	—	—	—	Ammonium sulphate	5.3	*pp*
—	106.40°	—	84 000 (1A + 5B)	84 000	2.55	PEG4000	—	*qq*
—	—	—	144 000 (4)	144 000	3.9	PEG4000	7.5	*rr*
—	—	—	70 000 (1)	70 000	2.02	PEG6000	6.0	*ss*

* Units are $Å^3$ $dalton^{-1}$.

Table 2 (*cont.*)

Protein	*Source*	*Space group*	*Cell dimensions*		
			a/Å	*b*/Å	*c*/Å
Isocitrate dehydrogenase	*Azotobacter vinelandii*	$P4_22_12$	122.1	122.1	163.9
Ricin [uu]	Castor seeds	$P2_12_12_1$	72.9	79.1	114.7
Bence-Jones protein Pav	Human urine	$P2_12_12_1$	93.6—95.1	92.7 (3)	72.8 (2)
Cytochrome *c'*	*Rhodopseudomonas capsulata*	$P6_2$ or $P6_4$	72.2	72.2	52.4
	Rhodospirillum molischianum	$P2_12_12_1$	56.5	72.5	75.7
Quinolinate phospho-ribosyltransferase	Pig liver	$P6_322$	120.9	120.9	95.0
C-Phycocyanin	*Anabaena variabilis*	$P6_3$	154.1 (1)	—	40.07 (6)
B-Phycoerythrin	*Porphyridium cruentum*	$R3$	188.7 (4)	—	60.0 (2)
Pepsinogen	Pig pancreas	$C2$	104.8	43.1	88.4
Ribulose-1,5-bisphosphate carboxylase-oxygenase	Tobacco	$P4_22_12$	149	149	138

(*a*) Changes from native $P2_122_1$ caused by soaking in thallium nitrate. (*b*) M. Yonei, Y. Mitsui, and Y. Iitaka, *J. Mol. Biol.*, 1977, **110**, 179. (*c*) G. V. Chapman, P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa, *ibid.*, p. 187. (*d*) Proteolytically degraded forms of fibrinogen. (*e*) N. M. Tooney and C. Cohen, *J. Mol. Biol.* 1977, **110**, p. 363. (*f*) C. H. Carlisle, P. F. Lindley, D. S. Moss, and C. Slingsby, *ibid.*, 1977, **110**, 417. (*g*) Different protein concentrations give the different crystal forms. (*h*) Yu. N. Chirgadze, S. V. Nikonov, M. B. Garber, and L. S. Reshetnikova, *J. Mol. Biol.* 1977, **110**, 619. (*i*) H. G. Mannherz, W. Kabsch, and R. Leberman, *F.E.B.S. Letters*, 1977, **73**, 141. (*j*) Tryptic digestion of the elongation factor. (*k*) W. H. Gast, W. Kabsch, A. Wittinhofer, and R. Leberman, *F.E.B.S. Letters*, 1977, **74**, 88. (*l*) D. L. Hwang, D. E. Foard, and C. H. Wei, *J. Biol. Chem.*, 1977, **252**, 1099. (*m*) E. N. Baker and S. V. Rumball, *J. Mol. Biol.*, 1977, **111**, 207. (*n*) M. L. Hackert, C. Abad-Zapatero, S. E. Stevens, and J. L. Fox, *ibid.*, p. 365. (*o*) Accessory pigment of blue-green algae. (*p*) P. J. Artymiuk, C. C. F. Blake, and P. J. Geary, *J. Mol. Biol.*, 1977, **111**, 203. (*q*) J. D. Robertus, A. F. Monzingo, and J. D. Irvin, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 775. (*r*) T. S. Baker, D. Eisenberg, and F. Eiserling, *Science*, 1977, **196**, 293. (*s*) F. Jurnak, A. Rich, L. Van Loon-Klaassen, H. Bloemendal, A. Taylor, and F. H. Carpenter, *J. Mol. Biol.*, 1977, **112**, 149. (*t*) D. C. Wiley and J. J. Skehel, *ibid.*, p. 343. (*u*) J. D. G. Smit, J. Pulver-Sladek, and J. N. Jansonius, *ibid.*, p. 491. (*v*) A. Arnone, P. H. Rogers, J. Schmidt, C.-N. Han, C. M. Harris, and D. E. Metzler, *ibid.*, p. 509. (*w*) A. Wolodawer, J. Roberts, and J. S. Holcenberg, *ibid.*, p. 515. (*x*) J. Lee, S. C. Chang, K. Hahm, A. J. Glaid, O. Gawron, B. C. Wang, C. S. Yoo, M. Sax, and J. Glusker, *ibid.*, p. 531. (*y*) P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, M. P. Venkatappa, and L. E. Vickery, *ibid.*, p. 649. (*z*) M. A. Holmes, S. J. Remington, B. Schwendimann, G. E. Christie, and B. W. Matthews, *ibid.*, p. 651. (*aa*) P. J. Hudson, H. Hengarner, and J.-I. Harris, *Biochem. Soc. Trans.*, 1977, 5, 725. (*bb*) γ-Subunit seems to be

Cell angles			Mol. wt. & No. of subunits	Mol. wt. of Asym. unit	V_m*	Precipitant	pH	Ref.
α	β	γ						
—	—	—	80 000 (1)	160 000	1.91	Ammonium sulphate, or phosphate buffer	6.5—8.0	*tt*
—	—	—	65 000 (1A + 1B)	65 000	2.54	PEG6000	4.75	*vv*
—	—	—	50 000 (2)	50 000	3.17	PEG4000 or ammonium sulphate	Wide range	*ww*
—	—	120°	28 000 (2)	28 000	2.37	Ammonium sulphate	—	*xx*
—	—	—	28 000 (2)	14 000	2.77	Ammonium sulphate	4.2	*xx*
—	—	—	172 000 (6)	172 000	3.59	Ammonium sulphate, 4 °C	7.5	*yy*
—	—	120°	70 000 [$(\alpha\beta)_2$]	70 000	2.0 (2)	Ammonium sulphate	7.3	*zz*
—	—	120°	240 000 [$(\alpha\beta)_6\gamma$]	240 000	2.8 (1)	Ammonium sulphate	6.9	*zz*
—	91.3°	—	39 600 (1)	39 600	2.52	Lithium sulphate	6.0	*aaa*
—	—	—	560 000 (8 large + 8 small)	140 000	2.71	Ammonium sulphate	5.0	*bbb*

* Units are $Å^3$ $dalton^{-1}$.

disordered. (*cc*) C. Abad-Zapatero, J. L. Fox, and M. L. Hackert, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 266. (*dd*) M. Pierrot, J. P. Astier, B. Abadie, and G. Marchis-Mouren, *F.E.B.S. Letters*, 1977, **79**, 105. (*ee*) I. Rayment, P. Argos, and J. E. Johnson, *J. Ultrastructure Res.*, 1977, **61**, 240. (*ff*) S. P. Wood, J. E. Pitts, T. L. Blundell, I. J. Tickle, and J. A. Jenkins, *European J. Biochem.*, 1977, **78**, 119. (*gg*) Yu. N. Chirgadze, M. B. Garber and S. V. Nikonov, *J. Mol. Biol.*, 1977, **113**, 443. (*hh*) Contains 17.5% carbohydrate, which may account for the 60% liquid content of the crystals. (*ii*) R. Aschaffenburg, C. C. F. Blake, J. M. Burridge, and M. P. Esnouf, *J. Mol. Biol.*, 1977, **114**, 575. (*jj*) R. Giege, D. Moras, and J. C. Thierry, *ibid.*, 1977, **115**, 91. (*kk*) H. Gehring, P. Christen, G. Eichele, M. Glor, J. N. Jansonius, A.-S. Reimer, J. D. G. Smit, and C. Thaller, *ibid.*, p. 97. (*ll*) F. Jurnak, A. Rich, and D. Miller, *ibid.*, p. 103. (*mm*) J. W. Becker, J. A. Ziffer, G. M. Edelman, and B. A. Cunningham, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3345. (*nn*) W. F. Anderson and B. W. Matthews, *J. Biol. Chem.*, 1977, **252**, 5556. (*oo*) Synthetic RNase S lacking residues 16—20 and, in some cases, with further substitutions. (*pp*) I. M. Chaiken, H. C. Taylor, and H. L. Ammon, *J. Biol. Chem.*, 1977, **252**, 5599. (*qq*) P. B. Sigler, M. E. Druyan, H. C. Kiefer, and R. A. Finkelstein, *Science*, 1977, **197**, 1277. (*rr*) A. McPherson, D. Burkey, and P. Stankiewicz, *J. Biol. Chem.*, 1977, **252**, 7031. (*ss*) K. A. Magnus and W. E. Love, *J. Mol. Biol.*, 1977, **116**, 171. (*tt*) E. W. Czerwinski, P. H. Bethge, F. Scott Matthews, and A. E. Chung, *ibid.*, p. 181. (*uu*) Anti-tumour glycoprotein Ricin II. (*vv*) J. E. Villafranca and J. D. Robertus, *J. Mol. Biol.*, p. 331. (*ww*) B. C. Wang, C. S. Yoo, R. Y. Hwan, M. Sax, W. E. Brown, and M. Michaels, *J. Mol. Biol.*, 1977, **116**, 619. (*xx*) P. Weber and F. R. Salemme, *ibid.*, 1977, **117**, 815. (*yy*) W. D. L. Musick, *ibid.*, p. 1101. (*zz*) R. M. Sweet, H. E. Fuchs, R. G. Fisher, and A. N. Glazer, *J. Biol. Chem.*, 1977, **252**, 8258. (*aaa*) S. N. Rao, S. N. Koszelak, and J. A. Hartsuck, *ibid.*, p. 8728. (*bbb*) T. S. Baker, S. W. Suh, and D. Eisenberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1037. (*ccc*) Polyethylene glycol.

Table 3 *Low-resolution structure determinations*

Protein	*Source*	*Resolution/Å*	*Comments*	*Ref.*
Tomato bushy stunt virus	—	5.5	Each subunit has two distinct domains, with only one obvious connection. Domain 1 is smaller and forms half of each of the dyad clustered protrusions. Domain 2 constitutes the polyhedral shell of the virion. The coat protein shows two distinct conformations dependent on its position being near a local or a true two-fold axis. The domains' structures are invariant and the two conformers are related by movements about the hinge	*a*
Myohaemerythrin	Sipunculan worm *Themiste zostericola*	5.5	Examination of the structure at low resolution has shown the existence of a pseudo-dyad through the molecule, relating two large domains of the protein	*b*
Tobacco mosaic virus	—	4.0	There are four recognizable regions in the structure: (i) the four packed 'radical helices' (ii) the RNA binding site – formed between two subunits (iii) the carboxyl cage – 8 residues (3 Glu, 3 Asp, 2 Arg) – a binding site for divalent cations? (iv) the vertical helices – form the inner wall and protect the RNA	*c*
6-Phosphogluconate dehydrogenase	Sheep liver	6.0	A dimer with a crystallographic two-fold axis. There is an indentation in each subunit, distant from the subunit interface, and several helices (up to 40 Å long) can be recognized	*d*
Glutathione reductase	Human erythrocytes	6.0	Three domains can be recognized. Domain I binds $NADP^+$ and glutathione binds to domain III with an 18 Å gap between the nicotinamide ring and the —SS— bridge of oxidized glutathione. It is assumed that FAD binds to domain II and bridges this gap	*e*

Nucleosome cores	Rat liver	25	The particle is approximately 110 × 110 × 57 Å, and is flat and somewhat wedge-shaped. There are 1.75 turns of DNA wound on a histone core	*f*
Elongation factor (EF-Tu)	*E. coli*	6	The molecule appears to be 75 × 50 × 35 Å and consists of a compact globular head (45 × 40 × 40 Å) and a curled tail (55 Å long × 25 Å diameter). The molecule shows a groove in the centre which could accommodate an RNA double-helix	*g*
Protamine/t-RNA	Salmon sperm/yeast	5.4	The protein is composed of four α-helices joined by three partially flexible hinge regions. Each helix is long enough to perform double-helix cross-linking. The flexible joints will allow repositioning of the helices in such a way that DNA molecules in a variety of orientations can be condensed	*h*
Pancreatic polypeptide	Chicken or turkey pancreas	4.5	The molecules are partly α-helical and are arranged as compact dimers about the crystallographic two-fold axes	*i*

(*a*) F. K. Winkler, C. E. Schutt, S. C. Harrison, and G. Bricogne, *Nature*, 1977, **265**, 509. (*b*) W. A. Hendrickson and K. B. Ward, *J. Biol. Chem.*, 1977, **252**, 3012. (*c*) G. Stubbs, S. Warren, and K. Holmes, *Nature*, 1977, **267**, 216. (*d*) M. J. Adams, J. R. Helliwell, and C. E. Bugg, *J. Mol. Biol.*, 1977, **112**, 183. (*e*) H. A. Zappe, G. Krohne-Ehrich, and G. E. Schulz, *ibid.*, 1977, **113**, 141. (*f*) J. T. Finch, L. C. Lutter, D. Rhodes, R. S. Brown, B. Rushton, M. Levitt, and A. Klug, *Nature*, 1977, **269**, 29. (*g*) W. Kabcsh, W. H. Gast, G. E. Schulz, and R. Leberman, *J. Mol. Biol.*, 1977, **117**, 999. (*h*) R. Wade Warrant and S.-H. Kim, *Nature*, 1977, **271**, 130. (*i*) S. P. Wood, J. E. Pitts, T. L. Blundell, I. J. Tickle, and J. A. Jenkins, *European J. Biochem.*, 1977, **78**, 119.

Table 4 *Low-angle X-ray scattering*

Structure	*Source*	*Mol. wt.*	R_G/Å	V/Å^3	*Comments*	*Ref.*
β-Haemocyanin	*Helix pomatia*	700 000 350 000	90.0 75.0	1.35×10^6 0.635×10^6	Dissociation products of the β-haemocyanin molecule (mol. wt. 9×10^6). These two products are thought to consist of 16 and 8 subunits respectively	*a*
β-Haemocyanin	*Helix pomatia*	9×10^6	184	14×10^6	Molecule suggested to be a hollow cylinder, with relative dimensions of height : outer, diameter : inner diameter of 1.2 : 1.0 : 0.45	*b*
Antithrombin III (+heparin)	Human	65 000 (+10 700)	29.7	—	Antithrombin behaves as an ellipsoid with semi-axes 19, 37, and 52 Å. Little change on binding heparin, suggesting that it may bind in a long deep cleft	*c*
Fatty acid synthetase	Pig liver	490 000	69.0	0.88×10^6	Best fit to the scattering curve suggests a highly anisotropic structure, and the possibility is that the growing fatty-acid chain is passed from one half of the molecule to the other during each step of the synthetic cycle	*d*
Immunoglobulin KOL	Human	150 000	58.4	0.33×10^6	Molecule appears to be more extended in solution than is seen in the crystal	*e*
Aspartate transcarbamylase	—	—	—	—	Binding of transition-state analogue gives substantial changes	*f*
D-Riboluse-1,5-diphosphate carboxylase	*Dasycladus* (green alga)	535 000	45.5	0.74×10^6	Purified enzyme and redissolved crystals give identical values. Molecule appears to be a sphere of radius 56 Å	*g*
Malate synthase	Yeast	186 000	39.6	0.343×10^6	Molecule appears to be a cylinder 54 × 36 Å high. Small structural changes on substrate binding	*h*
ε-Subunit of chloroplast coupling factor	Spinach	11 900	11.8	17 000	In solution, ε-subunit is a prolate ellipsoid, with half axes of $a = b = 12.7$ Å, $c = 25.4$ Å	*i*
Turnip yellow mosaic virus	—	5.53×10^6	111	—	Small-angle neutron-scattering work, using the contrast-variation method	*j*

Southern bean mottle virus	—	6.69×10^6	123	—		
Cucumber mosaic virus	—	5.78×10^6	127	—		
Bromegrass mosaic virus	—	4.70×10^6	113 or 134	—	Best model has low electron density at the centre of the particle. Protein appears to be 56—84 Å from the centre of the particle, with the RNA 14—56 Å from the centre and ordered	*k*
Ribosomal L7/12 + L10 complex	*E. coli*	60 000	45	101 800	Complex appears to consist of two L7/L12 dimers + one L10 molecule. L7 and L12 appear to be highly elongated	*l*
Ribosomal protein S4	*E. coli*	23 800	42	37 700	Molecule appears to be a flattened, elongated disc	*m*
L18 + 5*S* RNA	*E. coli*	50 100	38.5	—	L18 molecule seems to extend well away from the RNA	*n*
50*S* ribosome subparticle	*E. coli*	—	66 (RNA) 102 (protein)	—	Tight packing of RNA with protein on the periphery	*o*
25.5*S* ribosome particle	*E. coli*	—	72	—	30*S* particle stripped of 10—12 proteins. Since the shape hardly changes, it seems that these proteins do not maintain the compact structure	*p*
S4-RNA	*E. coli*	136 000	43.5	—	Appears to be an oblate ellipsoid 132 × 132 × 32 Å	*q*
Myosin subfragment S1	—	115 000	32.0	—	Particle shown to be non-spherical	*r*

(*a*) J. Berger, I. Pilz, R. Witters, and R. Lontie, *European J. Biochem.*, 1977, **73**, 247. (*b*) J. Berger, I. Pilz, R. Witters, and R. Lontie, *ibid.*, 1977, **80**, 79. (*c*) B. Furugren, L.-O. Andersson, and R. Einarsson, *Arch. Biochem. Biophys.*, 1977, **178**, 419. (*d*) W. Folkhard, B. Felser, I. Pilz, O. Kratky, H. Dutler, and H. Vogel, *European J. Biochem.*, 1977, **81**, 173. (*e*) I. Pilz, E. Schwarz, and W. Palm, *ibid.*, 1977, **75**, 195. (*f*) M. F. Moody, *Biophys. J.*, 1977, **17**, 181a. (*g*) H. H. Paradies, B. Zimmer, and G. Werz, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 397. (*h*) P. Zipper and H. Durchschlag, *ibid.*, 1977, **75**, 394. (*i*) U. D. Schmidt and H. H. Paradies, *ibid.*, 1977, **78**, 383. (*j*) B. Jacrot, C. Chauvin, and J. Witz, *Nature*, 1977, **266**, 417. (*k*) B. Sjöberg, *European J. Biochem.*, 1977, **81**, 277. (*l*) R. Osterberg, B. Sjöberg, I. Pettersson, A. Liljas, and C. G. Kurland, *ibid.*, 1977, **73**, 22. (*m*) R. Osterberg, B. Sjöberg, R. A. Garrett, and J. Littlechild, *ibid.*, p. 25. (*n*) R. Osterberg and R. A. Garrett, *ibid.*, 1977, **79**, 67. (*o*) I. N. Serdyuk and A. K. Grenader, *ibid.*, p. 495. (*p*) I. N. Serdyuk, A. K. Grenader, and V. E. Koteliansky, *ibid.*, p. 505. (*q*) R. Osterberg, B. Sjöberg, R. A. Garrett, and E. Ungewickeell, *ibid.*, 1977, **80**, 169. (*r*) K. M. Kretzschmar, R. A. Mendelson, and M. F. Morales, *Biophys. J.*, 1977, **17**, 36a.

method of mounting Southern bean mosaic virus crystals which allows the crystals to be mounted wet while simultaneously minimizing crystal slippage. Denne[11] discusses the use of the W/θ scan on diffractometers as an efficient monochromation technique and has demonstrated that it is quite straightforward to work with the $K\alpha_1$ copper line for medium- or high-angle reflections. Several papers have appeared on new ways of applying the absorption correction to *X*-ray intensity data.[12–14] An improved method for combining isomorphous replacement and anomalous scattering data from biological macromolecules has been communicated by Einstein.[15] Tanaka[16] has reported a method of representing the fast-rotation function in a polar co-ordinate system, allowing much faster calculations to be performed, while Sygusch[17] has described a theory for the simultaneous least-squares refinement of protein phases and heavy-atom parameters. A new translation function, a generalization of the function introduced by Rossmann, Blow, Harding, and Coller, has been defined[18] and has been used in a study of bovine liver catalase.

The interpretation of low-resolution electron-density maps for large molecules frequently proves to be very difficult and, usually, no progress can be made with phase extension by direct methods. Podjarny and Yonath[19] describe an iterative procedure for map modification which allows the use of matrix methods. The technique has been applied to extend phase information from 5 Å to 3 Å for yeast $tRNA^{Phe}$. Since this range of resolution is often characterized by a failure of isomorphous replacement to provide sufficiently good phases, it may well be widely applicable, especially to proteins.

The structure of sickling deer type III haemoglobin has been solved by means of the rotation and translation functions.[20] The molecules pack to form a distorted hexagonal structure with open solvent channels, the network of molecules bearing an unmistakable relation to the fibres seen in human sickled cells. The structure was later refined, using a restrained least-squares refinement procedure.[21] The initial co-ordinates with an *R* factor of 0.43 (4—9 Å data) have been subjected to 16 cycles of refinement and the *R* factor has been reduced to 0.28 (1.98—5 Å data). Interpretation of the electron-density maps was carried out with a graphics display system. Chambers and Stroud[22] have reported the refinement of diisopropyl fluorophosphate-inhibited bovine trypsin at 1.5 Å. The process has been carried out almost entirely on a minicomputer and has lowered the *R* factor from 0.472 at 2.7 Å to 0.235 at 1.5 Å resolution; the authors show that substantial changes (by several Å) have been necessary in some areas. Sussman and co-

[11] W. A. Denne, *Acta Cryst.*, 1977, **A33**, 987.
[12] B. K. Lee and J. R. Ruble, *Acta Cryst.*, 1977, **A33**, 629.
[13] B. K. Lee and J. R. Ruble, *Acta Cryst.*, 1977, **A33**, 637.
[14] H. D. Flack, *Acta Cryst.*, 1977, **A33**, 890.
[15] J. R. Einstein, *Acta Cryst.*, 1977, **A33**, 75.
[16] N. Tanaka, *Acta Cryst.*, 1977, **A33**, 191.
[17] J. Sygusch, *Acta Cryst.*, 1977, **A33**, 512.
[18] D. B. Litvin, *Acta Cryst.*, 1977, **A33**, 62.
[19] A. D. Podjarny and A. Yonath, *Acta Cryst.*, 1977, **A33**, 655.
[20] W. C. Schmidt, R. L. Girling, T. E. Houston, G. D. Sproul, E. L. Amma, and T. H. J. Huisman, *Acta Cryst.*, 1977, **B33**, 335.
[21] W. C. Schmidt, R. L. Girling, and E. L. Amma, *Acta Cryst.*, 1977, **B33**, 3618.
[22] J. L. Chambers and R. M. Stroud, *Acta Cryst.*, 1977, **B33**, 1824.

workers [23] have described the refinement of yeast $tRNA^{Phe}$, using a method of structure factor least-squares refinement. The method involves constrained groups linked by distance restraints and has been used on yeast $tRNA^{Phe}$, lowering the *R* factor from 0.42 to 0.25 at 2.7 Å resolution. This procedure is directly applicable to protein structures. A phase-extension technique specifically for proteins has been reported by Agarwal and Isaacs.[24] This method involves refinement, by least-squares methods, of the positional and thermal parameters of a set of dummy atoms placed in the initial low-resolution electron-density map so as to minimize the discrepancy between the calculated and observed structure factors. Phases calculated using the refined atomic co-ordinates are used to extend the resolution and improve the quality of the electron-density map. The method uses a new least-squares algorithm which has a large radius of convergence and has been used successfully to phase the structure factors of 2-zinc insulin at 2 Å and 1.5 Å, starting from a set of isomorphous phases at 3 Å resolution. Since refinements always require several calculations of structure factors, the fast Fourier-transform method described by Ten Eyck [25] is of great interest. The cost (in computer terms) of this method compared to standard methods has been presented, and it has been shown that the fast Fourier-transform technique is 3½ to 7 times less expensive than conventional methods for non-centrosymmetric space groups.

Work on the use of synchrotron radiation has continued in the past year. The design and use of a camera for low-angle *X*-ray diffraction experiments have been reported.[26] All important adjustments can be carried out remotely, with television monitoring, and a comparison is drawn with conventional *X*-ray sources. The operating conditions of the synchrotron, the NINA facility at the Daresbury Laboratory, are described in an appendix. Two papers on the design and construction of *X*-ray monochromators have been published.[27, 28] The direct application of synchrotron radiation to protein crystallography has been reported by Phillips and co-workers.[29] Precession photographs were recorded from rubredoxin crystals at seven discrete wavelengths, some above and some below the iron atom's *K* absorption edge. The iron site could be correctly located from difference Patterson and difference Fourier maps based on either the real or the imaginary part of the anomalous scattering, and in an appendix the authors describe a method for the Lorentz-polarization correction of data from precession photographs recorded using synchrotron radiation.

Excess small-angle *X*-ray scattering from dilute solutions of macromolecules is usually interpreted with the assumption that the electron density within the macromolecule is constant. Carpenter and Mattice [30] have examined seven models for the relative effects of shape and distribution of electron density. These

[23] J. L. Sussman, S. R. Holbrook, G. M. Church, and S.-H. Kim, *Acta Cryst.*, 1977, **A33**, 800.
[24] R. C. Agarwal and N. W. Isaacs, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2835.
[25] L. F. Ten Eyck, *Acta Cryst.*, 1977, **A33**, 486.
[26] J. C. Haselgrove, A. R. Faruqi, H. E. Huxley, and U. W. Arndt, *J. Phys.* (*E*), 1977, **10**, 1035.
[27] J. B. Hastings, *J. Appl. Phys.*, 1977, **48**, 1576.
[28] N. G. Webb, S. Samson, R. M. Stroud, R. C. Gamble, and J. D. Baldeschwieler, *J. Appl. Cryst.*, 1977, **10**, 104.
[29] J. C. Phillips, A. Wlodawer, J. M. Goodfellow, K. D. Watenpaugh, L. C. Sieker, L. H. Jensen, and K. O. Hodgson, *Acta Cryst.*, 1977, **A33**, 445.
[30] D. K. Carpenter and W. L. Mattice, *Biopolymers*, 1977, **16**, 67.

results were then used in an analysis of 16 globular proteins to compare observed and calculated radii of gyration.[31] Fedorov and Denesyuk [32] have described results obtained for the large-angle *X*-ray diffuse scattering of some globular proteins, and presented a new rapid method for calculating diffuse scattering curves. Analysis of Soule–Porod plots of protein small-angle scattering data,[33] a new method for determining the radius of gyration from small-angle data,[34] and corrections for the angle dependence of Lorentz polarization and structure factors in *X*-ray diffraction-line profiles [35] have been published. Methods for the improvement of the alignment in diffuse fibre diffraction patterns which allow the pattern to be sharpened azimuthally have been reported by Lovell and Windle,[36] and Bacon and co-workers [37] have analysed the systematic variations in the position of Bragg peaks in neutron diffraction. Scheringer [38] has investigated the extent to which the peak heights in difference Fourier maps are reduced by termination errors in the Fourier series and thermal smearing, and also describes deconvolution procedures which allow reconstruction of the true peaks.

The successor to MULTAN, namely MAGLIN, has been described by Woolfson,[39] who predicts that MAGLIN will not only be considerably faster than MULTAN but will be able to solve the more complicated structures which MULTAN cannot handle. The theory of magic integers, applicable to both MULTAN and MAGLIN, has been reported by Main.[40]

The display and interpretation of electron-density maps using the optical comparator is a time-consuming, error-prone, and laborious procedure. An optical method [41] has been devised in which the data, phased by conventional techniques, are subjected to a one-dimensional Fourier transformation and the results are transferred to a photographic transparency. By shining coherent laser light through the transparency, a three-dimensional image of the molecule is obtained. There have also been several computer graphics systems described in which the Richards' box and model-building components have been replaced by high-performance computer-controlled visual display units. Tsernoglou and co-workers [42] have reported the fitting of the 2.2 Å resolution map of snake venom neurotoxin, the entire process being carried out on a computer-controlled graphics system; it takes only 60 h for two people to produce co-ordinates for all the atoms. The co-ordinates produced gave an *R* factor of 0.47, which is comparable to *R* factors for conventionally built models. Nir and co-workers [43] describe a graphics system for building, manipulating, and displaying molecules. They present examples of its use in the study of the template properties of polynucleotides and

[31] W. L. Mattice, and D. K. Carpenter, *Biopolymers*, 1977, **16**, 81.
[32] B. A. Fedorov and A. I. Denesyuk, *Acta Cryst.*, 1977, **B33**, 3198.
[33] N. S. Murthy and J. R. Knox, *J. Appl. Cryst.*, 1977, **10**, 137.
[34] O. Glatter, *J. Appl. Cryst.*, 1977, **10**, 415.
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[36] R. Lovell and A. H. Windle, *Acta Cryst.*, 1977, **A33**, 390.
[37] G. E. Bacon, N. Cowlam, and A. G. Self, *Acta Cryst.*, 1977, **A33**, 46.
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[40] P. Main, *Acta Cryst.*, 1977, **A33**, 750.
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their specific interactions with polypeptides. A third system has been reported by Jack [44] which, though not a graphics system, is of considerable use in the interactive fitting of co-ordinates to a skeletal wire model. Only two or three co-ordinates need to be measured for each residue and the program then adjusts the torsion angles to fit the guide points as well as possible, and produces co-ordinates for all the atoms in the residue. The analysis of the protein structures known to date has been greatly assisted by recent advances at the computer-based Protein Data Bank.[45, 46] Atomic co-ordinates and connectivities are stored in a standard format and each entry contains information on the protein and its structure determination. There are also files of structure factors and phases, together with torsion angles for some of the entries, and all the data stored in the bank are available on magnetic tape for public distribution.

3 Immunoglobulins

Crystal structure determinations on fragments of immunoglobulins have provided the basic information on the conformation of immunoglobulins and have allowed inferences to be drawn as to the functioning of the intact antibody molecules. The structure of the dimer of the variable domains of the Bence-Jones protein ROY has been reported.[47] The structure was solved by Patterson-function search procedures, using the known structure of the Bence-Jones protein REI, the rotation functions being calculated for both a dimer and a monomer model of the REI protein. The monomer search gave a maximum overlap with a peak height 1.25 times above the highest noise peak, the overlap for the dimer search giving a peak height 1.40 times noise at approximately the same position. Refinements, including overall temperature factor and scale factor, have given an R factor of 0.336 for the 780 strongest reflections from 6.5 to 3.0 Å resolution. A difference Fourier map showed two large positive peaks corresponding to the side-chains of Leu-96, which was excluded from F_{calc}, and Tyr-49, which, even though it was included in F_{calc}, undergoes a conformational change relative to REI. Density was also seen for several side-chains that are known to be different in the REI and ROY sequences and omitted from F_{calc}. Overall, the ROY protein is seen to be more similar to the AU protein than it is to the REI protein.

The structure of an intact human immunoglobulin, IgGDob, has been analysed,[48] using the known domain co-ordinations from investigations of immunoglobulin fragments and a previously calculated low-resolution electron-density map. Co-ordinates for the domain under study were systematically rotated and translated in the electron-density map, and the degree of fit was calculated, based on the number of transformed co-ordinates which overlapped density. Fitting of the F_c domains was relatively easy but the quality of the F_{ab} region of the map

[44] A. Jack, *Acta Cryst.*, 1977, **A33**, 497.
[45] F. C. Bernstein, T. F. Koetzle, G. J. B. Williams, E. F. Meyer, jun., M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi, and M. Tasumi, *J. Mol. Biol.*, 1977, **112**, 535.
[46] F. C. Bernstein, T. F. Koetzle, G. J. B. Williams, E. F. Meyer, jun., M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi, and M. Tasumi, *European J. Biochem.*, 1977, **80**, 319.
[47] P. M. Colman, H. J. Schramm, and J. M. Guss, *J. Mol. Biol.*, 1977, **116**, 73.
[48] E. W. Silverton, M. A. Navia, and D. R. Davies, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 5140.

was poorer, resulting in broad peaks in the fitting profiles. The derived structure is T-shaped, with the relative orientation of the F_c domains being similar to that seen in the isolated F_c structure. The C_H2 domains of the F_c structures have no direct contacts, being separated by the carbohydrate chains, whose predicted position corresponds to a branched chain of strong density in the map.

The structures of the variable and constant F_{ab} domains were sufficiently different to allow one to distinguish between these regions on the basis of overlap count, and the hinge region between the F_{ab} and F_c domains, in this protein only seven residues long, was fitted in a region of strong electron density. Although the variable and constant regions of the F_{ab} were fitted separately, the ends of the two chains which were to be joined fell within 5 Å of each other, allowing connection to be made by simple adjustments in the switch region.

Although the method cannot give any of the finer details of the individual domain structures, it has given, for the first time, a three-dimensional picture of an IgG molecule in its entirety, particularly the relationship of F_{ab} to F_c. The important role of the complex carbohydrate in the formation of the interface between the two C_H2 domains and also between C_H2 and C_H1 has also been demonstrated.

4 Electron Transport and Redox Reactions

Myoglobin.—The structures of metmyoglobin [49] and deoxymyoglobin [50] have been refined at 2 Å resolution, using new data collected on a four-circle diffractometer. For the metmyoglobin refinement [49] the first electron-density map calculated used the previously determined multiple isomorphous replacement phases, with the newly collected amplitudes. The known structure of metmyoglobin was plotted onto this map and the ill-defined residues were adjusted to give the best fit to the density. After real-space refinement these co-ordinates were used to calculate phases and used in subsequent Fourier and difference Fourier syntheses. The refined model, with an *R* factor of 23.5%, has a root-mean-square difference in atomic positions of 0.93 Å, and 111 of the 145 atoms of uncertain position in the original co-ordinate list have been placed. The exact conformations of the *N*-terminal valine residue and the *C*-terminal glycine residue are still uncertain, as the electron density for these two residues is weak. The electron density for the haem group was very clear, and the displacement of the iron atom from the porphyrin plane towards His-8F could be clearly seen. This displacement, 0.40 Å, is in good agreement with the 0.3 Å reported previously for myoglobin and for other iron–porphyrin compounds. The separation of the plane of the four pyrrole ring nitrogens from the mean haem plane is 0.13 Å. When real-space refinement allowed the pyrrole rings to turn, it was seen that pyrroles I, II, and III became tilted towards His-8F while pyrrole IV moved the other way. A difference Fourier map, calculated for a flat iron–porphyrin compound to check this strange geometry, indicated that pyrroles I and II should be bent to bring their nitrogen atoms closer to His-8F, while pyrroles III and IV showed no preference. This map also confirmed the displacement of the iron atom from the

[49] T. Takano, *J. Mol. Biol.*, 1977, **110**, 537.
[50] T. Takano, *J. Mol. Biol.*, 1977, **110**, 569.

plane. There were 83 inter-atomic contacts, excluding hydrogens, of less than 4 Å between the haem group and its surrounding residues. Some 82 solvent molecules have been placed, and most are interpreted as waters bound to the surface hydrophilic side-chains. Comparison with horse methaemoglobin gave root-mean-square differences in atomic positions for the main chain and common side-chains of 1.94 Å for the α-chain and 1.85 Å for the β-chain, while the haem group is seen to have rotated around the bond between histidine nitrogen and the iron atom, causing it to move away from helix G.

The structure of deoxymyoglobin has been determined,[50] using newly observed structure amplitudes and phases calculated from the metmyoglobin model.[49] Starting from a difference Fourier synthesis, real-space and Fourier refinement were carried out, giving a final *R* factor of 23.3%. The mean atomic shift between deoxy- and met-myoglobin is 0.23 Å. The iron atom's displacement from the porphyrin plane increases from 0.40 to 0.55 Å, the movement of the iron atom appearing to be at 60° to the haem plane normal axis. Since this would destroy the four-fold co-ordination symmetry, the movement was constrained to the haem normal. His-8F moves in approximately the same direction, the sideways shift of 0.15 Å for His-8F increasing the angle between the haem axis and the iron–histidine nitrogen bond from 3.9 to 10.7°. The number of inter-atomic contacts between the haem and the surrounding residues decreases from 83 to 74, part of this decrease being due to the removal of the water molecule. The changes seen in going from met- to deoxy-myoglobin are seen to be similar to those seen in haemoglobin, but occur on a smaller scale.

Haemoglobin.—The structure of human foetal deoxyhaemoglobin FII has been solved at a resolution of 2.5 Å,[51] using a single isomorphous derivative and the known structure of human adult deoxyhaemoglobin. The difference Fourier synthesis between the foetal and adult structures shows all the amino-acid substitutions clearly, together with several solvent molecules, even though they had not been included in the structure factor calculation. The α-subunits are seen to be very similar to the structure observed in the adult haemoglobin structure, and for the most part the γ-chains of foetal haemoglobin and β-chains of adult haemoglobin are identical except for the *N*-terminal region (the first three nonhelical residues) and helix A, which moves closer to the molecular dyad. This movement is similar to that seen on the binding of 2,3-diphosphoglycerate or inositol hexaphosphate to adult deoxyhaemoglobin (lowering its oxygen affinity) and in the foetal haemoglobin it may be caused by the replacement of Glu-A4β by an aspartate. This movement of the *N*-terminus also increases the distance of His-2γ from the two phosphate groups of 2,3-diphosphoglycerate, which may explain the lowered binding affinity.

Although the adult and foetal haemoglobins crystallize in different space groups, both crystals are composed of molecules stacked along the molecular *x*-axis. Except for the weakening of one electrostatic interaction, the inter-molecular contacts are the same, but this one change may explain the increased solubility and anti-sickling effect of the foetal haemoglobin.

[51] J. A. Frier and M. F. Perutz, *J. Mol. Biol.*, 1977, **112**, 97.

The structure of horse methaemoglobin reconstituted with mesohaem, in which the haem vinyl groups are converted into ethyl groups, has been reported.[52] The quaternary structures of metmesohaemoglobin and native methaemoglobin are identical, but extensive movements in the tertiary structure, propagating out from the haem pocket, are seen. In the α-subunits the ethyl group at position 2 is rotated relative to the vinyl group it replaces and the pyrrole rings move towards the proximal side of the haem, pyrroles II and III moving further than I and IV. These changes give a distinct change in the tilt of the α-haem group. The changes in the β-subunits are almost identical. In addition to several motions of individual residues in the haem pocket, extensive rigid-body motions are seen. In both the α- and β-subunits the E-helix follows the tilting haem group, while the G-helix is forced back by the haem tucking deeper into the pocket. Several movements of the F-helix towards the α_1–β_2 interface are seen. These alterations in structure are much greater than those seen for met-deuterohaemoglobin, in which the vinyl groups are totally removed. Since the main effect is the movement of the ethyl groups further out of the porphyrin plane than is seen for the vinyls, it is difficult to see how the overall differences in structure can be caused by steric factors alone. The movements may well be generated by subtle changes in the co-ordination of the iron atom in response to altered electron density, caused by an electronic contribution from the ethyl groups.

The structural alterations seen in deoxyhaemoglobin Tacoma have been described.[53] Despite the loss of two arginine residues, haemoglobin Tacoma has an unaltered electrophoretic mobility. This is found to be due to the carboxylate of Glu-B8β, which interacts with the arginine Arg-B12β in the native haemoglobin, moving to form hydrogen bonds to His-G18β and His-G19β, and so raising the p*K* values of the histidines that their positive charges compensate for the loss of the guanidinium groups. Since the Arg-B12β side-chains in the native structure make several hydrogen bonds in the $\alpha_1\beta_1$ contact, their removal accounts for the decreased stability of haemoglobin Tacoma and a lower oxygen equilibrium constant. The diminished alkaline Bohr effect cannot yet be explained in terms of the structural changes.

The structural changes involved in the binding of inositol hexaphosphate, IHP, to human fluoromethaemoglobin have been described.[54] The structure was investigated by means of difference Fourier synthesis against deoxyhaemoglobin A at 3.5 Å resolution, the high degree of isomorphism showing that the fluoromethaemoglobin molecule undergoes a transition to the deoxy-form on binding IHP. The IHP is bound on the molecular dyad at the entrance to the central cavity between the β-chains. The iron atom with its liganded fluoride ion is seen to move from the proximal side of the haem plane towards the distal side by between 0.6 and 1.4 Å, and His-F8 follows the movement of the iron. There are slight shifts in the F- and E-helices in the same direction for the α-subunits.

The modification of human deoxyhaemoglobin by bound pyridoxal compounds has been reported by Arnone and co-workers.[55] When pyridoxal 5′-sulphate is

[52] D. W. Seybert and K. Moffat, *J. Mol. Biol.*, 1977, **113**, 419.
[53] P. W. Tucker and M. F. Perutz, *J. Mol. Biol.*, 1977, **114**, 415.
[54] G. Fermi and M. F. Perutz, *J. Mol. Biol.*, 1977, **114**, 421.
[55] A. Arnone, R. E. Benesch, and R. Benesch, *J. Mol. Biol.*, 1977, **115**, 627.

bound to the amino-terminal valines of the α-chains, its sulphate group replaces a weakly bound inorganic anion and forms salt bridges with Val-1α and with Arg-141 on the other α-chain. The pyridoxal ring interacts with residues on the H-helix of the α-chain to which it is attached. With pyridoxal 5′-phosphate groups attached to the amino-termini of the β-chains, the phosphate groups are seen to be located very near the positions occupied by the phosphate groups of 2,3-diphosphoglycerate, and serve as permanently bound counterions for the side-chains of His-143β, His-2β, and Lys-82β of both β-subunits. Each pyridoxal ring displaces a tightly bound inorganic anion and interacts with residues on the EF corner of the β-chain that it is attached to.

Cross-linking the β-chain termini with 2-nor-2-formylpyridoxal 5′-phosphate gives an asymmetric link from the 2′ and 4′ carbons of the ligand to the amino nitrogens of Lys-82β_1 and Val-1β_2 respectively. The phosphate group replaces the tightly bound inorganic anion and the 3-oxygen of the pyridoxal ring interacts with the side-chain of His-143β_1. Only the cross-linked derivative shows large tertiary structure changes, and these are confined to one β-chain.

The redetermination of the structure of horse methaemoglobin by phase extension and refinement has been described.[56] Using newly collected 2.0 Å resolution data, the known co-ordinates were subjected to successive cycles of real-space refinement into electron-density maps calculated using the observed structure factors and the phases calculated from the latest refined model. The *R* factor was lowered from 45 to 23% and the root-mean-square error in atomic positions was 0.32 Å. When refinement was complete, a difference Fourier synthesis showed 41 bound water molecules in each asymmetric unit and also showed five errors in the amino-acid sequence, one of which was confirmed chemically. Several of the helical segments are seen to be irregular, and the *N*- and *C*-terminal residues are disordered in the crystal. The inter-subunit contacts are seen much more clearly in this current map. The $\alpha_1\beta_1$ contact has 17 or 19 hydrogen bonds (compared to five found in the 2.8 Å map) and the $\alpha_1\beta_2$ has six or seven (compared to two). Fifteen water molecules are seen at the $\alpha_1\beta_2$ contact and four at the $\alpha_1\beta_2$ contact, the subunit contacts being more polar than previously thought. The resolution of the map is not sufficient to determine the exact geometry of the porphyrin rings, but, assuming flat porphyrins, the iron atoms are displaced from the planes of the rings towards the proximal histidines by 0.07 Å in the α-subunits and by 0.21 Å in the β-subunits. On the change from met- to deoxy-haemoglobin, several amino-acids in contact with the haem group move relative to it, the directions of movement being similar in the α- and β-subunits.

The structure of sickling deer haemoglobin Type III has been determined by molecular replacement [20] and its refinement by a restrained least-squares procedure [21] has been reported. The molecules were seen to form a distorted honeycomb structure with open solvent channels [20] very similar to those seen in human sickled cells. The *R* factor for the data at 3.5 Å resolution was 43%. After 16 cycles of the least-squares refinement, the *R* factor was lowered to 28% for the 1.98—5.00 Å data.[21]

[56] R. C. Ladner, E. J. Heidner, and M. F. Perutz, *J. Mol. Biol.*, 1977, **114**, 385.

Gelin and Karplus[57] have proposed a mechanism for the tertiary structural change seen on ligand binding by haemoglobin. Using the known deoxyhaemoglobin geometry, empirical energy calculations were performed which showed that the initial effect was the 'undoming' of the haem group, resulting in a repulsive histidine–porphyrin contact. The effects of the resultant tilting of the haem group are transmitted to the surface of the subunit by a well localized pathway in which Val-FG5 is a key residue.

Cytochromes.—The crystal structures at 2 Å resolution of tuna ferricytochrome *c*[58] and ferrocytochrome *c*[59] have been reported. The tuna ferricytochrome *c* crystallizes in space group $P4_3$, with two independent cytochrome *c* molecules in the asymmetric unit. Four isomorphous derivatives were used in the structure analysis. The polypeptide chain could be clearly followed in each subunit, and the side-chains, especially those of the haem group, are well resolved. The folding of the main chain is very similar to the previously reported horse ferricytochrome structure except for the position of Phe-82. The aromatic ring of Phe-82 lies next to the haem group and roughly parallel to it, in the same position as previously reported for tuna ferrocytochrome *c*. The previous report that the side-chain of Phe-82 was positioned differently in the oxidized and reduced forms was therefore incorrect. This mistake was due to the poorer quality of the resolution map two derivative 2.8 Å of the horse ferricytochrome *c* structure.

The structural analysis of ferrocytochrome *c* from tuna heart has been extended from 2.45 to 2.0 Å resolution.[59] The overall chain folding differs from the 2.45 Å model in two areas only; the methyl groups on the bridge between the haem group and the sulphur atoms of Cys-14 and Cys-17, and the loop containing residues 21—23. In each case, fitting the 2.0 Å density map gives excellent agreement with the ferricytochrome *c* structure.[58] Using a re-oxidized ferrocytochrome *c* crystal, three-dimensional data were collected and a difference Fourier map was calculated. The map was almost completely featureless, and of especial interest was the lack of any movement seen for the side-chain of Phe-82 or the iron atom. The high degree of conservation of glycines in the known cytochrome *c* sequences can now be explained. Six of the ten are necessary, since they occur in Type II 3_{10} bends. Three of the others occur at positions where larger side-chains could just be accommodated. The exposed nature of the conserved glycine at position 1 suggests that it might be necessary for proper chain folding or haem attachment by another enzyme during the synthesis of the cytochrome molecule.

The co-ordinates of the ferro- and ferri-cytochrome *c* structures have been optimized by making the best fit of models with standard bond lengths and angles to the electron-density maps.[60] The ferrocytochrome *c* and the two independent

[57] B. R. Gelin and M. Karplus, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 801.
[58] R. Swanson, B. L. Trus, N. Mandel, G. Mandel, O. B. Kallai, and R. E. Dickerson, *J. Biol.* 1977, **252**, 759.
[59] T. Takano, B. L. Trus, N. Mandel, G. Mandel, O. B. Kallai, R. Swanson, and R. E. Dickerson, *J. Biol. Chem.*, 1977, **252**, 776.
[60] N. Mandel, G. Mandel, B. L. Trus, J. Rosenberg, G. Carlson, and R. E. Dickerson, *J. Biol. Chem.*, 1977, **252**, 4619.

ferricytochrome *c* molecules were refined individually. The chain folding in all three molecules is generally the same, with a mean difference in the positions of main chain atoms of 1.0 Å or less in the three structures. Many of the differences seen in side-chain orientation are due to the altered packing of molecules in the two crystal forms. Difference Fourier maps were largely featureless within the molecular boundaries, and only very slight differences were seen for the surface residues of the molecules. The structure of the reduced and oxidized forms of cytochrome *c* at this higher resolution shows that, contrary to previous results, the functioning of cytochrome *c* involves no conformational changes.

Rubredoxin.—The structure of the iron–sulphur protein rubredoxin, from *Desulphovibrio vulgaris*, has been determined by the molecular replacement method.[61] The starting model was based on the structure of the protein from *Clostridium pasteurianum*, the two proteins showing 17 amino-acid substitutions, and the *D. vulgaris* protein having two residues deleted from the *C*-terminus. The calculation of the rotation function and structure factors was based on those atoms common to the two proteins and gave a starting *R* factor of 45% for the data from 10 to 4 Å. After fully constrained real-space refinement, the *R* factor fell to 40% for the data to 2 Å resolution. Interpretation of the electron-density map shows that 14 of the side-chains excluded from the structure-factor calculation could be seen, and the overall polypeptide folding and hydrogen-bonding were seen to be the same as in the *Clostridium* protein. The iron–sulphur complex can be clearly seen, and the precision in the determination of its geometry is about 0.1 Å in bond length and 2—3° in bond angles. The Fe—S bonds range in length from 2.15 to 2.35 Å, and the S—Fe—S bond angles range from 102 to 124°, indicating a possible deviation from the tetrahedral value. The distribution of bound water is similar in the two structures except where modified by packing considerations or side-chain substitution. The solvent areas of the map appear highly ordered, with many peaks of 0.5 e $Å^{-3}$ and higher, and this is consistent with the relative hardness of the crystals.

Flavodoxin.—The crystal structure of the semiquinone form of flavodoxin from *Clostridium MP* has been determined at 2.0 Å by multiple isomorphous replacement.[62] By a combination of difference-Fourier, real-space, and reciprocal-space methods, the structure has been refined at 1.8 Å to a final *R* factor of 19.4% and the refined structure used to explore in detail the conformation of the flavin-mononucleotide binding site. Comparison of the structure with that of oxidized flavodoxin showed several changes in conformation accompanying the one-electron reduction. Residues 56—59, which are responsible for binding the isoalloxazine ring, change appreciably, with the carbonyl of the peptide connecting Gly-57 to Asp-58 forming a hydrogen bond to the flavin N(5) in the semiquinone form. This bond is not present in the oxidized form. In both the oxidized and semiquinone forms of clostridial flavodoxin the isoalloxazine ring is essentially planar, the N(5)—N(10) bonding angles being approximately 0° for the oxidized

[61] E. T. Adman, L. C. Sieker, L. H. Jensen, M. Bruschi, and J. Legall, *J. Mol. Biol.*, 1977, **112**, 113.

[62] W. W. Smith, R. M. Burnett, G. D. Darling, and M. L. Ludwig, *J. Mol. Biol.*, 1977, **117**, 195.

form and 2.5° for the semiquinone form. The intensity changes seen in the interconversion of the oxidized form to the semiquinone form have been shown to be partly due to alterations in the packing of the molecules and the resulting changes in the residues involved in intermolecular contacts. The root-mean-square distance between the 523 backbone atoms, excluding the sequences 56—59 and 89—91, is 0.31 Å.

5 Lysozyme

Two papers on the binding of substrates to lysozyme have been published. Sarma and Bott [63] have extended their structure analysis of turkey egg-white lysozyme, by the molecular replacement method, to 2.8 Å resolution. The preliminary 2.8 Å model gave an *R* factor of 0.467 for the data between 10 and 2.8 Å. The preliminary electron-density map was calculated, using the observed structure amplitudes for turkey lysozyme and the phases calculated using the co-ordinates of the main-chain atoms and the beta carbons of the rotated and translated hen lysozyme molecule. Many side-chains and two of the four disulphide bridges were apparent in this map, and after the co-ordinates were idealized gave an *R* factor of 0.452 for 10—2.8 Å resolution data. The molecules pack in the unit cell with the active centre cleft in the vicinity of the six-fold screw axis, and the clefts are not blocked by any neighbouring molecules. The co-ordinates of the main-chain atoms for the turkey lysozyme, when compared to hen lysozyme, have a root-mean-square deviation of about 1 Å. A difference Fourier analysis was calculated, using phases derived from the final model and structure amplitude differences between a native and a disaccharide-soaked crystal. The disaccharide, *N*-acetylglucosamine-*N*-acetylmuramic acid, binds in sites C and D, the peak for sub-site D being considerably weaker than the C sub-site. This could be due to unfavourable interactions or to the displacement of ions from site D. There are two strong peaks in the cleft beyond site D, the first of these being close to the predicted site E. The second peak, however, does not fit site F, lying closer to Phe-34 rather than Glu-57, and the sugar in this site, thought to be the *N*-acetylmuramic acid, can only make two hydrogen bonds with the enzyme molecule. The second paper on lysozyme substrate binding [64] reports conformational energy calculations on the binding of oligosaccharides to the rigid active site of hen egg-white lysozyme.

The effects of denaturants on triclinic crystals of lysozyme have been reported.[65–67] Glutaraldehyde-cross-linked crystals that had been treated with denaturants of increasing concentration showed an increase in cell volume and a decrease in the minimum observable *X*-ray spacings.[66] Two distinct classes of effect are seen. Detergent-type reagents having both hydrophilic and hydrophobic chemical groups, including sodium dodecyl sulphate and bromoethanol, cause very little expansion in cell volume until a critical molarity is reached, after which

[63] R. Sarma and R. Bott, *J. Mol. Biol.*, 1977, **113**, 555.
[64] M. R. Pincus, S. S. Zimmerman, and H. A. Scheraga, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2629.
[65] W. Traub, A. Yonath, A. Podjarny, A. Sielecki, B. Honig, and J. Moult, *Biophys. J.*, 1977, **17**, 134a.
[66] A. Yonath, A. Sielecki, J. Moult, A. Podjarny, and W. Traub, *Biochemistry*, 1977, **16**, 1413.
[67] A. Yonath, A. Podjarny, B. Honig, A. Sielecki, and W. Traub, *Biochemistry*, 1977, **16**, 1418.

the volume increases very rapidly. The hydrophilic reagents, including potassium thiocyanate and urea, cause gradual increases in the crystal volume and give total cell-volume increases much less than that seen for the detergents. Guanidinium hydrochloride and guanidine thiocyanate show an intermediate behaviour. Loss of the *X*-ray diffraction pattern follows the change in cell volume, being sharp and gradual for detergents and salts respectively. With detergent-like reagents, renaturation follows a rather less sharp transition than denaturation. This recovery is sharper and more complete than for crystals treated with hydrophilic reagents. The structural effects of the denaturation by sodium dodecyl sulphate have been more fully investigated.[67] Two denatured cross-linked crystals, soaked in 1.1M sodium dodecyl sulphate, were renatured in solutions of 0.35M and 0.0M sodium dodecyl sulphate. Both crystals diffracted to 2.9 Å resolution, but data were only collected to 3.2 Å, to avoid the necessity of using more than one crystal for each data set. The loss in crystallinity and the differences in overall temperature factor suggest that the re-folding process results in somewhat disordered crystals of a protein whose conformation is similar but not identical with that of native lysozyme. In both difference maps, three sodium dodecyl sulphate molecules could be placed, and the most obvious feature is the radial displacement of the two wings of the molecule (wing 1 residues 1—38 and 103—129 and wing 2 residues 43—100) away from the hydrophobic core, the wings moving as two rigid bodies. The similarity of the two maps shows that the two renatured crystals are very similar, but, since the sodium dodecyl sulphate cannot be totally removed, this is not really a true renaturation. Although the structure can reasonably be identified as an intermediate in the unfolding process, this has not been demonstrated conclusively.

6 Proteolytic Enzymes

A 2.8 Å resolution electron-density map of actinidin, a sulphydryl protease, has been described by Baker.[68] The two derivative isomorphous replacement map proved very easy to interpret, and, despite the fact that the sequence was not totally known, about 40% of the residues could be identified with some certainty, 22 of the aromatic side-chains being very well resolved. The molecule has been interpreted as a chain of 218 residues, sequencing work suggesting that there are two more residues at the *C*-terminal end of the chain. The protein is folded into two domains, domain I containing residues 19—115 and 214—218 and domain II residues 1—18 and 116—213. Domain I contains no significant β-structure but has four pieces of α-helix, one of these being five turns long (residues 25—42) and forming the interface between the two domains. Domain II contains only one stretch of α-helix, much of the remainder of the molecule consisting of an extensive anti-parallel β-sheet. This sheet, which is mostly buried in the interior of the molecule, contributes many of the side-chains which make up the core of domain II.

Comparison with papain shows a striking similarity between the two molecules, despite the fact that about 50% of the residues are different. The eight additional residues and one deletion in actinidin all occur at the surface of the molecule, and

[68] E. N. Baker, *J. Mol. Biol.*, 1977, **115**, 263.

none appears to affect the conformation significantly. Only one substantial difference in main-chain conformations can be seen, the axes of the α-helical turns at residues 99—104 being almost at right angles to each other. Examination of the active-site regions shows that the single histidine residue in actinidin occupies a position very similar to that of the imidazole group in papain. Several main-chain groups are directed into the active site, and could be involved in substrate binding. The non-polar binding pocket in papain is somewhat occluded by a methionine and a tyrosine side-chain, making it shorter and more closed-in, and the three aromatic residues lining it are replaced by arginine, threonine, and serine residues. These changes may well be concerned with changed specificity for the actinidin molecule.

The three-dimensional structure of the acid protease penicillopepsin has been reported.[69] The enzyme is an extracellular acid protease, and from partial sequence comparisons it has been suggested that it is an evolutionary homologue of pepsin and chymosin. It is also the first acid protease for which the whole polypeptide chain can be traced unambiguously. The molecule is asymmetric, being 65 × 49 × 39 Å, and this agrees with low-resolution studies on other acid proteases.[70] The molecule is bilobal, the two lobes being separated by a deep cleft approximately perpendicular to the 65 Å molecular dimension. The larger lobe is composed of the *N*-terminal portion of the chain and the *C*-terminal part forms the smaller lobe. The main structural feature is an 18-strand mixed β-sheet, which twists, in the usual anticlockwise manner, by 540° from one end to the other. There are four small helices, and the single disulphide bridge is located near the surface of the molecule. The two additional disulphide bridges seen in pepsin occur at positions where the corresponding residues in penicillopepsin are sufficiently close to form the bonds. The two aspartate residues implicated in the mechanism occur close to each other at one end of the cleft, and they may share a proton. This evidence, together with the fact that the low-resolution structure of a related acid protease with bound inhibitor has also implicated the groove as the active site,[70] suggests that the 30 Å cleft is the substrate-binding site. Despite mechanistic studies which suggest that pepsin and carboxypeptidase A should have similar structures, it is seen that the structure of penicillopepsin is distinctly different from that of carboxypeptidase A. The use of a specific inhibitor, *viz.* 1,2-epoxy-3-(*p*-nitrophenoxy)propane, has shown the two active-site aspartic acid residues to be Asp-32 and Asp-215, and it has been proposed that the proton shared between these two residues is the electrophilic component of catalysis.[71] A tyrosine residue donates its proton to the amide nitrogen of the scissile bond while a hydroxyl ion bound between the substrate and the carboxy-group of Asp-32 attacks the carbonyl carbon atom. The tyrosine involved, Tyr-75, is located on a hairpin loop, and its movement on binding the inhibitor is analogous to the movement of the active-site tyrosine residue in carboxy-peptidase A.

The structures of the two fungal acid proteases have now also been reported at

[69] I.-N. Hsu, L. T. J. Delbaere, M. N. G. James, and T. Hofmann, *Nature*, 1977, **266**, 140.
[70] E. Subramanian I. D. A. Swan, M. Liu, D. R. Davies, J. A. Jenkins, I. J. Tickle, and T. L. Blundell, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 556.
[71] M. N. G. James, I.-N. Hsu, and L. T. J. Delbaere, *Nature*, 1977, **267**, 808.

high resolution.[70] Though complete amino-acid sequences are not available for either of the two enzymes, the overall shape and backbone chain conformations of the two enzymes are very similar both to each other and to that of penicillopepsin. The binding of a microbial hexapeptide, pepstatin, to the *Rhizopus chinensis* protease shows that the inhibitor binds in the cleft between the two lobes of the molecule and appears to make contacts with the enzyme along its length of 20 Å. It seems from these three structures that the acid proteases will be shown to exhibit structural homologies similar to those of the serine proteases.

A refinement of the structure of bovine trypsinogen at 1.8 Å has been reported,[72] the starting model being produced by locating trypsin molecules in the trigonal trypsinogen unit cell, using Patterson search techniques. An electron-density map at 1.9 Å of bovine trypsinogen, solved by multiple isomorphous replacement, has also been reported.[73] The active site containing Asp-102, His-57, and Ser-195 is similar to that seen in trypsin, showing a similar hydrogen-bonded network. Activation of the zymogen may well be due to stabilization of the chains forming the specificity pocket, several of these regions showing little or no density in either of the maps. The side-chain of Asp-194, which forms a salt bridge to the *N*-terminus of Ile-16 in trypsin, cannot form this link since the activation peptide lies on the surface of the molecule. There is some ambiguity in the two structures reported as to the position of the carboxy-group of Asp-194. One group reports that it forms a hydrogen bond to the side-chain of His-40 [72] while the other group identifies it as hydrogen-bonding to internal solvent molecules.[73]

A refinement of di-isopropyl-fluorophosphate-inhibited bovine trypsin has been described,[22] the average positional change being 0.9 Å for alpha-carbon atoms and 1.2 Å for all atoms. The largest movements occurred for residues on the surface of the molecule. In the active site the side-chains of His-57 and Asp-102 are seen to have tilted such that the δ-N of His-57 is closer to one of the carboxyl oxygens than the other.

The binding of inhibitors to thermolysin has been used to elucidate a mechanism for the enzyme,[74, 75] and the structure of thermolysin has been compared with that of carboxypeptidase A.[76] The binding of phosphoramidon [74] and of several dipeptides [75] suggests that the substrate binds to thermolysin, with the carbonyl oxygen of the scissile bond displacing a water molecule and becoming the fourth zinc ligand. The side-chain of Glu-143 and a neighbouring water molecule approach the carbonyl carbon of the scissile peptide. This carboxy-group may act as a general base, catalysing the attack by the water molecule on the scissile bond. The imidazole group of His-231 moves close to the scissile peptide nitrogen atom, to which it may donate its proton, forming a tetrahedral intermediate which breaks down to yield the products. In this mechanism Glu-143 appears to be a counterpart of Glu-270 in carboxypeptidase A, while the role of His-231 is analogous to that of Tyr-248. These possible similarities in mechanism have been analysed in structural terms [76] and it has been seen that, although the overall

[72] H. Fehlhammer, W. Bode, and R. Huber, *J. Mol. Biol.*, 1977, **111**, 415.
[73] A. A. Kossiakoff, J. L. Chambers, L. M. Kay, and R. M. Stroud, *Biochemistry*, 1977, **16**, 654.
[74] L. H. Weaver, W. R. Kester, and B. W. Matthews, *J. Mol. Biol.*, 1977, **114**, 119.
[75] W. R. Kester and B. W. Matthews, *Biochemistry*, 1977, **16**, 2506.
[76] W. R. Kester and B. W. Matthews, *J. Biol. Chem.*, 1977, **252**, 7704.

foldings of thermolysin and carboxypeptidase are quite different, the active sites have several features in common. Many of the differences seen can be attributed to the fact that carboxypeptidase A is an exopeptidase while thermolysin is an endopeptidase.

A refinement of the structure of native subtilisin has been reported,[77] from which it may be seen that the position of the active-centre serine residue, Ser-221, had been incorrectly assigned. Comparison of the structure at pH 5.9 and pH 7.5 shows no movement of the side-chain for Ser-221 or His-64, but the side-chain of buried aspartate, Asp-32, moves 0.1 Å closer to His-64 at the higher pH. In common with the other serine proteases, the hydrogen bond from Ser-221 to His-64 (if present) is severely distorted, whereas in all cases the hydrogen bond between His-64 and Asp-32 is a strong bond, with normal geometry. It is suggested that the catalytic function of the His–Asp couple is to act either as a binding site for the proton in the tetrahedral transition state or as a proton relay station. A mechanism in which the histidine side-chain moves cannot, however, be discounted.

7 Glycolytic Enzymes

Phosphorylase *a*.—The locations of the binding sites for substrates and effectors on the glycogen phosphorylase *a* molecule have been reported.[78, 79] A four derivative 3.0 Å resolution electron-density map has been calculated which agrees well with the previous two derivative map. A strong peak 6 Å from the glucose-binding site, and located inside the protomer, has been interpreted as the pyridoxal 5′-phosphate, the phosphate moiety being clearly tetrahedral in shape. It is proposed that this is the active site, and the phosphoryl-binding site, where AMP also binds, is a regulator binding site. The close proximity of the pyridoxal 5′-phosphate to the substrate suggests that protons exchanging from the 5′-phosphate could either participate directly in general acid–base catalysis or transfer *via* the nearby side-chain of Lys-289. The binding of maltoheptaose, a glycogen analogue, shows a 25 Å closest approach to the glucose-1-phosphate site.[79]

Hexokinase.—The high-resolution crystal structures of yeast hexokinase complexed with substrates, activators, and inhibitors for both the monomer and the dimer forms have been reported.[80, 81] All of the sugar substrates and inhibitors examined bound at a single site in both the monomer and dimer forms, this site lying in the deep cleft that separates the monomer into two lobes. Sugar-binding gives extensive changes in the protein conformation in both the monomer and the dimer, the changes depending on the sugar used. ATP binds at a site between the two subunits (the I site) and the electron density can be fitted best by an ATP molecule in the extended conformation. The γ-phosphate of this ATP molecule

[77] D. A. Matthews, R. A. Alden, J. J. Birktoft, S. T. Freer, and J. Kraut, *J. Biol. Chem.*, 1977, **252**, 8875.
[78] J. Sygusch, N. B. Madsen, P. J. Kavinsky, and R. J. Fletterick, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4757.
[79] J. Sygusch, N. B. Madsen, and R. J. Fletterick, *Biophys. J.*, 1977, **17**, 111a.
[80] T. A. Steitz, C. Anderson, W. Bennett, R. McDonald, and R. Stenkamp, *Biochem. Soc. Trans.*, 1977, **5**, 620.
[81] T. A. Steitz, W. F. Anderson, R. J. Fletterick, and C. M. Anderson, *J. Biol. Chem.*, 1977, **252**, 4494.

is 20 Å from one glucose 6-hydroxy-group and 30 Å from the other, suggesting that it is not involved in catalysis. The binding of AMP has located the adenine-binding site (the A site), in which other small molecules, *e.g.* di-iodofluorescein and 5-iodosalicylate, also bind. An ATP molecule can be model-built into the A site, and when it is in the extended conformation the phosphorus of the γ-phosphate group lies about 6 Å from the 6-hydroxy-group of the bound glucose molecule and the γ-phosphate lies in one of the sulphate-binding sites. It is suggested that activators bind at the I site and stabilize the active dimer conformation.

Glucose-6-phosphate Isomerase.—The structure of this enzyme has now been reported at 3.5 Å resolution.[82] The molecule is approximately spherical, with a radius of 35 Å, each subunit consisting of two domains having a β-sheet core surrounded by α-helices. There is a slight cleft between the two domains, which are quite different in size. The smaller domain, of about 150 amino-acid residues, contains four parallel β-strands each connected through an α-helix, while the larger domain, of about 360 residues, contains six parallel β-strands, again interconnected through α-helices. The innermost strand of the larger domain is close to the molecular dyad axis, and this β-sheet structure is therefore continuous through the two large domains. Apart from a similarity in the connectivity of the small domain with half of the triose phosphate isomerase molecule there is no obvious resemblance to the other glycolytic enzymes. The very close association of the subunits is reflected in chemical studies which show that the dimer has a great resistance to dissociation.

Glyceraldehyde-3-phosphate Dehydrogenase.—The structure of glyceraldehyde-3-phosphate dehydrogenase from a thermophilic bacterium has been determined for the coenzyme-bound form of the enzyme.[83, 84] Unlike the lobster muscle enzyme, the tetrameric molecule has precise 222 symmetry, though the overall structures of the subunits for the two species are very similar. The two domains of the enzyme have separated functions, residues 1—148 forming the nucleotide-binding domain while residues 149—333 form the catalytic domain. An important feature of the latter domain is an irregular S-shaped loop of chain which interacts with the NAD^+ molecule and a helix of one symmetry-related subunit and with several amino-acids in another subunit. Slight changes in hydrogen bonding in the catalytic domain result in the burying of an aspartic acid residue which seems to be involved in thermostability by formation of a salt bridge with a residue on a symmetry-related subunit. The core of the thermophile enzyme is found to be no more hydrophobic than that of the mesophile enzyme. Coenzyme binding is identical in the four subunits, with the active-centre cysteine lying half-way between the nicotinamide ring and a histidine side-chain, and none of the asymmetry of subunit structure reported for the lobster enzyme has been found.

The structure of an abortive ternary complex of lobster hologlyceraldehyde-3-phosphate with trifluoroacetone has been reported.[85] The inhibitor is bound

[82] P. J. Shaw and H. Muirhead, *J. Mol. Biol.*, 1977, **109**, 475.
[83] G. Biesecker and A. J. Wonacott, *Biochem. Soc. Trans.*, 1977, **5**, 647.
[84] G. Biesecker, J. I. Harris, J. C. Thierry, J. E. Walker, and A. J. Wonacott, *Nature*, 1977, **266**, 328.
[85] R. M. Garavito, D. Berger, and M. G. Rossmann, *Biochemistry*, 1977, **16**, 4393.

covalently to the active-site cysteine residue and is structurally similar to the natural acyl intermediate formed with glyceraldehyde-3-phosphate. The difference electron-density map showed a large positive peak, corresponding to the bound trifluoroacetone group, and a negative peak at the anion-binding site shows that even though the analogue is not as long as the substrate, it still extends far enough to displace the bound sulphate ion. The features seen are not identical in all four subunits, but are related in pairs by the molecular Q-diad axis, the true two-fold axis in the human enzyme.

Pyruvate Kinase.—The structure determination of cat muscle pyruvate kinase has been extended from 6 to 3.1 Å resolution.[86, 87] The conformation of the four crystallographically related subunits of the enzyme was seen to be divided into three distinct domains. The two large domains, A and C, contain central cores of mainly parallel β-strands interconnected by α-helices. The small B domain, furthest from the molecular centre, appears to form a flexible part of the subunit. Intersubunit contacts include an extended β-sheet formation and helix interactions from the C domain, and interactions between a long irregular α-helix for the A domain. The active site is located in domain A at the carboxyl end of the β-sheet, with the substrates binding approximately perpendicular to the strands of the sheet.

Post Glycolytic Enzymes.—The three-dimensional structure of chicken muscle triose phosphate isomerase has been further analysed [88] and certain similarities with lactate dehydrogenase have been found. Substrate-binding studies have shown a loop of nine residues which moves towards the active site when phosphate or 2-phosphoglycollate bind at the active site, and the natural substrate, dihydroxyacetone phosphate, causes the movement of the loop together with conformational changes in both subunits. Preliminary work on the active centre of yeast phosphoglycerate kinase has been reported [89] and the residues involved in the active centre of yeast phosphoglycerate mutase, namely two histidines and an arginine, have been characterized.[90]

Other Glycolytic Dehydrogenases.—The stereochemistry of the active centre of lactate dehydrogenase has been correlated with the known amino-acid sequences of five isoenzymes, including M4 and H4 isozymes.[92] The major catalytic difference seen is the replacement of an alanine residue in the M chain by a glutamine residue in the H chain, this residue lying in the vicinity of the coenzyme phosphate binding site and possibly hydrogen bonding to the coenzyme.

Binding of substrates to alcohol dehydrogenase and the conformational changes

[86] D. K. Stammers, M. Levine, D. I. Stuart, and H. Muirhead, *Biochem. Soc. Trans.*, 1977, **5**, 654.
[87] D. K. Stammers and H. Muirhead, *J. Mol. Biol.*, 1977, **112**, 309.
[88] D. C. Phillips, P. S. Rivers, M. J. E. Sternberg, J. M. Thornton, and I. A. Wilson, *Biochem. Soc. Trans.*, 1977, **5**, 642.
[89] H. C. Watson, T. N. Bryant, N. P. C. Walker, P. J. Shaw, and P. L. Wendell, *Biochem. Soc. Trans.*, 1977, **5**, 652.
[90] S. I. Winn, H. C. Watson, L. A. Fothergill, and R. N. Harkins, *Biochem. Soc. Trans.*, 1977, **5**, 657.
[91] W. Eventoff, M. G. Rossmann, S. S. Taylor, H.-J. Torff, H. Meyer, W. Keil, and H.-H. Kiltz, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2677.
[92] C.-I. Bränden, *Biochem. Soc. Trans.*, 1977, **5**, 612.

induced by this binding have been reported. A comparison of the apoenzyme structure with a 4.5 Å resolution map of the enzyme–NAD^+–dimethyl sulphoxide complex has been published [91] and some of the parts of the coenzyme and substrate which are responsible for inducing the conformational change have been characterized. The binding of the enzymatically active NAD^+ analogue 3-iodopyridine-adenine dinucleotide and of the inactive analogue pyridine-adenine dinucleotide have been described.[93] Both analogues bind in the same conformation, with the adenosine moiety bound similarly to the binding of NADH. The remaining portions of the analogues are bound very differently, with the pyridine ring lying at the surface of the crevice between the two domains of the subunit rather than lying in the nicotinamide pocket. The pyridine ring lies 15 Å away from the catalytically active zinc atom and close to a lysine residue which has been shown to be important in NADH dissociation. Binding studies with two inhibitors, *i.e.* imidazole and 1,10-phenanthroline,[94] show that they both bind close to the active-centre zinc atom and in so doing displace the water molecule, which is bound to the zinc in the apoenzyme structure. No other changes in structure are seen when the inhibitors bind.

8 Other Globular Proteins

Adenylate Kinase.—The specific interaction of adenylate kinase with the anaesthetic halothane has been determined at 6 Å resolution.[95] The difference Fourier map shows a single significant peak, about 8 × 7 × 5 Å, which corresponds well with the size of halothane at 6 Å resolution. The halothane-binding site is a hydrophobic pocket deep in the molecule, this pocket having been previously identified as the adenine-binding pocket. Kinetic experiments have shown that halothane is an inhibitor of the enzyme.

The determination of the structure of pig adenylate kinase in another crystal form has been reported.[96] This form, crystal form B, has been determined at 4.7 Å resolution, using four isomorphous derivatives. The electron-density map clearly showed the α-helices, which could be related to those seen in crystal form A, but there were large differences in the active site. The transition from form A to B is an overall shift in the molecules of 3 Å, with a negligible rotation. The change in conformation was determined from a difference Fourier synthesis and showed a movement of the loop, from residues 16 to 22, of 6 Å at residue 19. This movement is accompanied by a shift of the neighbouring helix by 2 Å, the residues 123—133 opening up a large hydrophobic pocket. Several other chain movements of 1—2 Å are also clearly seen, and involve about 15% of the residues. The pH at which the transition occurs, pH 6.4, implicates His-36 as an important residue, and the environment of this residue is different in the two forms, the disposition of the neighbouring residues Asp-93 and Cys-25 altering around the imidazole side-chain.

[93] J.-P. Samama, E. Zeppezauer, J.-F. Biellmann, and C.-I. Bränden, *European J. Biochem.*, 1977, **81**, 403.
[94] T. Boiwe and C.-I. Bränden, *European J. Biochem.*, 1977, **77**, 173.
[95] W. Sachsenheimer, E. F. Pai, G. E. Schulz, and R. H. Schirmer, *F.E.B.S. Letters*, 1977, **79**, 310.
[96] W. Sachsenheimer and G. E. Schulz, *J. Mol. Biol.*, 1977, **114**, 23.

The binding positions of ATP and AMP in pig adenylate kinase have been located [97] by difference Fourier syntheses at 6 Å resolution. The ATP-binding site was identified, using salicylate and P_1,P_5-di(adenosine-5′) pentaphosphate, and is located between the helices composed of residues 69—84 and 100—107. Decavanadate binds in the enzyme's conspicuous cleft, and this site has been identified as the phosphate site. It is lined by one lysine and six arginine side-chains, and in the native crystal structure of form A it contains three sulphate ions. The AMP site has been identified as the hydrophobic pocket which opens on the A to B transition.[96] It has been shown to bind 1-anilino-8-naphthalene-sulphonate and ATP + Mn^{2+}, the latter combination being an inhibitor in solution studies. With these assignments the transferred phosphoryl group is near Asp-93, with His-36 (known to be in the active site) also nearby. The results of these papers [96, 97] suggest that the known conformations of adenylate kinase reflect an induced fit of the enzyme. Conformation B is assumed to be the conformation of the free enzyme, and A the conformation induced by substrate binding.

Dihydrofolate Reductase.—Dihydrofolate reductase catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, the latter being an essential one-carbon carrier in several pathways, most importantly in purine and pyrimidine synthesis. Many inhibitors of the enzyme are useful as antibacterials, antiprotozoals, immunosuppressants, and antineoplastic agents. The structure of the enzyme from a methotrexate-resistant strain of *E. coli* has been determined at 2.5 Å resolution.[98] Two isomorphous derivatives were used and the data were collected on a multi-wire area detector diffractometer. The electron-density map showed that the two molecules in the asymmetric unit were almost identical, but several discrepancies with the published amino-acid sequence were found.

The overall folding of the polypeptide chain is dominated by an eight-stranded β-sheet consisting of 30% of the backbone chain. The sheet shows the usual left-handed twist, amounting to 130° from one end to the other. There are three helical regions, which compose 18% of the backbone chain. The methotrexate molecule is bound in a cleft, 15 Å deep, which cuts across the whole face of the enzyme. Thirteen residues are involved in binding the methotrexate molecule, which is draped like a saddle over an α-helix. The pyrimidine end of the drug is located in a deep hydrophobic pocket whereas the face of the pyrazine ring not in contact with the α-helix is completely exposed to solvent, allowing the close approach of an NADPH molecule for dihydrofolate reduction. The aromatic ring of the *p*-aminobenzoyl portion of the drug is bound in a second hydrophobic pocket. The glutamate portion is bound at the enzyme's surface by an arginine residue. The stronger binding of methotrexate when compared to dihydrofolate can be explained in structural terms. The pteridine ring of methotrexate will be protonated at position N-1 owing to the amino-group substitution on C-4, whereas this is not the case for folate, which has a hydroxy-group on C-4. The

[97] E. F. Pai, W. Sachsenheimer, R. H. Schirmer, and G. E. Schulz, *J. Mol. Biol.*, 1977, **114**, 37.
[98] D. A. Matthews, R. A. Alden, J. T. Bolin, S. T. Freer, R. Hamlin, N. Xuong, J. Kraut, M. Poe, M. Williams, and K. Hoogsteen, *Science*, 1977, **197**, 452.

highly conserved residue Asp-27, which is thought to be catalytically essential, lies with its two carboxy oxygens 2.7 Å away from the N-1 of the pteridine.

The β-sheet topology found for dihydrofolate reductase is unlike that of any other protein whose structure is known. However, a comparison of the geometries of dihydrofolate reductase and lactate dehydrogenase shows that the spatial arrangement (but not the connectivities) of the $(\beta\alpha\beta)_2$ structure is nearly the same for the two enzymes. This $(\beta\alpha\beta)_2$ structure is also the nucleotide-binding site in dihydrofolate reductase.

Wheat-germ Agglutinin.—The dimeric plant lectin wheat-germ agglutinin is known to agglutinate various types of animal cells, in particular malignant cells. The structure of this protein has been determined at 2.2 Å resolution, using the multiple isomorphous replacement method.[99] The wheat-germ agglutinin dimers, of dimensions 40 × 40 × 70 Å, consist of two closely associated protomers of 164 amino-acid residues each, which are centred about the crystallographic two-fold axes. Each protomer is folded into four distinct domains (A, B, C, D) and the two protomers in the asymmetric unit are identical. The four domains are folded very similarly, and each one has an intra-domain disulphide bond. The four domains which are thought to constitute a protomer make only limited contact with one another, through long-range side-chain interactions, but the contacts between the domains in the dimer are extensive. These inter-protomer contacts bury a large part of the protomer surface and are probably responsible for the great stability of the dimer. Structural homology and partial sequence homology, involving the eight cysteine and some of the glycine residues, suggest that the wheat-germ agglutinin chain evolved as a result of gene-quadruplication followed by divergent evolution. A 4 Å resolution analysis of the binding of the saccharide 4-methylumbelliferyl di-*N*-acetylchitobiose indicates a strongly occupied binding site lying in the crevice between domains C and D and suggests a minor binding site at the corresponding location in the crevice between domains A and B.

Ferritin.—Ferritin is found in a large number of higher organisms, where it performs the vital function of storing iron. The ferritin molecule is composed of 24 subunits, each containing about 160 amino-acids, and has a total molecular weight of 444 000. The internal cavity can contain up to 4500 iron atoms in a microcrystalline form. The structure of apoferritin at 2.8 Å has been reported [100] and shows that the subunits, which are arranged with 432 symmetry, form a hollow sphere of 130 Å and 75 Å external and internal diameters respectively. Each subunit is cylindrical, being 55 Å long and having a diameter of 27 Å, and contains four long helices, with their axes nearly parallel to each other. This structure is similar to that seen in haemerythrin and tobacco mosaic virus coat protein. Subunit packing suggests the formation of stable dimers, which then pack together. The four-fold axis packing generates channels 10 Å wide which are lined by the fifth short helix; this is perpendicular to the four long helices. Inter-subunit contacts at the three-fold axes are more extensive than those seen at the four-fold axes.

[99] C. S. Wright, *J. Mol. Biol.*, 1977, **111**, 439.
[100] S. H. Banyard, D. K. Stammers, and P. M. Harrison, *Nature*, 1977, **271**, 282.

Arabinose-binding Protein.—The crystal structure of the L-arabinose-binding protein, an essential component of the arabinose-transport system in *E. coli*, has been determined at 2.8 Å resolution.[101] The molecule is ellipsoidal, with overall dimensions 70 × 35 × 35 Å, and is folded into two distinct domains. The overall folding consists of 35% helix and 20% β-structure, and the two domains (which are of equal size) can be related by an approximate local two-fold axis. Each domain is constructed from a long continuous chain portion and a part from a crossover portion which is shared between the two domains. Each domain contains a central six-strand β-structure, which shows a left-hand twist of 70—80°, and has four α-helices, two on each side of the sheet. This folding is reminiscent of the nucleotide-binding fold in the dehydrogenases and kinases. Soaking in solutions of L-arabinose and D-galactose has failed to locate the sugar-binding site but the structure may have been solved with sugar bound. The single essential cysteine residue, Cys-64, is located in the deep cleft between the two domains, and an unexplained electron-density peak is located adjacent to Cys-64 and a tryptophan residue. This peak may represent a bound L-arabinose molecule.

Miscellaneous.—The structure of neurotoxin *a* from the Philippines sea snake and its comparison with that of neurotoxin *b* have been reported.[102, 103] The two toxins vary at a single site, the histidine residue at position 26 in neurotoxin *b* being replaced by an amino-acid with a smaller side-chain. This change has no effect on the rest of the structure, even on the protruding loop which is thought to interact with the acetylcholine receptor.

The refinement of prealbumin at 1.8 Å resolution has been described.[104] The prealbumin monomer has eight β-strands, organized into two four-stranded sheets, these sheets being the major points of subunit interaction. Packing into tetramers yields a structure of four eight-stranded β-sheets, *i.e.* an 'inner' pair sandwiched between an 'outer' pair. An open channel runs through the molecule, this channel taking the form of a 16-stranded β-cylinder about 50 Å long with two crystallographically related binding sites for thyroxine lying in this channel. The outer eight-stranded β-sheet structures form two concave cylindrical structures about 40 Å long, of diameter 25 Å, and with a helical pitch of about 33 Å. Using an interactive graphics facility, a DNA molecule was model-built into these sites, and could be located without steric interference. The proposed DNA-binding site is rich in ionic side-chains, containing ten lysines, four histidines, eight glutamates, and six aspartates, all of which are positioned such that they could interact with DNA.

The aggregation of bacteriochlorophyll protein seen in electron micrographs has been re-examined in terms of the known structure of the protein.[105] The previously reported packing arrangement is confirmed, although the molecules are shown to be packed as trimers rather than tetramers.

The structure of ribonuclease *S* with a synthetic *S*-peptide that contains

[101] F. A. Quiocho, G. L. Gilliland, and G. N. Phillips, jun., *J. Biol. Chem.*, 1977, **252**, 5142.
[102] D. Tsernoglou and G. A. Petsko, *Biophys. J.*, 1977, **17**, 224a.
[103] D. Tsernoglou and G. A. Petsko, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 971.
[104] C. C. F. Blake and S. J. Oatley, *Nature*, 1977, **268**, 115.
[105] B. W. Matthews, R. E. Fenna, and S. J. Remington, *J. Ultrastruct. Res.*, 1977, **58**, 316.

ornithine at residue 10 has been reported.[106] This analogue, which shows 45% enzymatic activity when compared to the native enzyme, shows small but detectable changes in several regions of the structure, including the active site.

The structure of 2-keto-3-deoxy-6-phosphogluconate aldolase from *Pseudomonas putida* has been described [107] and the enzyme shown to be a trimer. The crystallization of guinea-pig haemoglobin in the gut of a blood-feeding tick and the isomorphousness of the crystals with those grown *in vitro* has been reported.[108]

Two structure determinations by n.m.r. spectroscopy have been reported. The binding site for dinitrophenol on an IgA molecule has been determined by model building and n.m.r. from known *X*-ray structures,[109] and the structure of melanostatin in solution has been compared with its known *X*-ray structure.[110]

9 Protein Conformation

Two papers [111, 112] comparing insulin with relaxin, a polypeptide hormone produced in the corpus luteum, have been published. Porcine relaxin, mol. wt. about 5600, consists of one A chain and one B chain, whose amino-acid sequences are consistent with inter-chain and intra-chain disulphide bridges of the same disposition as those of insulin. Only five other residues are identical in the two sequences. By manual model-building [111] and using a computer graphics system,[112] the two groups produce very similar final conformations, with one of the tryptophans buried in the hydrophobic core while the other is exposed at the surface.

Comparison of homologous proteins has often been used to determine important catalytic residues. Lenstra and co-workers [113] have examined 24 homologous ribonucleases which differ in up to 34% of their sequence to see which features are essential for the three-dimensional structure. The results obtained by the structure-prediction methods of Chou and Fasman, Burgess *et al.*, and Lim have been compared, and they showed that all residues which, according to Lim's method, are essential for the formation of secondary structure are invariant in the 24 proteins studied. The invariability observed for the distribution of the hydrophobic residues suggests an important role in determining the structure, and the function of these residues has been examined. Dufton and Hider [114] have applied a modified Chou and Fasman method to predict the structures of 57 snake venom toxins described as toxic or cytotoxic. Despite a range of toxicities, a common secondary structure distribution was detected, and the results highlight the differences between short and long toxins and between neurotoxins and cytotoxins. The differing specificities of the toxins can be traced to certain regions of the toxin in question.

[106] G. Valle, G. Zanotti, B. Filippi, and A. Del Pra, *Biopolymers*, 1977, **16**, 1371.
[107] W. A. Wood, *Trends Biochem. Sci.*, 1977, 223.
[108] J. D. G. Smit, O. Grandjean, R. Guggenheim, and K. H. Winterhalter, *Nature*, 1977, **266**, 536.
[109] R. A. Dwek, S. Wain-Hobson, S. Dower, P. Gettins, B. Sutton, S. J. Perkins, and D. Givol, *Nature*, 1977, **266**, 31.
[110] R. Deslauriers, R. L. Somorjai, and E. Ralston, *Nature*, 1977, **266**, 746.
[111] S. Bedarkar, W. G. Turner, T. L. Blundell, and C. Schwabe, *Nature*, 1977, **270**, 449.
[112] N. Isaacs, R. James, H. Niall, G. Bryant-Greenwood, G. Dodson, A. Evans, and A. C. T. North, *Nature*, 1978, **271**, 278.
[113] J. A. Lenstra, J. Hofsteenge, and J. J. Beintema, *J. Mol. Biol.*, 1977, **109**, 185.
[114] M. J. Dufton and R. C. Hider, *J. Mol. Biol.*, 1977, **115**, 177.

An analysis of the structural complementarity between double-stranded DNA and a two-stranded antiparallel β-sheet in proteins has been reported.[115] Contact occurs in the narrow groove of the DNA, and the symmetry elements (as well as the repeat distances) of the DNA and β-ribbon provide favourable contacts.

Rossmann and Argos [116] pose three questions which must be considered when comparing protein structures.

(1) Can the two proteins be oriented to superimpose a significant number of secondary structural elements? – structural equivalence.

(2) Are these structures directed and sequenced along the polypeptide chain in the same sequential order? – topological equivalence.

(3) How large are the insertions/deletions between the equivalent sections when compared to the total polypeptide chain?

Quantitative comparisons of lactate dehydrogenase with glyceraldehyde-3-phosphate dehydrogenase, of cytochrome b_5 with haemoglobin β-chain, and of hen egg-white lysozyme with bacteriophage *T*4 lysozyme are reported and the probability of convergence or divergence is examined in relationship to the various criteria of similarity. Argos and Rossmann have also described [117] a stable super-secondary structure composed of four roughly parallel α-helices which they have observed in haemerythrin, tobacco mosaic virus protein, and tyrosyl t-RNA synthetase. Since the latter two interact with RNA, it is proposed that this four-helical arrangement might be a nucleic-acid-binding super-secondary structure, and be common to other proteins with this function.

Two groups have reported comparisons of proteins to evaluate the possibility of convergence of active-centre geometries.[118, 119] A comparison of lactate dehydrogenase with glyceraldehyde-3-phosphate dehydrogenase [118] has shown that, once the conformation of the substrate with respect to the functional groups in the enzyme's active site has been fixed, the A- or B-side specificity of the nicotinamide ring is predetermined, since the C-4 atom of the nicotinamide has to approach the reactive carbon of the substrate. Divergently evolving dehydrogenases with a common precursor must therefore maintain their nicotinamide specificity if the fold of the catalytic domain is unaltered. Further comparisons suggest that the serine proteases (subtilisin and the chymotrypsin family) show one hand in the structure of the acyl intermediate while the cysteine enzymes (papain and glyceraldehyde-3-phosphate dehydrogenase) show the other hand. The second group of workers have devised a graphical representation which correlates the pattern of allowed single base substitutions with the associated changes in the structural properties of the encoded amino-acids.[119] The results show that in general there is little tendency for the code to be structurally conservative where a single base change results in an amino-acid substitution, and the authors discuss the consequences in relation to protein evolutionary processes.

Two computer programs for identifying secondary structure, one which finds

[115] G. M. Church, J. L. Sussman, and S.-H. Kim, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1458.
[116] M. G. Rossmann and P. Argos, *J. Mol. Biol.*, 1977, **109**, 99.
[117] P. Argos, M. G. Rossmann, and J. E. Johnson, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 83.
[118] R. M. Garavito, M. G. Rossmann, P. Argos, and W. Eventoff, *Biochemistry*, 1977, **16**, 5065.
[119] F. R. Salemme, M. D. Miller, and S. R. Jordan, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2820.

peptide chain-turns specifically [120] and one which can identify α-helix, β-sheet, and reverse-turn structures,[121] have been reported.

The turn-finding program requires only the α-carbon co-ordinates of the residues, and when applied to a group of test proteins gave results in excellent agreement with visual turn identifications from physical models.[120] The more general program [121] performs an analysis based on patterns of peptide hydrogen bonds, inter-C_α distances, and inter-C_α torsion angles, and it has been used to analyse secondary structure in 62 proteins. This program also uses α-carbon co-ordinates only, and the authors provide an objective and consistent compilation of α-helix, β-sheet, and reverse-turn secondary structure in almost all globular proteins of known tertiary structure.

Several papers presenting models for the packing of secondary structures and protein folding have been published.[122–131] Packing considerations have been used to derive rules for the interactions of α-helices and β-sheets [122] which, though they do not include information as to the exact size and shape of individual side-chains, give excellent results. The method could be extended to include specific side-chain effects. Nagano has analysed the preferred handedness of the β-strand–α-helix–β-strand super-secondary structure [123] and has presented results which can explain the preferred right-handed sense of the structure. In a later paper [124] Nagano tests his prediction method, based on doublet analysis, on the structures of adenylate kinase and bacteriophage T4 lysozyme. The results for lysozyme show that certain structures were not predicted correctly unless some triplet information was included, the effect of this inclusion being an increase in the strength of helix prediction as a whole rather than an increase in the percentage of residues correctly predicted as helix.

Sternberg and Thornton have presented a series of four papers [125–128] on the properties of β-sheets in proteins. In the first paper [125] they analyse the handedness of the connection between two parallel β-strands and find that, irrespective of the secondary structure in the connection, the unit is nearly always right-handed. The rotation between the strands, the length of the loop regions, and the observed main-chain torsion angles are examined and shown to fit their proposed explanation. General patterns in the folding of β-sheet structures when they are analysed for the number of strands in a sheet, the type of connection between two sequential intra-sheet strands, the ordering of the strands with particular reference to the *N*- and *C*-terminal strands, and the number of residues in the strands and their connecting regions have been reported.[126] The results also suggest that the final structure is very stable, and as such may be expected to be seen in different

[120] G. D. Rose and J. P. Seltzer, *J. Mol. Biol.*, 1977, **113**, 153.
[121] M. Levitt and J. Greer, *J. Mol. Biol.*, 1977, **114**, 181.
[122] C. Chothia, M. Levitt, and D. Richardson, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4130.
[123] K. Nagano, *J. Mol. Biol.*, 1977, **109**, 235.
[124] K. Nagano, *J. Mol. Biol.*, 1977, **109**, 251.
[125] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1977, **110**, 269.
[126] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1977, **110**, 285.
[127] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1977, **113**, 401.
[128] M. J. E. Sternberg and J. M. Thornton, *J. Mol. Biol.*, 1977, **115**, 1.
[129] J. S. Richardson, *Nature*, 1977, **268**, 495.
[130] P. Y. Chou and G. D. Fasman, *J. Mol. Biol.*, 1977, **115**, 135.
[131] G. Von Heijne and C. Blomberg, *J. Mol. Biol.*, 1977, **117**, 821.

proteins, similarities not necessarily implying evolutionary relationships. This analysis has been extended to determine the folding parameters, which can be used to calculate probable β-sheet structure,[127] and a computer program has been written to calculate the probabilities for given sequences. For about half the β-sheet structures known, the observed arrangement is the most probable, and Sternberg and Thornton's approach, unlike conventional methods, can predict probable sheet structures of large globular proteins from the results of a successful prediction of the secondary structure alone. In the fourth paper [128] Sternberg and Thornton have determined the influence of hydrophobic free energy on the ordering of strands in β-sheet structures. By comparing the total hydrophobicity of each strand in a sheet they have shown that in 30 out of 39 sheets studied the more hydrophobic strands occur near the centre, and in 20 of these 30 sheets the most hydrophobic strand is buried, with the other strands, which are arranged in order of decreasing hydrophobicity, located outwards in each direction. Since longer strands are potentially more hydrophobic, they occur most frequently at the centre of the sheets, and the results suggest that hydrophobicity is an important factor in determining strand order. The hydrophobicity on each side of the sheet is also analysed. Richardson [129] has surveyed the connectivities of β-sheets in the known protein structures and produced simplified topological diagrams for the 37 distinct β-sheet structures. Except for one connection in subtilisin, all others are right-handed, and it is observed that no parallel β-sheet occurs with less than five strands, suggesting that considerable co-operativity is necessary to stabilize this structure. The occurrence of pure parallel or pure antiparallel is seen to be very much greater than would be expected from a random distribution, and only two knotted topologies, both in carbonic anhydrase C and both at the extremities of the polypeptide chain, are observed. The β-structure in 19 proteins has been analysed for residue–residue correlations, and the results show that hydrophobic residues tend to occur together as inter-strand nearest-neighbours. This work also suggests that hydrophobic nucleation may be important in sheet structures.

Chou and Fasman [130] have examined 29 proteins of known sequence and structure, and they found 459 β-turns in regions of chain reversal. They have characterized a β-turn as a tetrapeptide where the $C\alpha_i$ to $C\alpha_{i+3}$ distance is below 7 Å and the residues are not in a helical region. The bend is considered to have hydrogen bonding if the O_i to N_{i+3} distance is less than 3.5 Å. Examination of the torsion angles of 421 β-turns allowed them to be grouped into 11 types, and analysis of the sequences has allowed the residues occurring most frequently in each of the four positions to be determined. Hydrophobic residues show the lowest potential to be in a β-turn but tend to occur more frequently in the region just beyond the turn. The correlaton between β-strands has been analysed for paired residue–residue interactrase.[131]

10 Muscle

Crustacean striated muscles given equatorial *X*-ray reflections attributable to the hexagonal array of myofilaments in the same way as vertebrate striated muscles. The intensity distributions are, however, quite different, the crustacean pattern showing higher-order reflections with intensities comparable to the innermost two

reflections (the 1, 0 and 1, 1 reflections). The patterns obtained from crab leg flexor muscle and crayfish abdominal extensor muscle have been interpreted [132] as showing that the thick filaments are hollow. The best fit to the observed intensities was obtained when the internal and external radii of the thick filaments were assumed to be 57 Å and 95 Å respectively for the crab and 45 Å and 75 Å for the crayfish.

The changes seen in the diffraction pattern before and after a slow stretch have been examined for frog muscle [133] and have been interpreted as suggesting that the number of cross-linkages is smaller after stretch than before stretch. The *X*-ray equatorial reflections from frog muscle have also been studied by Yu and co-workers.[134] They have observed a weak reflection between the 1, 0 and 1, 1 peaks which does not index on the hexagonal filament lattice. The spacing of this reflection varied in direct proportion to that of the 1, 0 peaks for sarcomere lengths between 2.0 and 3.0 μm, but its intensity appeared relatively insensitive to length changes. They suggest that the Z-disc structure is the major source of this non-indexible reflection. *X*-Ray diffraction studies on contracting dog heart muscle have also been reported.[135] The equatorial *X*-ray reflections were recorded at different phases of the cardiac cycle and the ratio of the 1, 0 and 1, 1 reflections was determined at these different phases. Correlation of this ratio with the relative mass of the thick to the thin filaments suggests that as a quiescent muscle goes into rigor there is a transfer of myosin projections from the vicinity of the thick filaments to that of the thin filaments. The results also suggest that there is a radial transfer of the myosin projections between the systolic and diastolic phases, and that some actin–myosin interaction remains during the diastolic phase.

Two new crystal forms of tropomyosin have been reported.[136, 137] One of the reported forms [136] is crystallized from the α-tropomyosin component after purification, and shows a higher protein density in the crystals, which may well suit them for *X*-ray diffraction studies.

11 Other Biological Structures

Sparling and Klug [138] have obtained *X*-ray diffraction photographs from gels of chromatin prepared by brief digestion of rat-liver nuclei with nuclease. They observe a sharp pattern of peaks at apparent spacings of 110 Å, which, they propose, is due to the spacing between turns of the solenoidal structures seen in electron-microscope studies rather than from the spacing of nucleosomes along the nucleofilament.

Complexes of DNA and various protamines have been studied by *X*-ray diffraction,[139] showing that all the protamines studied, despite having considerable differences in molecular weight and amino-acid compositions, stabilize the

[132] N. Yagi and I. Matsubara, *J. Mol. Biol.*, 1977, **117**, 797.
[133] H. Sugi, Y. Amemiya, and H. Hashizume, *Proc. Japanese Acad.*, 1977, **53B**, 178.
[134] L. C. Yu, R. W. Lymn, and R. J. Podolsky, *J. Mol. Biol.*, 1977, **115**, 455.
[135] I. Matsubara, A. Kamiyama, and H. Suga, *J. Mol. Biol.*, 1977, **111**, 121.
[136] M. L. Greaser, M. Yamaguchi, and G. Vanderkooi, *J. Mol. Biol.*, 1977, **116**, 883.
[137] W. Longley, *J. Mol. Biol.*, 1977, **115**, 381.
[138] L. Sperling and A. Klug, *J. Mol. Biol.*, 1977, **112**, 253.
[139] P. Suau and J. A. Subirana, *J. Mol. Biol.*, 1977, **117**, 909.

DNA in the B form. Salts of DNA with arginine and L-arginyl-L-arginine were also studied.

The packing of ribosomes in crystalline sheets seen in certain lizard oocytes has been reported [140] and a low-resolution three-dimensional map has been obtained.[141] The ribosomes pack as tetramers, around a four-fold axis, with both the large and small ribosomal subunits adjacent to the membrane surface; attachment to the membrane is accomplished by a protrusion from the large subunit.

A low-resolution structure of oriented hydrated cytoplasmic microtubules has been described.[142] This model shows the microtubule wall to extend from 70 to 150 Å in radius, with a homogeneous central region. The outside surface of the tubule is subdivided by vertical grooves separating the 13 protofilaments and by a steep 10-fold family of grooves. Low-angle scattering data confirm the overall dimensions,[143] and both groups of workers explain why these values are greater than those seen in the electron microscope.

12 Fibrous Proteins

Hulmes and co-workers [144] have presented a review of the various attempts to interpret the low-angle meridional *X*-ray diffraction patterns of tendon to yield the axially projected electron density of collagen fibrils. The validity of the assumptions used in these interpretations has been tested by producing models for the electron density based on the known amino-acid sequence of collagen and comparing the observed diffraction patterns with those calculated for the models.[144, 145] The myofibrillar structure of collagen and the importance of hydrophobic interactions in its structure have been studied,[146] as has the axial relationship between the collagen and mineral components in calcified tendon.[147] A simple three-dimensional molecular crystal model for the collagen fibril has been reported [148] and has been shown to account for certain features of the near-equatorial low-angle diffraction pattern of rat-tail tendon. Two other models for the collagen fibril have been proposed.[149, 150]

X-Ray analysis of the structure of chicken scale keratin has shown it to consist of long 30 Å diameter filaments embedded in an amorphous matrix.[151] The filaments are made up of a helical array of protein chains having a β-conformation. By a combination of known structural rules and the meridional and equatorial *X*-ray data, Huggins [152] has proposed a structure for α-keratin which explains some experimental observations that the current 'rope' models do not.

[140] P. N. T. Unwin and C. Taddei, *J. Mol. Biol.*, 1977, **114**, 491.
[141] P. N. T. Unwin, *Nature*, 1977, **269**, 118.
[142] E. Mandelkow, J. Thomas, and C. Cohen, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3370.
[143] B. A. Fedorov, I. L. Shpungin, V. I. Gelfand, V. A. Rosenblat, G. Damaschun, H. Damaschun, and M. Papst, *F.E.B.S. Letters*, 1977, **84**, 153.
[144] D. J. S. Hulmes, A. Miller, S. W. White, and B. B. Doyle, *J. Mol. Biol.*, 1977, **110**, 643.
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[146] K. A. Piez and B. L. Trus, *J. Mol. Biol.*, 1977, **110**, 701.
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[148] J. Woodhead-Galloway, *Acta Cryst.*, 1977, **B33**, 1212.
[149] D. W. L. Hukins, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 335.
[150] K. Okuyama, M. Takayanagi, T. Ashida, and M. Kakudo, *Polymer J.*, 1977, **9**, 341.
[151] M. Stewart, *J. Ultrastruct. Res.*, 1977, **60**, 27.
[152] M. L. Huggins, *Macromolecules*, 1977, **10**, 893.

The structure of the chitin fibrils of a diatom has also been reported,[153] and shows that the polysaccharide fibrils are highly crystalline.

13 Membranes and Lipoproteins

Examination of the effects of calcium ions on phosphatidylserine bilayers by X-ray diffraction has shown that the replacement of sodium by calcium causes the polar groups to bind tighter together.[154] This tight packing is transmitted to the hydrocarbon chains, leading to crystallinity in that region.

Diffraction patterns from stacked haemolysed erythrocyte ghosts give an asymmetrical electron-density profile with an excess of positive density on the inner, cytoplasmic side.[155] It is suggested that this excess density represents the loosely bound protein components spectrin and actin. Membranes from *E. coli* have been examined by high-angle *X*-ray diffraction after treatments which cause segregation of the bound proteins into localized areas, and the extent of the segregations has been quantified.[156]

The thermal transition of human plasma low-density lipoprotein has been studied by small-angle *X*-ray diffraction[157] and seems to be due to a smectic–disordered transition of the cholesterol esters in the core of the particle. Diffraction experiments on human lipoprotein X suggest that the underlying structural feature is the lipid bilayer, with the protein content partly bound within the polar head-group regions and partly in soluble form in the vesicle interior.[158]

The arrangements of cytochrome oxidase molecules in two-dimensional vesicle crystals and their structure at 30 Å resolution in stained preparations and at 12 Å resolution in unstained material have been reported.[159] These structures differ greatly, but suggest that an interpretable structure will be obtained from a three-dimensional analysis of unstained material. *X*-Ray diffraction and electron microscope studies of membranes from the electric skate's electroplax have shown that the acetylcholine-receptor molecules traverse the endplate membrane.[160] The receptors have an overall length of 110 Å normal to the membrane and extend about 15 Å from one side of the membrane and 55 Å from the other. Burge and co-workers have reported results on the structure of the peptidoglycan of cell walls from *Staphylococcus aureus* and *Bacillus licheniformis*, showing them to be very similar.[161]

14 Synthetic Peptides

X-Ray fibre diffraction techniques have been applied to study the structures of

[153] W. Herth and P. Zugenmaier, *J. Ultrastruct. Res.*, 1977, **61**, 230.
[154] H. Hauser, E. G. Finer, and A. Darke, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 267.
[155] E. H. Pape, K. Klott, and W. Kreutz, *Biophys. J.*, 1977, **19**, 141.
[156] L. Letellier, H. Moudden, and E. Schechter, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 452.
[157] D. Atkinson, R. J. Deckelbaum, D. M. Small, and G. G. Shipley, *Proc. Nat. Acad. Sci., USA.*, 1977, **74**, 1042.
[158] P. Laggner, O. Glatter, K. Müller, O. Kratky, G. Kostner, and A. Holasek, *European J. Biochem.*, 1977, **77**, 165.
[159] R. Henderson, R. A. Capaldi, and J. S. Leigh, *J. Mol. Biol.*, 1977, **112**, 631.
[160] M. J. Ross, M. W. Klymkowsky, D. A. Agard, and R. M. Stroud, *J. Mol. Biol.*, 1977, **116**, 635.
[161] R. E. Burge, R. Adams, H. H. M. Balyuzi, and D. A. Reaveley, *J. Mol. Biol.*, 1977, **117**, 955.

poly(L-lysine hydrobromide)[162] and poly-N^5-(4-hydroxybutyl)-L-glutamine.[163] The conformations of poly(L-tyrosine) in DMF, studied by small-angle *X*-ray diffraction,[164] and the transition of poly(γ-methyl D-glutamate), β-form, in the temperature range -80 to $+120$ °C[165] have been reported. Studies on the conformations of poly-N^5-(3-hydroxypropyl)-L-glutamine,[166] poly(γ-benzyl L-glutamate),[167] and solid films of poly(γ-methyl D-glutamate)[168] have been published.

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

edited by R. H. Pain, with contributions by T. Brittain, D. P. E. Dickson, D. J. Osguthorpe, H. W. E. Rattle, B. Robson, R. M. Stephens, R. Thomas, and E. J. Wood.

1 Theoretical Aspects of Protein Conformation

Contributed by D. J. Osguthorpe and B. Robson

In previous Reports in this series we have given particular emphasis to topics related to the problem of how proteins fold up, and to studies of water structure which relate to the structure and stability of proteins. In our last Report,[1] we made a critical appraisal of a discussion by Schultz[2] concerning the status of our understanding of protein structure at the physicochemical level. It is probably true to say that recent papers have brought these issues to a head, so here we undertake to examine the latest work in the context of the earlier ideas and to summarize the new status of the theoretical approach. To do this, we depart from our normal style of presentation and select for discussion some papers which highlight the critical issues.

Solvent Behaviour.—Experimental studies of proteins and smaller analogues in solution[3–9] continue to be popular, and theoretical studies of pure water struc-

[162] M. Suwalsky and A. Llanos, *Biopolymers*, 1977, **16**, 403.
[163] D. R. Rueda, P. Spadon, A. S. Verdini, and A. Del Pra, *Biopolymers*, 1977, **16**, 2347.
[164] F. Hamada, Y. Ishimuro, T. Hayashi, and A. Nakajima, *Biopolymers*, 1977, **16**, 2351.
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[166] D. R. Rueda, A. S. Verdini, and A. Del Pra, *Macromolecules*, 1977, **10**, 715.
[167] A. Shoji, Y. Yamamoto, T. Ozaki, M. Ida, and Y. Saito, *Macromolecules*, 1977, **10**, 723.
[168] J. Watanabe, S. Sasaki, and I. Uematsu, *Polymer J.*, 1977, **9**, 337.
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[2] G. E. Schulz, *Angew. Chem. Internat. Edn.*, 1977, **16**, 23.
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[8] J. N. Spencer, J. R. Sweigart, M. E. Brown, R. L. Bessing, T. L. Hassinger, W. Kelly, D. L. Housel, G. W. Reisinger, D. S. Reifsnyider, J. E. Gleim, and J. L. Peiper, *J. Phys. Chem.*, 1977, **81**, 2237.
[9] M. Morcellet and C. Louchex, *Polymer*, 1977, **18**, 1082.

ture [10–13] are still in evidence. Theoretical analyses of the hydrophobic effect [14–17] also reflect the interest in aspects of water behaviour important for biological systems. As the term 'hydrophobic effect' is most currently used, it relates to a linear relationship between some measure of the bulk of the solute molecule and its free energy of partitioning between water and a non-polar solvent. This free energy is such as to favour a non-polar environment for non-polar solute molecules, and hence is widely held to explain the coming together of non-polar side-chains when a protein folds up in water. A paper by Hermann [18] reflects the belief in a linear relationship and continues an old argument with the Tanford school which is essentially about the quantitative details of this relationship.

Cramer,[19] however, discusses some doubts on the assumptions underlying the hydrophobic effect: in a sense, he denies the existence of any hydrophobic effect as behaviour qualitatively distinct from solute–solvent interactions in general. Cramer interprets the principal underlying assumption to be that the origin of the effect is in a kind of thermodynamic repulsion between non-polar solute and water, the non-polar solvent phase playing a relatively neutral role. He then criticizes this assumption by presenting data which relate not to the transfer free energy between solvent phases, but to the solvation free energy of solutes by the aqueous and non-polar phases. In other words, the transfer-free energy of solute from water to the vapour phase, and from non-polar solvent to the vapour phase, are considered separately. If the interaction between solute and water were the dominant and characteristic contribution, then a plot of molecular bulk of solute against transfer free energy out of water would be expected to be similar whether the receptor phase was non-polar solvent or simply solute vapour. In fact, it is the solvation by non-polar solvent which is most consistent with transfer between solvents, while the plot for transfer out of water to the vapour phase is relatively 'featureless'. Cramer finds these findings hard to reconcile with the hydrophobic interpretation of partitioning phenomena.

In the most general terms, Cramer's point that hydrophobicity is an artificial concept in that it is only quantitatively different from other solvent effects is sound, and the only true description is in terms of what each water molecule is, on average, doing. In the past it has been widely held that such a description, though desirable in principle, is far beyond current computational facilities. Hagler and Moult [20] have shown that this is not true, and, using the Monte Carlo method, have simulated the behaviour of water in hydrated crystals of lysozyme and a cyclic peptide. Those water molecules which were observed in the *X*-ray analysis usually corresponded to those water molecules in the simulation with a high probability of occupying one position. The Monte Carlo technique seems

[10] J. C. Owicki and H. A. Scheraga, *J. Amer. Chem. Soc.*, 1977, **99**, 7403.
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[19] R. D. Cramer, *J. Amer. Chem. Soc.*, 1977, **99**, 5408.
[20] J. Moult and A. T. Hagler, *Nature*, 1978, **272**, 222.

quite capable of handling 300—400 water molecules, the solute being assumed rigid in this particular study. Of course, if only a finite number of water molecules are considered, there would be the serious problem of special effects at the solvent–vacuum interface, *i.e.* it would be a droplet of solution which is being considered, and not a continuous phase. However, the advantage of a crystal is not only that it provides data about water positions which can be checked against theory, but that it consists of repeating unit cells. These unit cells were retained in the calculation, so that the behaviour of water in each cell was copied in all the other cells. This technique has been widely employed in the guise of 'periodic boundary conditions' in Monte Carlo analysis of pure water and simple solutions, but there is always the slight danger, unless great care is taken, of introducing the effects of long-range order into the calculation. In this case, however, the periodic boundary conditions have real meaning as crystallographic repeat distances, so that any long-range order effects are relevant to the system studied.

The implications of this work and its relation to other studies have been discussed by Robson and Jones.[21] The most significant finding (in agreement with experiment) is that there is an innermost solvation shell which, in the vicinity of charged and hydrogen-bonding protein groups, is highly ordered. On the other hand, much water in the vicinity of the protein is extremely labile, although its behaviour may not be identical to that of free water and may contribute a significant entropy. In principle, solvation-free energies may be directly calculable by this approach, although a lot more computation time would be required for accurate results. Robson and Jones note that, particularly for non-crystalline systems, the water molecules further out could be replaced by a bulk effect in the form of a continuum reaction field, and this would considerably simplify and speed calculations of this kind.

Although Moult and Hagler [20] considered that their method would also be applicable to the denatured form of the protein, Robson and Jones [21] felt pessimistic on the grounds that a correct account of the denatured form includes many conformations in equilibrium. Hence the technique is still not immediately applicable to measure the stability of the native state with respect to the disordered form, nor for similar reasons is it applicable to studies of the folding process and the genesis of structural features in proteins.

Protein and Polypeptide Behaviour.—Current calculations of polypeptide dynamic behaviour are carried out either *in vacuo* or with crude representations of the solvent effect (such as the introduction of a dielectric constant).[22–26] Most studies of this type involve variations on the Monte Carlo technique, although the use of Molecular Dynamics is likely to become increasingly popular. Briefly, the difference is that Monte Carlo is essentially a technique for sampling a large number of conformations, whereas Molecular Dynamics actually simulates molecular motion by taking into account Newton's laws of motion for atoms in

[21] B. Robson and M. N. Jones, *Nature*, 1978, **272**, 206.
[22] S. Premilat and B. Maigret, *J. Chem. Phys.*, 1977, **66**, 3418.
[23] G. Allegra, *J. Chem. Phys.*, 1977, **66**, 5453.
[24] D. E. Neves and R. A. Scott, *Macromolecules*, 1977, **10**, 339.
[25] W. L. Mattice, *Macromolecules*, 1977, **10**, 511.
[26] M. Lautout-Magat, *Macromolecules*, 1977, **10**, 1375.

the system. The latter has been used in order to simulate conformational fluctuations of the native state of the small protein pancreatic trypsin inhibitor.[27] In contrast to the rigid picture of the native state suggested by *X*-ray crystallography, this work reveals a 'rich variety of motional phenomena that occur on the atomic level at ordinary temperatures'. The average root mean square fluctuation of all atoms from mean positions was 0.9 Å, although larger displacements of 3 Å or more occurred at some regions on the protein surface. Regions of α-helix and β-pleated sheet were relatively rigid, although even these displayed oscillatory behaviour which, in the case of the pleated sheet, was described as a 'rippling motion'. Robson [30] emphasized that it was fortunate that the simulation did not wander too far from the native structure, or the neglect of the solvent could have been serious.

Improvement of Energy Functions.—Improvement of energy functions is usually called 'refinement', a word which implies that it is hoped that the functions were not too bad to start off with. In most cases the functional form is assumed and, if the energy functions are being derived *de novo*, the parameters are adjusted by a least-squares method to give optimal agreement with experiment; this constitutes 'parameterization'. Energy functions obtained in this kind of way are 'empirical', since conformational energies may also be calculated by the more time-consuming *ab initio* method, *i.e.* by a parameter-free quantum mechanical calculation. There is also an intermediate type of calculation which is termed 'semi-empirical': this is quantum mechanical in concept but saves time by assuming some parameters with which various approximations are made. Because of the speed of empirical methods of calculation, parameterization and refinement of empirical functions continues to be popular.[28–34] A large number of calculations on simple analogues of peptides and proteins illustrate both the popularity of this approach and the sort of circumstances in which further refinement is possible, and in some cases even necessary, to bring about agreement between theory and experiment.[35–56]

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The energy surface of an analogue of the glycyl dipeptide has recently been calculated with the use of quality basis sets and without building up the calculation from molecular fragments. Robson *et al.*[57] found that the energy surface was in reasonable agreement with that calculated by empirical functions, although not in agreement with some semi-empirical calculations. The greatest discrepancies between the *ab initio* and empirical calculations involved a conformational barrier whose energy is dominated by a *cis* nitrogen–nitrogen interaction and an N···H—N hydrogen bond. The *ab initio* energy surface was otherwise in particularly good agreement with energy functions parameterized by Hagler *et al.*[58] Both the *ab initio* and solvation calculations provide further, if circumstantial, evidence for the applicability of empirical functions. Unfortunately, the pertinent experimental data for peptides relate to equilibrium, rather than kinetic, situations. Hence they cannot be used to assess the height of conformational barriers, one such barrier being the greatest point of disagreement between the *ab initio* calculation and the functions of Hagler *et al.*

Protein Folding.—All the above studies concerning solvent behaviour, protein behaviour, and refinement of energy calculations may be considered as steps towards the goal of predicting protein structure from the amino-acid sequence alone. Simulation of the folding process is a particular technique for predicting protein structure, based on the notion that predictions can only be made in reasonable computer time if some attempt is made to duplicate the process by which an unfolded protein in the test-tube recovers its native structure so rapidly. The idea that the speed of the natural process is due to nucleation steps remains widespread. This nucleation process is suspected to be the initial formation of secondary structure features such as α-helix, around which the rest of the protein wraps itself. Numerous publications relate to the analysis, stability, and prediction of secondary structure.[59–78] An analysis of the success and implications of

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simple secondary structure methods has been carried out.[79] However, it is probably fair to say that all these papers are basically refinements and applications of earlier investigations.

Since secondary structure appears to determine tertiary structure, at least in part, there is increasing interest in their inter-relationship. One viewpoint is that there are strong interactions between secondary structure features, usually the α-helical and β-pleated sheet regions of a protein, which lead to special packing arrangements. The conglomerate structures so formed are often referred to as super-secondary structures.[80–83] Such studies are still very much in the analytical stage, and that such conclusions can lead to useful predictions of super-secondary structures is only beginning to be demonstrated.[84] A recent simulation by Tanaka and Scheraga [82] will probably be fairly typical of work in the near future in that it exploits initial calculations for secondary structure formation and then incorporates these directly into a calculation of tertiary interactions without explicit consideration of super-secondary interactions as divorced from any other kind of tertiary interaction.

The bulk of the recent publications which relate to tertiary structure has not been concerned with prediction of tertiary structure in the sense of a three-dimensional arrangement of atoms. Methods of statistical analysis of amino-acid sequence and conformation have been extended to include analyses of the types of side-chain which prefer to come together [85] and the types of side-chain which tend to combine to form hydrophobic protein cores.[86] These could probably be usefully employed in folding simulations.

Other theoretical [87–98] and experimental studies of theoretical interest [99–107] may help in analysis and interpretation of tertiary structure and folding but do not

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always represent, or have direct relevance to, folding simulations. However, some of them certainly may be relevant when simulations of protein folding become sufficiently successful and practicable that comparison with experimental folding studies becomes feasible. Brandts *et al.*[107] have extended their interesting argument that proline residues in a protein are strong determinants of folding rates, because they are capable of undergoing conformational transitions over a fairly high energy barrier and must occur in the correct conformational form during folding. Creighton's continuing experimental study[100] of disulphide bridge formation and rearrangements during folding also provides information about intermediate steps in the folding process which could be either taken into account in folding simulations or, more reasonably, compared with the calculated folding pathway.

Conclusions.—In the past year there have been considerable advances in the theoretical treatment of solvent behaviour and in the behaviour of the native state of the protein molecule. On the other hand, it is probably true to say that there have been no dramatic advances towards the central problem, namely how a globular protein folds up to its tertiary structure. The reason for this is clear. The significance of the advances in the former fields is that these problems are now shown to be tractable by exact methods, whereas the folding problem is not yet so. Recent progress puts the central theme in the review of Schultz,[2] which we expanded in the previous Report in this series,[1] in a new light. Schultz concluded that the accumulated data about protein structures 'challenges us to understand the architecture of the observed structures and thus to trace biology back to its physical roots on a much more fundamental scale than was previously possible'. In fact, it is clear that we still do not have the computing power to do this, and so observations on native structures are still essential in order to derive principles of protein architecture which can be used to bridge the gap between the physical roots of the folding process and the native structure.

2 Mechanisms of Folding in Globular Proteins

Contributed by R. M. Thomas

An extensive review of the mechanisms of the folding of globular proteins has been published[108] and theoretical approaches to the formation of secondary structure

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[108] G. Némethy and H. A. Scheraga, *Quart. Rev. Biophys.*, 1977, **10**, 239.

and folding are discussed elsewhere in this volume.[109] The correlation between the primary sequence and tertiary structure of proteins has also been considered.[110]

Stability.—*Thermodynamics.* Conformational, hydrophobic, and vibrational effects are of the greatest importance in a consideration of the stabilization and interaction of proteins, and the relative contributions of these to the heat capacity and entropy changes have recently been clarified.[111] It is concluded from studies on the self-association of glucagon that the heat capacity change is the principal characteristic of hydrophobic interactions.[112] Thermal denaturation of phosphoglycerate kinase from *Thermus thermophilus* shows that the heat capacity and enthalpy changes associated with the transition are negligibly small and an explanation is suggested for the unusual thermostability of the protein.[113] Similar studies allow the maximum stability of cytochrome *c* from various sources to be calculated [114] and different energetic states of differing thermal stability of NAD-dependent dehydrogenases to be identified.[115]

The thermodynamic processes involved in folding and unfolding transitions are given detailed consideration in the individual studies.

Stabilization against Denaturation. Differential scanning calorimetry studies on the thermal behaviour of lactate dehydrogenase show a highly co-operative unfolding process against which the protein is stabilized by co-factor or sucrose but not by pyruvate.[116] Immobilized trypsin in which autolysis does not occur is markedly stabilized to urea denaturation by calcium ion,[117] possibly by co-ordination of the metal to side-chains of the tertiary structure. Transfer of α-chymotrypsin from water to cyclohexane in the presence of methyltrioctylammonium chloride does not result in extensive denaturation of the protein in the organic phase.[118] Carboxylated chymotrypsin A is less susceptible to both thermal and sodium dodecyl sulphate (SDS)-induced denaturation than the native protein,[119] and significant alteration of the stability of phage T4 lysozyme is produced by substitution of tyrosine for Trp 138.[120] The structure of cobra cardiotoxin is resistant to disruption by solvents of lower polarity than water, but can be denatured by 6M-guanidinium chloride (GuCl),[121] whereas even at this denaturant concentration the water-soluble trypsin inhibitor from soybean (Kunitz) takes 2 weeks to unfold to a disulphide-cross-linked random coil.[122] The resistance to denaturation of this protein is discussed with reference to its biological function.

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Effects of Solvents on Protein Conformation.—*Denaturation and Partially Denatured States.* The thermodynamics of the interaction of β-lactoglobulin with GuCl and urea has been studied.[123] Interaction enthalpies and free energies have been determined, as have the volume changes accompanying the interaction of the protein and denaturant. The physical nature of the thermally and GuCl perturbed states of β-lactoglobulin A have been established and parameters for the transitions estimated,[124] the thermally denatured species retaining 15—20% residual structure which is abolished on the addition of GuCl. The denaturation of δ-crystallin [125] and α-globulin [126] by urea and GuCl has been investigated. The urea-denatured state of α-globulin is more ordered than that induced by GuCl while the thermally induced two-state transition of bovine cardiac G-actin results in a less disordered state than with that produced by either denaturant.[127] Reduction of rabbit uteroglobin does not disrupt the globular structure but the reduced form is more susceptible to urea denaturation than the native protein.[128] The structures of various phospholipases and their zymogens have been shown to be resistant to acid but sensitive to both SDS and alkali denaturation.[129] Fluorescence studies of the fruit protein monellin reveal that the half-width of the total fluorescence emission band provides an index of the overall conformation of the protein [130] and reflects its 'sweetness'.

The specific effects of various inorganic denaturants on the conformation of α-lactalbumin have been followed by circular dichroism (c.d.) and ultraviolet (u.v.) absorption measurements.[131] Lithium and sodium perchlorate produce the same structure as is found on acid denaturation and in which helical structures are unaffected. A three-state transition is produced by lithium chloride. One of the two denatured states is less unfolded than the acid-denatured state, and the other is identical to the state produced by GuCl and other organic denaturants. Additivity of polar and hydrophobic group contributions gives the best account of experimental data determined from the effect of substituted formamide denaturants on globular proteins,[132] denaturant efficiency increasing with increasing chain length or hydrocarbon content of the substituent alkyl group. An estimation of changes in the hydrophobic contribution to the free energy of lysozyme caused by the addition of quaternary ammonium homologues has been used to predict resultant changes in the thermal denaturation temperature.[133] Theory and experiment only agree if it is assumed that many hydrophobic groups are still buried in the thermally denatured form. Cross-linked triclinic lysozyme has been denatured with a variety of compounds.[134] Hydrophilic denaturants, such as

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[130] J. G. Brand and R. H. Cagan, *Biochim. Biophys. Acta*, 1977, **493**, 178.
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[133] R. A. Badley, L. Carruthers, and M. C. Philips, *Biochim. Biophys. Acta*, 1977, **495**, 110.
[134] A. Yonath, A. Sielecki, J. Moult, A. Podjarni, and W. Traub, *Biochemistry*, 1977, **16**, 1413.

potassium thiocyanate and urea, interact mainly with surface polar groups causing gradual structural changes. Detergents, which disrupt the hydrophobic interior, take effect only after a critical denaturant concentration is reached. Refolding of SDS-denatured lysozyme produces a near-native structure in which an SDS molecule is trapped in the interior between the two 'wings' of the protein. This is taken to reflect the independent formation of two large structural regions prior to the final adoption of the native structure.[135] Microcalorimetric studies of the effect of SDS on trypsin and reduced trypsin reveal that the two forms of the protein unfold along different thermochemical pathways.[136]

Structure-enhancing Solvents. Solvents that induce the formation of more ordered structures have been used in conformational studies. β-Sheet formation is induced in stratum corneum by organic sulphoxides[137] and c.d. and viscosity measurements have been made on the conformation of lysozyme at low pH in the presence of dioxan,[138] which induces the formation of α-helix. Myelin basic protein extracted by deoxycholate has 8—14% more helical structure than that extracted by acid or organic solvents and may adopt a more ordered structure in myelin, possessing activity not seen in the water-soluble unordered conformation.[139]

Protein Fragments and Complementation.—The inactive carboxypeptidase fragment, residues 1—102, of the small ribonuclease barnase has been shown to regain nuclease activity when complemented with tryptic fragments 88—110 or 95—110 and with the succinylated peptide 88—110 but not with the terminal octapeptide 102—110. This suggests retention of a native structure by the major fragment but one which requires a tightly held intact sequence in the region His 102 and Tyr 103 to regain activity.[140] Complementation of two overlapping fragments of staphylococcal nuclease simultaneously forms two alternative, enzymically active, ordered structures termed type I and II complexes. The ratio of I to II formed in 2 min after mixing the fragments is approximately 0.3 and appears to be independent of temperature and the presence or absence of ligands. The equilibrium populations of the complexes are determined by the drop in free energy between the unfolded state and each complex, and although this decrease is greater for type I complex it is initially less populated than type II, indicating that the probability of following a particular pathway is not related to the decrease in free energy from the unfolded to the folded state.[141] Biologically active complexes have been formed from overlapping fragments of horse cytochrome *c*. The complexes each consist of a haem-containing fragment and one of a variety of apofragments. An ordered ferric complex is detected by cytochrome b_2 assay and fluorescence quenching by the haem group.[142] Limited tryptic digestion of cytochrome *c* produces two peptides which can form an active non-covalent complex[143] which has also been successfully modified at the position of Lys 39.

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Cyanogen bromide (CNBr) cleavage of the exopenicillinase from *Staphylococcus aureus* produces three major peptides which aggregate to form a compact globular complex, penicillinase-C.[144] Sedimentation, gel filtration, c.d., and optical rotatory dispersion behaviour of penicillinase and penicillinase-C are remarkably similar although there is a small increase in aperiodic structure and greater conformational mobility of tyrosine residues in the complex. Penicillinase-C may be dissociated by GuCl or urea, the component polypeptides adopting a random coil configuration. The complete reversibility of this process reflects the specificity of interior interactions in the native protein.

Protein magnetic resonance and c.d. studies on peptides produced by the acid hydrolysis of chicken erythrocyte histone H5 [145] indicate that the two *C*-terminal peptides are unable to form secondary or tertiary structures. The *N*-terminal peptides form both types of organized structure, peptides 1—99 possessing a similar number of helical residues to the intact histone. In contrast, investigation of histone IV and its CNBr-cleavage fragments by c.d. and spectrophotometric titration reveal the existence of stable secondary structure in the *C*-terminal fragment which is perturbed only by 8M-GuCl or 6M-urea,[146] and n.m.r. studies show that the basic *N*-terminal regions remain free when the molecule self aggregates.[147] A reversible, acid-induced transition of human serum albumin (HSA) affects the yields of the large fragments produced by peptic digestion and it is suggested that the *C*-terminal region of the protein unfolds or separates from the rest of the molecule during this process.[148] Immunological investigation of an isolated peptide of myoglobin suggests that 1% of the peptide molecules adopt native-like conformation in aqueous solution.[149]

Folding and Folding Pathways.—*Theoretical.* The application of catastrophe theory to the folding of proteins has been generally criticized on the basis, amongst others, that the process is inherently continuous and cannot be represented by discontinuous jumps.[150]

A common folding pathway for the globins has been proposed.[151] An analysis of the amino-acid sequence of trypsinogen reveals symmetrically arranged peptide pairs which are expected to fold in a similar way to each other,[152] possibly allowing prediction of the folding pathway. Previously reported studies of lysozyme and cytochrome *c* have been re-examined using a general kinetic analysis.[153] In both cases the transitions can be described by a three-species model

$$U_1 \rightleftharpoons U_2 \rightleftharpoons N$$

[144] E. A. Carrey and R. H. Pain, *Biochem. Soc. Trans.*, 1977, **5**, 689.
[145] C. Crane-Robinson, G. Briand, P. Sautière, and M. Champagne, *Biochim. Biophys. Acta*, 1977, **493**, 283.
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(U_1, U_2 are unfolded forms and N the native protein). It is proposed that this model provides a general description of the folding processes of small proteins.

Experimental Investigations of Pathways and Intermediates. α-Lactalbumin undergoes a three-state folding transition. A helical intermediate, A, in which long-range specific interactions are not required for stability, relaxes rapidly to the fully unfolded state. It is deduced that folding requires three steps, (i) formation of incipient helical structure, (ii) packing of helical segments and hydrophobic interaction, and (iii) final electrostatic stabilization.[154, 155] Studies on the α-lactalbumin transition include c.d. of the N → A transition [156] and the unfolding transition in the presence of small ligands.[157] Calorimetric results for the trans-conformation are compared with those for lysozyme.[158] A three-state process is also revealed by viscometric and spectral studies of the urea-induced transition of ovomucoid which involves an expanded intermediate with increased exposure of tyrosine residues.[159]

Partial elucidation of the pathway for the refolding of reduced ribonuclease A (RNAase A) has been proposed from c.d. observations.[160] A disulphide bond which induces favourable intra-chain interactions stabilizes an α-helical region, which may not otherwise persist, in one of the two 'wings' of the protein. The disulphide linkages in that wing reorganize by co-operative interactions of the polypeptide chain. Further β-structure appears as the other wing forms, followed by final adoption of the native structure (but see also ref. 178). Evidence that the unfolding of RNAase A is sequential has been established by electron paramagnetic resonance studies of the spin-labelled protein during thermal denaturation.[161] An immunological approach to the folding of RNAase indicates that a highly stable segment, residues 80—124, contains a nucleating site and demonstrates the presence of folding intermediates.[162] The ability to bind 2′-CMP has been used as a probe of RNAase structure.[163] Results show no native-like intermediate which does not bind the ligand, whereas similar studies on bovine carbonic anhydrase [164] indicate a quasi-native folding intermediate in which the binding site is sufficiently well developed to bind an inhibitor specific to the native protein. Differing amounts of residual secondary structure are found in rabbit muscle aldolase after denaturation in various solvents. These structures refold to the native protein in a manner consistent with the existence of a common folding intermediate.[165] A consumption pocket immunoelectrophoretic estimation of regain of antigenicity has been used to follow the refolding of reduced

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[156] K. Nitta, T. Segawa, K. Kuwajima, and S. Sugai, *Biopolymers*, 1977, **16**, 703.
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[158] T. Okuda and S. Sugai, *J. Biochem.*, 1977, **81**, 1051.
[159] A. Waheed, M. A. Qasim, and A. Salahuddin, *European J. Biochem.*, 1977, **76**, 383.
[160] S. Takahashi, T. Kontani, M. Yoneda, and T. Ooi, *J. Biochem.*, 1977, **82**, 1177.
[161] R. R. Matheson, H. Dugas, and H. A. Scheraga, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 869.
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[163] B. T. Nall and R. L. Baldwin, *Biochemistry*, 1977, **16**, 3572.
[164] B. P. N. Ko, A. Yazgan, P. L. Yeagle, S. C. Lottich, and R. W. Henkens, *Biochemistry*, 1977, **16**, 1720.
[165] J. Gerschitz, R. Rudolph, and R. Jaenicke, *Biophys. Structure Mech.*, 1977, **3**, 291.

human serum albumin [166] and indicates that certain areas of the molecule rapidly refold to the native structure but that incorrectly folded portions may persist for a considerable time after the initial refolding period. It is also shown that immunological reactivity is regained long before reformation of the disulphide bonds.

Domains. General. Trypsin cleavage of the β_2 subunit of *E. coli* tryptophan synthetase produces a partially functional dimer, the monomer of which consists of two non-overlapping fragments [167] which may be separated after denaturation. The isolated fragments refold to approximate the conformation of the corresponding fragments in the intact protein.[168] If the results of chemical modification of the lysine residues of fructose diphosphate aldolase (FDA) [169] are compared with those obtained with glyceraldehyde-3-phosphate dehydrogenase (GPD),[170] of which the three-dimensional structure is known, a possible folding domain between residues 107 and 227 in FDA may be proposed by analogy with the catalytic (subunit interaction) domain reported for GPD. It is also argued that intramolecular ionic interactions in buried hydrophobic pockets, as well as those at the protein surface, may contribute significantly to protein stability. Two domains are reported for the subunit of the GPD of *Bacillus stearothermophilus*, the first being involved in NAD binding.[171] Monomeric yeast phosphoglycerate kinase also consists of two independently folding domains as demonstrated by a study of the reversible folding transition induced by GuCl.[172]

The acid-induced expansion of bovine serum albumin (BSA) produces a state which retains globular regions but does not result in the formation of an open structure.[173] Immunological determination of the reappearance of native structure in the three domains of BSA shows that the *C*-terminal third of each domain refolds more rapidly than the *N*-terminal two-thirds [174] and indicates that folding of the *C*-terminal double loop, a possible nucleating region, is required for the subsequent folding of the domain. However, interdomain interaction restricts the ability to refold when the domain is considered as part of the intact protein.

Immunoglobulins. The thermally induced changes in IgG(κ) IVA, which, on the basis of c.d. measurements do not appreciably affect the β-sheet arrangement, were found to occur in the Fab fragments, and those induced in the kappa-type Bence-Jones protein IVA occur in the V_L domain.[175] The variable and constant regions have independently folded domains and it is probable that in the intact light chain they interact with weak non-covalent forces.[176] A pathway for the self-organization of IgG domains from antiparallel β-hairpins has been computed;

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[167] A. Hogberg-Raibaud and M. E. Goldberg, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 442.
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[171] G. Biesecker, J. I. Harris, J. C. Thierry, J. E. Walker, and A. J. Monacott, *Nature*, 1977, **266**, 328.
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[175] V. P. Zav'yalov, A. P. Demchenko, A. G. Suchomudrenko, and G. V. Troitsky, *Biochim. Biophys. Acta*, 1977, **491**, 7.
[176] V. P. Zav'yalov, G. V. Troitsky, N. N. Kechinashvili, and P. L. Privalov, *Biochim. Biophys. Acta*, 1977, **492**, 102.

one of the two most favourable structures arrived at in this way coincides with the native structure of the domains.[177]

Disulphide Bond Formation. The kinetics of refolding of RNAase A have been followed by the identification of protein molecules with differing numbers of the four possible disulphide bonds. Those with the full complement of these bonds do not necessarily adopt the native conformation and acquisition of native structure is apparently performed by disulphide interchange of molecules with three or four disulphide bonds.[178] Observations on the folding and unfolding of reduced [179] and urea- or GuCl-denatured [180] bovine pancreatic trypsin inhibitor show similarities to the results for RNAase and indicate that the refolding protein does not simply form sequential disulphide bonds.[181] Oxidation of reduced partially carboxymethylated lysozyme produces eight isomers each containing three 'native' disulphide bonds and one open disulphide. That all eight possible isomers are found is an indication that no one disulphide bond is obligatory in the formation of the other three.[182] Intracellular mechanisms for disulphide bond formation are also discussed.[183]

Proline Involvement. Cis-trans isomerization of proline residues may be responsible for the slow kinetic step seen in unfolding and refolding reactions, with the important corollary that the actual folding step is very fast. Thermodynamic measurements of the process in model compounds by ^{13}C n.m.r. have been reported.[184] Although kinetic studies on carp parvalbumins, which lack proline, reveal a biphasic folding and unfolding transition, it is argued that, as the slower step is fast compared with that seen in other proteins, these results are compatible with the central role of the isomerization process.[185]

3 Nuclear Magnetic Resonance

Contributed by H. W. E. Rattle

Biological n.m.r. now seems firmly established as a growth industry; continuing developments in instrumentation combine with the general advance of biochemistry to encourage expansion, though the vast majority of papers, not surprisingly, still come from the relatively few laboratories which specialize in n.m.r. Looking back over the past few years of this Report, the numbers of references quoted are: 1971, 55; 1972, 84; 1973, 89; 1974, 94; 1975, 154; 1976, 181; and 1977, 227. The rapid expansion which has followed the introduction of high-field superconducting machines in 1973—5 looks set to be repeated as a new generation is introduced over the next few years; with frequencies of 450 MHz and a ten-fold increase in sensitivity (up to 3000 : 1 signal to noise being claimed for new monolithic detectors), coupled with yet more powerful computational facilities

[177] V. P. Zav'yalov, *Biochim. Biophys. Acta*, 1977, **490**, 506.
[178] T. E. Creighton, *J. Mol. Biol.*, 1977, **113**, 329.
[179] T. E. Creighton, *J. Mol. Biol.*, 1977, **113**, 295.
[180] T. E. Creighton, *J. Mol. Biol.*, 1977, **113**, 313.
[181] T. E. Creighton, *J. Mol. Biol.*, 1977, **113**, 275.
[182] A. S. Acharya and H. Taniuchi, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2362.
[183] D. M. Ziegler and L. C. Poulsen, *Trends Biochem. Sci.*, 1977, **2**, 79.
[184] H. N. Cheng and F. A. Bovey, *Biopolymers*, 1977, **16**, 1465.
[185] J. F. Brandts, M. Brennan, and L.-N. Lin, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4178.

for the control of experiments and analysis of data, n.m.r. may grow still further out of its early reputation as 'the technique with the eternally rosy future'!

Turning to the 1977 papers, we find a general review of the state of biological n.m.r. in the report of the British Biophysical Society's Spring Meeting at Oxford [186] and a general review of ^{13}C studies of proteins.[187] An advanced technique which may find increasing use with very high-field spectrometers, that of two-dimensional *J*-resolved proton spectroscopy which has considerable potential in resolving and assigning individual spin multiplets in biopolymer spectra, is described.[188, 189] The use of the Carr–Purcell method A to determine T_2 seems to be subject to serious errors, particularly if $T_1 \geqslant 300$ ms, and a warning and suggestions for determining likely errors have been given,[190] while caution is also urged [191] in the use of the common shift reference DSS in the presence of aromatic solute molecules, since it appears that the trimethylsilyl group of the reference may be subject to ring-current shifts in the presence of, for example, ATP. Another paper of general interest concerns a simple n.m.r. method of determining levels of deuterium incorporation in partially deuteriated samples.[192]

Amino-acids and Smaller Peptides.—Not surprisingly, 'straight' studies of amino-acids are rare now, although one wide-line study of crystals is reported.[193] Much more interest is shown in the characterization of new compounds such as the imino-acid pyrrolidine-2,4-dicarboxylic acid [194] or a new cyclic metabolite of L-homocitrulline,[195] or in the behaviour of amino-acids under high radiation doses [196] or cross-linking with formaldehyde.[197] A possible pathway for the formation of amino-acids under primitive Earth conditions, by heating glycine with alumina, is illuminated by n.m.r.[198] The influence of hydrogen-bonding on the rotamer distribution of histidine in di- and tri-peptides has been discussed,[199] and the interaction of chloride with histidine, arginine, and lysine has been studied by ^{35}Cl resonance.[200] Other less common nuclei employed in amino-acid studies have been ^{15}N in histidines [201] and ^{31}P, employed in the measurement of ^{31}P chemical shielding tensors in L-O-serine phosphate and 3′-cytidine monophosphate.[202] Tritium resonance is employed in the analysis of the position and

[186] *Proc. Brit. Biophys. Soc.*, ed. R. Dwek, I. D. Campbell, and R. E. Richards, Academic Press, London, 1977, p. 381.
[187] W. Egan, H. Shindo, and J. S. Cohen, *Ann. Rev. Biophys. Bioeng.*, 1977, **6**, 383.
[188] K. Nagayama, K. Wüthrich, P. Bachmann, and R. R. Ernst, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 99.
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stereochemistry of tritium in labelled L-prolines,[203] while the *N*-terminal tryptic peptide blocking group of *Crithidia oncopelti* cytochrome *c*-557 was identified as dimethylproline using ^{1}H n.m.r.[204] The amount of connective tissue in meat may be rapidly determined using ^{13}C n.m.r. by determining the amount of L-hydroxyproline in its hydrolysate.[205]

Synthetic Polypeptides. A study of the ionic strength dependence of ^{1}H and ^{13}C chemical shifts in the spectra of poly-L-glutamic acid has appeared [206] and also a paper which points out the value of ^{15}N resonance in analysing the tacticity of some poly-DL-amino-acids,[207] while an old favourite, poly-β-benzyl-L-aspartate in trifluoroacetic acid–deuteriochloroform solvent mixtures, offers the interesting but on reflection not unexpected conclusion that conformational fixation of the side-chains occurs before the coil → helix transition of the backbone.[208] The dissociations of the carboxy- and amino-groups in glycine peptides up to $(Gly)_4$, and their effects on the methylene chemical shifts, are described.[209] N.m.r. has been found useful in the sequence determination of small peptides; a method using the lanthanide line-broadening probe Gd^{3+} has been described [210] and reviewed.[211] Other results which may well have value beyond small peptides include detailed studies of the exchange rate of the tryptophan indole nitrogen proton as a function of pH and temperature, in which all the observed effects could be described within a factor of 2 by the equilibrium constants $K_H = 100$, $K_{OH} = 10^8$ mol^{-1} s^{-1},[212] and a detailed analysis of amide proton titration shifts in tetrapeptide model compounds. It is suggested that few titration shifts are likely in random-coil peptides, the majority of observed shifts being due to conformational changes in folded chains.[213]

Cyclic peptides studied and reported in 1977 include *cyclo*-(D-Leu-L-His), which has a large hydrolytic activity towards the hydrophobic ester *p*-nitrophenyl laurate not shared by its analogue *cyclo*-(L-Leu-L-His),[214] *cyclo*-(Phe-MePhe), whose conformation was delineated,[215] and *cyclo*-(L-Pro-Gly)$_3$ in complex formation with chemically modified proline and valine salts.[216] A tripeptide, *N*-Ac-Gly--L-Val-Gly-OMe, which is a repeated peptide of tropoelastin, shows an 11-membered H-bonded ring of γ-turn conformation, and has been studied by ^{15}N resonance in which ^{15}N–^{1}H coupling constants reveal the non-planarity of the peptide moiety.[217] Other possible models for fragments of proteins are dis-

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cussed,[218, 219] in which the roles of sulphydryl and imidazole groups as metal-binding sites are investigated, particularly [219] as possible models for the blue Cu-binding centres of copper proteins. A very detailed study of the spin-couplings in fragments of five residues of tocinamide [220] yielded rotamer populations and opened a discussion of the possibilities for preferred conformations.

Naturally Occurring Peptides. In the latest instalments in a comprehensive study of the basic pancreatic trypsin inhibitor the emphasis is now shifting from low-field to high-field peaks. A 360 MHz proton study [221] permitted the assignment of the methyl resonances to their individual types of amino-acid and the identification of the methyl resonances of the *C*-terminal Ala-58 and the active-site Ala-16 residues. These methyl proton resonances were then related to the ^{13}C resonances of the methyl carbons in the molecule.[222] Nitration of the tyrosines was followed by chelation of Pr^{III} and Eu^{III} ions, leading to assignments of one α-CH and five amide proton resonances in the 270 MHz spectrum.[223]

Peptide hormones are a group of molecules of great importance whose study by n.m.r. has been limited by their limited availability. The story so far is summarized in a review,[224] while the backbone flexibility of luteinizing-hormone releasing hormone (luliberin) is discussed by two groups,[225, 226] one of which also considers the molecular dynamics of angiotensin II,[227] possibly of use in arriving at conformation models *via* individual ^{13}C T_1 values. Hydrogen–deuterium exchange studies of angiotensin II in deuteriated trifluoroethanol revealed more than three peptide hydrogens which do not exchange, consistent with other data which suggest a family of stable conformations, stabilized in trifluoroethanol by internal H-bonds.[228] For the enkephalins, apparent discrepancies in the published assignments of met-enkephalin are resolved by two papers [229, 230] which reveal that there are in fact two conformations, one cationic and one zwitterionic, although details of the structures are not yet available; further shifts and assignments are presented [231, 232] and it is proposed that leucine–enkephalin, too, has two or more conformations based on β-bends and which may exist in equilibrium in DMSO solution since the energy difference between them appears to be small.

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The most widely studied peptide hormone so far is oxytocin, and this shows in the increasing sophistication of the experiments being performed on it. Rotamer populations of the $C_\alpha-C_\beta$ bonds in the molecule have been investigated by ^1H n.m.r. in two laboratories,[233, 234] and substitution of D-alanine for L-alanine in positions 3 and 4 of the peptide-chain has enabled the role of these residues at corner positions of a reverse turn to be elucidated.[235] Two laboratories have synthesized oxytocins enriched at specific sites with ^{13}C and studied their interactions with bovine neurophysins. In one case [236, 237] it was concluded that the binding involved at least the first three but not the last three residues of the nonapeptide, and this conclusion has been extended,[238, 239] leading to a dynamic model of the interactions. Preliminary studies of the interaction between oxytocin and its competitive inhibitor (1-L-penicillamine)-oxytocin are also reported.[240]

Antibiotic and toxic peptides are this year represented by observations that modifications of gramicidin-S ornithine and phenylalanine side-chains, or substitution of glycine for proline in the chain, do not appear to affect its conformation.[241] Gramicidin-A′ molecules have been observed in phosphatidylcholine vesicles, with some indication from lanthanide probes that the tryptophan side-chains, which have some local mobility, may be located in the interfacial region of the bilayer.[242] The solution conformation of valinomycin was considerably refined by a good set of experiments involving double and triple heteronuclear resonance and $^{13}C-^{203, 205}Tl^+$ coupling constants, revealing a 'bracelet' structure formed by six fused β-turns in weakly polar media, and a 'propeller' conformation involving three type II β-turns in media of medium polarity.[243] Other antibiotics studied in some detail include cyclosporin C,[244] viomycin,[245] 2-deaminoacetinomycin D,[246] melanostatin,[247] and lophyrotomin, a new toxic octapeptide from the larvae of the sawfly *Lophyrotoma interrupta*.[248]

Enzymes and Related Proteins.—As the instrumental, as distinct from biochemical, need to restrict experiments to smaller peptides continues to diminish, the number of larger proteins studied increases at an accelerating rate. A general account of

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uses of paramagnetic probes to study the conformations of enzyme-bound substrates has been given.[249] Hen egg-white lysozyme continues to attract attention, with a detailed series of ring-current shift calculations [250] and a review of the interaction of lanthanide ions with the enzyme.[251] Much earlier work on the binding of lanthanides assumed that the magnetic susceptibility tensor of the metals is axially symmetric. A new study [252] based on the resonance shifting effects of Nd^{3+} and Ce^{3+} rejects the axially symmetric model with a high degree of probability. A continuing series of studies, using natural-abundance ^{13}C resonance, leads to the conclusion that the relaxation of non-protonated carbon nuclei is due to chemical shift anisotropy at high fields (>40 kG) [253] using relaxation probes to assign non-protonated aromatic carbon signals.[254] Considerable weakening of the binding of Gd^{3+} to lysozyme is found to follow the linking of one of its binding sites (Glu-35) *via* an ester linkage to modified Trp-108.[255] Unlike intact lysozyme, the modified protein does not show evidence of aggregation at neutral pH, and a similar reduction in self-association is seen when lanthanides are bound (near Asp-52 and Glu-35) to normal lysozyme, in which an intermolecular contact lifetime of 1—2 ms is found, with an alteration in the chemical shift of the C_γ of Trp-62 but not of other non-protonated aromatic carbons.[256] This is somewhat at variance with the results of another published study of lysozyme self-association, in which perturbations of Trp-108 signals were also found, together with evidence that the region around His-15, possibly including $\geqslant 1$ positively charged residues, takes part in a head-to-tail self-association.[257] The possibility of specific ^{13}C labelling of lysozyme is pursued in a study [258] in which *in vivo* incorporations of tritiated methionine were followed, showing a 5—8% incorporation which is very worth while, though the effort needed to produce it goes far beyond mere chickenfeed! Other studies of lysozyme include relaxation measurements on deuterium-labelled substrates bound to the protein [259] and studies of the relaxation of water protons in lysozyme crystals,[260] powder,[261] and in the water layer in the immediate vicinity of spin-labelled lysozyme molecules in solution.[262]

The enzyme–substrate interactions of chymotrypsin have been studied by ^{19}F,[263] ^{31}P,[264] and ^{13}C [265] resonance, in each case only fairly general conclusions

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being possible. The replacement of Zn by Mn at the active site of thermolysin enabled studies of the ionizable groups in the active-site region to be undertaken, and led to the notion that two molecules of the inhibitor *N*-trifluoroacetyl-D-phenylalanine will bind to the enzyme.[266] Unfolding of ribonuclease A has been investigated again,[267] as have the histidine residues of ribonuclease S-peptide and S-protein, this time *via* the hydrogen–deuterium exchange rates.[268]

The mechanism of carbonic anhydrase continues to attract interest. A comparative study of the histidine resonances in human, bovine, and rhesus carbonic anhydrases revealed a pattern of extensive homologies which are discussed in relation to various models for the catalytic mechanism,[269] while specific labelling of His-200 with ^{13}C revealed that although this residue is sensitive to active-site events [270] it is not the critical group in controlling catalytic activity.[271] Changes in the resonances of ^{199}Hg [272] and ^{113}Cd [273] bound in place of the Zn ion at the active sites of carbonic anhydrase were related to anion concentration, with the interesting result that the lines were split into widely spaced doublets by binding of $^{13}CN^-$, showing direct binding between the anion carbon atom and the active-site metal ion. Very close binding between substrate HCO_3^-, CO_2, and the metal of carbonic anhydrase is also favoured [274] following measurements made on cobalt-substituted enzyme and its effects on the longitudinal relaxation times of ^{13}C-labelled substrate. The relaxation behaviour of water in copper-carbonic anhydrase solutions [275] and frozen manganese-carbonic anhydrase solutions [276] is also reported.

Substrate and inhibitor binding to dihydrofolate reductase has been reviewed.[277] Experiments continue with ^{31}P measurements of the binding of 2′-AMP, a fragment of the coenzyme NADPH, which confirm the importance of the 2′-phosphate group to the binding. A preference for the dianionic form (1.6 kcal mol^{-1}) suggested an interaction with two positive charges in the enzyme, possibly on an arginine residue.[278] Binding of *p*-aminobenzoyl-L-glutamate to the protein may occur at two sites, one of which is competitive with methotrexate and at which the binding affects three histidine residues.[279] Extensive labelling of *Lactobacillus*

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casei dihydrofolate reductase aromatic residues with deuterium [280] and ^{19}F [281] has been used to study methotrexate binding and to show that substrate and inhibitor bind differently to the protein. Binding of the coenzyme NADPH appears to produce chemical shift changes *via* conformational changes of the whole molecule. It should be interesting to compare these results with those from studies of dihydrofolate reductase from a methotrexate-resistant strain of *L. casei*, at present being sequenced,[282] and another interesting pathway may be opened by the apparent success of ^{13}C labelling of the guanidine groups of arginine in reductase from *Streptococcus faecium*, giving five well resolved resonances which changed on the addition of ligands.[283]

The binding of NAD to the tetrameric glyceraldehyde-3-phosphate dehydrogenase isolated from sturgeon muscle shows negative co-operativity. Total and partial alkylation of the SH groups at the four active sites [284] coupled with ^{19}F labelling revealed that this co-operativity is probably due to induced interactions between these subunits which are at least partially mediated by the active-site SH residues. Similar experiments [285] quote *X*-ray evidence in support of these conclusions. A continuing study of complex formation in TPN-dependent isocitrate dehydrogenase [286] *via* proton relaxation rates of solvent water indicates that Mn^{II} acts as an electrophilic centre for the binding of substrate. On the specificity of substrate binding to glutamate dehydrogenase,[287] it is reported that whereas little difference is found in the binary complex formation between the enzyme and various dicarboxylic acid substrate analogues, the coenzyme would only form a strong ternary complex in the presence of the correct substrate.

A novel nuclear relaxation approach for the estimation of the distance between enzyme and nucleotide-bound metal ions has been described.[288] It depends on cross-relaxation of the unpaired electron spins of the metals and the effect that this has on the relaxation of inner-sphere water protons, and is used here to estimate a distance of 5.2 Å between Mn^{2+} and Cr^{3+} in the pyruvate kinase–Mn^{2+}–ATP–Cr^{3+} complex; this distance is consistent with a van der Waals contact between the hydration spheres of the metal ions. ^{31}P Resonance was used to infer that reversible co-ordination of the transferred phosphoryl group by the nucleotide-bound metal occurs during the phosphoryl transfer reaction of pyruvate kinase,[289] and the possibility is shown [290] of using $^{7}Li^{+}$ as a resonance probe of the univalent cation site on this enzyme. The coupled action of pyruvate kinase and adenylate kinase on adenosine 5′-phosphorothioate is shown to result

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in a specific diastereoisomer of adenosine 5′-*O*-(1-thiodiphosphate).[291] Another novel relaxation method, involving the transfer of selectively inverted spins between chemical environments, was used to study the 2ADP ⇋ ATP + AMP reaction catalysed by adenylate kinase.[292] The ^{31}P resonance of fully bound substrates of the arginine kinase reaction shows that the β-phosphate of ADP in a substrate analogue assumes a structure approaching that of bound ATP.[293] Other studies include proton relaxation rates for phosphoglycerate kinase [294] and creatine kinase,[295] ^{19}F studies of thymidilate synthetase,[296] and the identification of trimethyl-lysine in a rat testis calcium-dependent regulatory protein of cyclic nucleotide phosphodiesterase.[297]

The binding of dihydroxyacetone phosphate to triose phosphate isomerase, studied by ^{31}P resonance, has been shown to be strong only in the free keto form, thus removing any correction that might have had to be made to the kinetic parameters to allow for binding of the hydrate.[298] A combination of ^{13}C with ^{31}P resonance studies of the same enzyme resulted in the suggestion that 2-phosphoglycolate binds as the trianion and echoes the difference between free keto and hydrate forms of dihydroxyacetone phosphate in binding.[299] *Staphylcoccus aureus* has a phosphocarrier protein system which is also amenable to ^{31}P studies. It appears to have a relatively rigid structure, although not stabilized by disulphide bridges, and its histidine titrations [300] and tyrosine titration and denaturation behaviour [301] have been reported. The covalently bound PO_4^{2-} group at the active site of phosphoglucomutase is another prime candidate for ^{31}P resonance.[302] Among the detailed results discussed is evidence that the bound Mg^{2+} activator is close to, but not directly co-ordinated to, the active-site phosphoserine. Allosteric interactions between metal ion and phosphate at the active sites of the dimeric zinc metalloenzyme alkaline phosphatase of *E. coli* are reported [303] to be responsible for the negative co-operativity of phosphate binding to this enzyme; in this case ^{113}Cd resonance was used to reinforce the results of phosphorus resonance experiments. Phosphorus was again used in studies of rabbit muscle

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glycogen phosphorylase binding of pyridoxal-5′-phosphate[304] and glucose 1-phosphate.[305]

The cystine residues of aspartate transaminase may be labelled with ^{19}F; in the resulting spectrum there are three signals, one corresponding to residue 82 which shows a single ionization shift, another which shows two such shifts, and a third which is particularly affected by substrate binding.[306] The linewidth/pH and shift/pH of the ^{19}F signals from difluoro-oxaloacetate bound to the aldimine form of aspartate transaminase show inflections in both the pH 5 and pH 8 regions, the pH 5 inflection being absent in complexes with the apoenzyme, indicating that it is due to ionization of a group associated with the pyridoxal phosphate cofactor.[307] The binding of succinate inhibitor to aspartate transcarbamylase, studied by ^{1}H n.m.r., indicates that three forms of this enzyme exist, differing in their states of protonation.[308]

Preliminary studies which, it is hoped, are the precursors of a fascinating and very important series may be found in the preparation of selectively deuteriated analogues of the lac repressor. Those who have had the courage to embark on such an ambitious study of this large but vital protein surely deserve success, although the rewards are likely to come slowly.[309, 310]

On a slightly simpler level, we may note studies of the interaction between human serum albumin and tryptophan,[311] L-isoleucine and its binding protein from *E. coli*,[312] and histidine and its binding protein J from *Salmonella typhimurium*.[313] Brief mention may also be made of studies on histidine decarboxylase,[314] glutamine synthetase,[315] indolyl-3-alkane-α-hydrolase,[316] rabbit muscle aldolase,[317] ubiquitin,[318] reduced keratin,[319] papain,[320] serine hydroxymethylase,[321] and rabbit uteroglobin,[322] all of which used n.m.r. to a fairly large extent.

In an interesting first step in the use of n.m.r. to study the structures and

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functions of venom toxins,[323] the n.m.r. spectra of a neurotoxin and a cardiotoxin from *Naja mossambica mossambica* were compared. Although the spectra indicate considerable structural similarities between the proteins, the cardiotoxin was shown by the amide proton exchange rates to be much more flexible in solution than the neurotoxin. The suggestion is made that this may be connected with the different functions of the toxins.

The binding of calcium to specific calcium-binding proteins is open to n.m.r. investigations: in one protein from bovine dentine [324] which has about 40 residue % aspartic acid and 40 residue % of phosphoserine, ^{31}P spectra revealed a specific Ca^{2+}–orthophosphate interaction in addition to the Ca^{2+}–carboxylate interactions indicated by i.r. spectroscopy. Sodium resonance was used to monitor the displacement of Na^+ by Ca^{2+} at the calcium-binding site of pike parvalbumin,[325] and in a ^1H n.m.r. study of calcium binding by rabbit muscle troponin-C the large spectral changes consequent upon calcium complexation indicate that binding to the two high-affinity sites both directs and stabilizes much of the structure; other changes in the spectrum on Ca^{2+} binding to low-affinity sites may be associated with the mechanism of contraction.[326]

The structure of the antibody–hapten binding site is clearly of very great importance, though difficult to pursue. Three papers describing experiments on variants of mouse myeloma protein appeared in 1977. In two of these the hapten was phosphorylcholine [327, 328] which was labelled at the methyl group with ^{13}C; the similarities between n.m.r. spectra on binding to several myeloma proteins indicated a significant degree of conservation of important hapten binding site interactions and suggest two main interaction subsites with different dissociation rate constants. The evidence also supports the view that the heavy chains of these proteins predominate in the interaction. Considerable detail of the binding site of the dinitrophenyl-binding IgA mouse myeloma protein MOPC 315 has been determined by a variety of techniques.[329]

Membrane Proteins and Lipoproteins.—We find three studies have appeared of the integral membrane enzyme cytochrome *c* oxidase. In the first [330] the lipids were labelled with a difluoro-group at the 1-position of the hydrocarbon chain and the resultant ^{19}F spectra used to establish that the protein has an ordering effect on the lipid bilayer in reconstituted membranes well beyond those lipids in direct contact with the enzyme surface. An improvement in structural homogeneity in phosphatidylcholine vesicles on binding of cytochrome *c* oxidase is reported;[331] in this paper it is also pointed out that the protein appears to span the bilayer, although with

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statistical orientation. In a quite different set of experiments [332] on low-temperature flash photolysis of detergent-solubilized cytochrome *c* oxidase and its CO binding kinetics, n.m.r. studies support the presence of mobile regions well below the macroscopic freezing point of the solution. In the calcium ion transport ATPase–sarcoplasmic reticulum system, proton studies have established that the protein is highly mobile compared with other membrane-bound proteins such as rhodopsin;[333] in another interesting series of experiments,[334] the Ca^{2+} ATPase was reactivated with $^{13}C/^{14}C$-labelled oleic and linoleic acids and lysolecithins, retaining up to half its original activity. Only half of the fatty acids of the phospholipids are bound to the apoenzyme, the rest forming the membrane bilayer and being highly mobile in it. Something like 20% of the phospholipid is found to be protein-bound in human low-density lipoprotein;[335] the interactions are with the B peptide of the protein. Natural-abundance ^{13}C resonance has been used to investigate the structure of plasma lipoproteins [336] in which it is stated that the apoproteins have a 'limited role' in determining the dynamics of the lipid components, and ^{13}C labelling has been employed in studies of human [337] and *E. coli* [338] lipoprotein systems. Lipoprotein complexes between glucagon and dimyristoylphosphatidylcholine [339] and the glycophorin A phospholipid system [340] are also reported.

In experiments on the phosphatidylcholine exchange protein [341] it was found that, in the presence of this protein, between 50 and 85% of the *N*-methyl deuteriated phosphatidylcholine molecules in the outer layer of single-lamellar vesicles were replaced by protonated molecules from erythrocyte ghost membrane in 24 h, but that decay of the transbilayer compositional asymmetry within the vesicles themselves was a much slower process with a half-time of 26 days.

Copper Proteins.—Structural changes in azurin on conversion from the oxidized to the reduced form cause marked changes in the spectrum both in linewidth (broader lines in the oxidized form) [342] and in chemical shift. In addition to comparisons with the Hg^{II}-substituted protein which has a structure similar to that of the oxidized form, it is shown that several well resolved resonances between -9.5 and -12.5 p.p.m. in the spectrum of the reduced form are not detectable in the paramagnetic protein.[343] The same workers used optically detected magnetic resonance to study triplet states of tyrosine and tryptophan in azurin.[344] The copper–zinc-containing protein superoxide dismutase contains eight histidines, all of which give resolvable resonances which with one exception

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have pH-sensitive shifts at pH 6.8.[345] Of these histidines, His-41 is particularly labile to deuterium exchange,[346, 347] while it appears that the Zn^{2+} ion alone organizes the active site; if the ion is present, only His-19 is susceptible to chemical modification with diethyl pyrocarbonate, whereas all eight readily react in the apoenzyme.[348]

The difference between oxidized and reduced forms of copper proteins, noted for azurin, is emphasized again in the case of spinach plastocyanin, in which 16 of the 128 separate resolved natural-abundance ^{13}C resonances in the reduced form become undetectable in the oxidized form, while the other peaks show little change, indicating that there is no substantial conformational change.[349] Relaxation studies of hog kidney diamine oxidase [350] indicate that the active-site Cu^{II} is not located near the oxidizing site of the enzyme, but is located near the non-oxidized end of the binding substrate.

Iron-containing Proteins.—A proton resonance study of a series of high-spin synthetic porphyrin complexes suggests,[351] that the shifts of the axial imidazole, rather than those of the porphyrin, will be useful as indicators of histidine Fe tension. These proximal histidine proton resonances are assigned for sperm whale deoxymyoglobin and deoxyhaemoglobin A.[352] A large amount of data on the titration behaviour of individual histidines of myoglobins, based on ^{13}C resonance experiments at natural abundance, is presented [353] along with a considerable number of assignments; from the same group [354] comes a note concerning the titration of the *N*-terminal glycine residue. ^{15}N Studies of haem proteins are now being undertaken using labels at backbone nitrogens, in this case of glycine in haemoglobin,[355] giving a large number of ^{15}N resonances whose shifts were affected by denaturation, but less so (unfortunately) by ligand binding or dissociation into α- and β-subunits. Another approach is to label the nitrogen in CN^-, which then gives two resonances, assigned to Fe-bound $C^{15}N^-$ in the α- and β-subunits, for haemoglobin [356] and only a single resonance for other haemoproteins, these being found to have very pH-sensitive chemical shifts.[357] ^{13}C Labelling of the carbon in CO was linked to experiments in which a number of substituents were attached to the periphery of the haem group in various proteins;[358] the different protein structures impose differences between the haemo-

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globin α- and β-subunits and between myoglobin and haemoglobin which are largely independent of the substituents.[358] Another of the possible approaches to haem proteins is to use the many ring-current and hyperfine shifted resonances; one such study, of a variant haemoglobin which has permanently oxidized haem groups in the β-subunits, produces results consistent with a model of haemoglobin oxidation which is sequential, rather than the simpler two-state concerted allosteric model which is more usually propounded.[359] The field and temperature dependence of the hyperfine shifted resonance linewidths were also investigated,[360] revealing an apparent modulation of the dipole–dipole interaction between the Fe electron spin system and the haem methyl proton spins by rotational diffusion. Preliminary studies of plant leghaemoglobins are presented for lupine [361] and soybean [362] proteins. The binding of various ligands to haem proteins, including the interactions of formate with metmyoglobin,[363] bis-arylhydroxysulphonic acids with haemoglobin,[364] and ATP and 2,3-diphosphoglycerate [365, 366] with human haemoglobin, is also reported. The longitudinal relaxation time of protons in haem solutions is relatively easy to measure and can be quite informative: its field dependence is used to calculate that the electron spin relaxation time of high-spin Fe^{III} is 6×10^{-11} s.[367] The haem Fe accessibility to solvent molecules, using the relaxation of methanol protons in D_2O solution, is discussed,[368] as are other examples.[369–371]

The structural basis for the variation in redox potential of various cytochromes is discussed.[372] Although axial ligands dominate the potential, there is another factor, and variations in the chemical shifts of methionine resonances for various cytochromes indicate that different redox potentials are associated with different lengths of the Fe—S bond, a 0.1 Å shortening of this bond being associated with a reduction in redox potential of ~400 mV. The binding of this methionine sixth ligand to the iron is also discussed with reference to oxidation [373] and, with much other detail, in a comprehensive pH titration study.[374] Two conformers of ferricytochrome *c*, differing at the sixth ligand, are described,[374, 375] and the presence of a single trimethyllysine residue in the cytochrome *c* of *Candida krusei* has been

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established.[376] Two methods of chemical modification of cytochrome *c* which may be of interest to others are described; all the 19 lysine residues were guanidylated [377] (converted into homoarginine) with ^{13}C-labelled groups, two of which gave discrete resonances, and in the other the tyrosine at position 74 was iodinated,[378] the modification apparently having no effect on the spectral and other properties of the protein.

The cytochrome *c*-551 of *Pseudomonas aeruginosa* has been compared with mammalian cytochrome *c*'s.[379] Rather more detail is contained in reports of 360 MHz studies of cytochrome *c*-552 from *Euglena gracilis;* the intermolecular electron exchange rate, at 5×10^6 mol^{-1} s^{-1}, is about 1000 times faster than that in mammalian cytochrome *c*'s and one of the Fe ligands is shown to be His-14.[380, 381] Solvent proton magnetic relaxation in solutions of rabbit liver cytochrome P 450 leads to the conclusion [382] that the haem environment can accommodate several H_2O molecules in fast exchange with the bulk solvent. The low-spin $\rightleftharpoons$ high-spin transition of camphor-bound cytochrome P450 has been studied.[383]

The tetrameric protein ferredoxin has a marked effect on the n.m.r. spectrum of cytochrome c_3.[384] Further investigation of the ferredoxin (from *Desulfovibrio gigas*) reveals two stable trimeric states in addition to the tetramer, the three oligomers not being magnetically equivalent.[385] Many resonances of the spectrum of ferredoxin from *Clostridium acid-urici* are assigned,[386] and it is pointed out that other ferredoxins give very similar spectra which may be assigned by analogy.

The effect of metal binding on the structure of the cyclic hexapeptide ferrichrome has been studied [387] by comparing the ^{13}C spin–lattice relaxation of deferriferrichrome and alumichrome. In the context of iron-containing proteins we may also mention studies of fluoride and formate interaction with beef liver catalase,[388] of ATPase and substrate binding sites in the enzyme system nitrogenase from *Azobacter vinelandii*,[389] and of the haem environment [390] and interactions with indolepropionic acid [391] of horseradish peroxidase. A histidyl residue is probably responsible for the ligand-binding properties of the enzyme, and the alkali-induced transition of the molecule is due to direct co-ordination of an amino-acid

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residue to the haem Fe rather than to the proton dissociation of an Fe-bound water molecule as is the case in metoxyoglobin.[390]

Proteins Associated with Nucleic Acids.—The gene V protein from bacteriophage M13 has been shown to unwind a small double-helical fragment of DNA, d(pC-G-C-G), even at 0 °C. Exchange of protein between nucleic acid substrates is fast, and it is suggested that tyrosines take part in the protein–nucleic acid interaction, and that the unwinding is determined by fluctuations in the DNA structure.[392] Binding of the very lysine-rich histone H1 to DNA is considerably influenced by phosphorylation at either or both of Ser-37 and Ser-105, and phosphorylation of Ser-105 which is located in the globular region of H1 was found to reduce the enthalpy of structure formation from 24 to 13 kcal mol^{-1}.[393] The globular region itself, from residues 35—120, was prepared by limited trypsin digestion,[394] and a similar series of studies on fragments of histone H5 show that the *N*-terminal peptide 1—99 contains most, but not all, of the residues forming the globular region of the protein.[395] Fragmentation studies have also been made of the core histone H4, revealing that the self-aggregation of this protein leaves the basic *N*-terminal region of the histone free, presumably for interaction with DNA.[396] The core histones H2A, H2B, H3, and H4 are found complexed together in the nucleosome, and so there is considerable interest in their cross-interactions. Detailed fragmentation studies of the H3–H4 complex show that the critical regions for complex formation are residues 42—120 in histone H3 and 38—102 in H4.[397] ^{19}F Studies of trifluoroacetonylated H3 and of its interactions with H4 and in an equimolar core–histone mixture are reported,[398] with a series of ^{13}C and ^{31}P measurements[399, 400] which show that the globular regions of the individual core histones form a complex aggregate in the whole nucleosome, with freely moving *N*-terminal regions able to interact with the DNA double helix.

Moving on to RNA, two studies of RNA polymerase have been published. A single manganese ion may be bound to the catalytic site, providing a paramagnetic reference point[401] for kinetic measurements which are extended[402] to the conformation of enzyme bound adenyl-uridine and ATP. Ribosomal proteins were the subject of three papers, two of which[403, 404] appear to indicate that acid-urea

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extraction of these proteins retains less of the tertiary structure than salt extraction, while the third concentrates on the (salt-extracted!) protein S4, showing a specific tertiary fold which disappears on lowering pH below 5 or heating above 30 °C, both processes being reversible.[405]

Magnetic resonance studies of whole systems will undoubtedly be a major growth region in the next few years; it is worth pointing out ^{15}N spectra of whole cells,[406] spin-echo measurements of metabolism in human erythrocytes,[407] chromaffin granules,[408] a review of ^{31}P resonance in whole muscle studies,[409] and ^{1}H n.m.r. of intact normal and cancerous mouse cells.[410] Measurements of the state of water in cell and other systems are now less numerous, and are represented by studies of water in collagen [411] and in protein and tobacco mosaic virus solutions [412] and of water binding by the interesting antifreeze proteins from antarctic fish.[413]

4 Infrared Spectroscopy

Contributed by R. M. Stephens

Model Compounds.—The infrared spectra of 20 glycine- and alanine-containing di- and tri-peptides in D_2O showed that the zwitterion and hydrochloride forms of the tripeptides have the C^5 conformation, whereas in alkaline solution the hydrogen bond responsible for this conformation is broken. The end groups, CO_2^- or CO_2D, possess stretching vibrational frequencies which are characteristic of the terminal amino-acid. Bending vibrational frequencies of the CH_2 or Me groups were shown to be dependent on their position in the peptide chain.[414] The spectra of glycine, alanine, phenylalanine, glycine, HCl, and K glycinate have been recorded at 4 K. The authors also measured the i.r. spectra of glycine in aqueous solution between pH 0.5 and pH 13.30, and it was concluded that glycine, HCl was converted into glycine when the pH was between 2.34 and 9.60, and that K glycinate was formed between pH 9.60 and pH 14.[415] A study of the solid-state vibrational spectra of deuterium-substituted L-glutamic acid hydrochloride showed that, from the analysis of the band shifts resulting from deuteriation, interatomic bond distances remained virtually unchanged. The cell volume expanded by only 0.05% upon deuteriation, whilst the macroscopic, linear, and non-linear polarizabilities were unaffected.[416] Solid-state and solution conformation studies have been made on *N*-butylcarboxycarbonyl-L-prolylglycine using i.r. spectroscopy and *X*-ray diffraction. The results indicate that in the solid-state

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form intramolecular hydrogen-bonded ten-membered oxy-analogues were present. These were absent, however, in polar solvents. Cotton effects observed at 225 nm indicated that a type of folding might be present in low-polarity solvents.[417] The amide A, I, II, and V bands from the oligopeptides H-$(X)_7$-OMeHCl, where X = Ala, Nva, Leu, Cys(Me), Met, Val, Ile, or Phe, have been recorded and structural postulations made. Most oligopeptides adopted an antiparallel β-sheet structure but it was suggested that when X = (Val, Ile, or Phe) the oligopeptides had the unusual parallel β-sheet conformation.[418] The i.r. spectra of 13 metal(II) complexes of glycylglycine have been recorded and discussed in relationship to the known or probable structures of the complexes. It was possible to distinguish various structures on the basis of the spectral differences.[419]

Using i.r. and magnetic spectroscopy, the interaction between the amide-group and copper(II) and nickel(II) has been studied.[420] The spectra of primary amides in the ν_{NH} region have indicated that $AchNH_2$ forms self-association groups having a *cis* conformation (referring to the position of the hydrogen atom involved with respect to the Me group) but forms *trans* association with other proton acceptors. Studies were also made on bound NH groups in binary and tertiary hydrogen complexes.[421, 422] Complicated ν_{NH} and ν_{ND} spectra from hetero-associated and self-associated acetamide were accounted for by Fermi perturbations and by the *cis–trans* isomerism of the H-bonds. The two protons in the $CONH_2$ group are different with respect to hetero- and self-association processes, and the cyclic structure of the acetamide dimer was proved. Frequencies and intensities of the ν_{NH} stretching vibrational frequency in the H-complexes and the corresponding H-bond strengths were evaluated.[423] The polarized Raman and far-i.r. spectra have been recorded from crystalline $^+NH_3CH_2CO_2^-$ and $^+ND_3CH_2CO_2^-$. A normal co-ordinate analysis of the optically active intra- and inter-molecular vibrations was performed. A modified Urey–Bradley intramolecular potential was used. The group factor splittings were elucidated using an intermolecular potential including exchange repulsion–dispersion interaction between non-bonded atoms and the Coulomb interaction. It was also concluded that the H-bond stretching potential contributed to lattice frequencies.[424] The *N*-terminal region of histone H4 and of various related oligopeptides Ac-Ser(Ac)-Gly-Arg-$(Gly\text{-}Lys\text{-}Gly)_2$-Leu-Gly-Lys-Gly-Gly-Ala-Lys-OMe, H-$(Gly\text{-}Lys\text{-}Gly)_3$-Leu-Gly-Lys-Gly-Gly-Ala-Lys-OMe, H-(Gly-Lys-Gly-Leu-Gly-Lys-Lys-Gly-Gly-Ala-Lys-OMe, and H-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys-OMe, have been studied using i.r. and c.d. spectroscopy. All four peptides possessed the polyproline II-type extended left-handed helical conformation in acidic and neutral solution, in moderate (0.15 mol. l^{-1}) ionic strength, in 80% EtOH, in 0.2 M-

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sodium dodecylsulphate, in 8M-urea, and in 5M-guanidinium,HCl. However, the conformation was sensitive to temperature changes and calcium chloride concentrations.[425]

Polypeptides.—The conformation of collapsed, oriented, molecular monolayers of poly-(α-aminoisobutyric acid) has been studied using polarized i.r. and electron diffraction. Strong evidence is produced to suggest that the structure is that of the 3_{10}-helix rather than the α-helix. The i.r. dichroisms of the amide-group vibrations are similar to those from the α-helix, showing that the orientations of the amide-group in the two conformations are similar. However, the amide A and amide V frequencies occur at 3275 and 694 cm^{-1} and these are significantly different from the corresponding frequencies obtained from type α-helical structures.[426] Large single crystals of poly-(γ-benzyl-L-glutamate) have been formed from concentrated chloroform solutions. The crystals (approximately $2 \times 0.3 \times 0.05$ cm) had an ordered structure but the i.r. spectra indicated that it was not helical and further structural studies are planned.[427] A series of sequential oligopeptides and a peptide containing the sequence L-Leu-L-Ala-L-Leu-Gly were prepared by fragment condensation methods and poly-condensation methods respectively. The conformations of the peptides were studied by far-i.r. and *X*-ray powder diffraction techniques. The polypeptides and hexadecapeptides from α-helical structures and the dodeca- and lower oligo-peptides form β-structures in the solid state. The critical chain length for the formation of an α-helix in the solid state was found to be 16 residues.[428] Poly[Glu(OBzl)-Glu]$_n$, poly(Glu-Gly)$_n$, poly[Gly-Glu(OBzl)-Gly], and poly[Gly-Glu-Gly] were prepared from the pentachlorophenyl esters of the sequential monomer. Polymers containing free glutamic acid residues were soluble in water, as were the lower molecular weight fractions of the polytripeptides containing the benzyl ester. I.r. and c.d. studies suggested that a helical structure (though not an α-helix) was favoured for the polydipeptide. The polytripeptides possibly formed two different types of helix (one similar to that found for the polydipeptide), but this was found to be solvent dependent.[429] Using force field characteristics obtained from β-polypeptides the normal vibrational frequencies of α-helical poly-(L-alanine) and *N*-deuteriated poly-(L-alanine) have been calculated. The agreement between calculated and observed Raman and i.r. frequencies is quite good.[430] A force field has been refined for the antiparallel chain rippled sheet structure of poly-glycine I in which transition dipole coupling and hydrogen-bonding are taken into account. Amide I and amide II mode splittings are accounted for and quantitative explanations of the amide A and B mode frequencies and intensities are provided. This force field is transferable to other β-polypeptides even though they have antiparallel chain pleated sheet structure. The $\nu(0\pi)$ amide II frequencies from poly-glycine I, poly-(L-alanylglycine), poly-

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(L-alanine), and poly-(L-valine) were calculated in order to evaluate the sensitivity of the amide II frequency to structure.[431] The normal vibrational frequencies of poly-(L-alanine) and poly-(L-alanylglycine) in the antiparallel chain pleated structure have been calculated using a force field obtained for poly-glycine I[431] plus additional force constants for the methyl-group. The agreement between the expected and observed i.r. and Raman frequencies was good. This substantiated the transferability of the force field in this case since poly-glycine I has a rippled sheet structure. The amide I and II mode splittings are accounted for by transition dipole coupling.[432]

Proteins.—I.r. absorption spectroscopy has been adapted to determine very quickly elevated triglyceride levels in human serum. Good quantitative agreement was found between this i.r. technique and two other independent biochemical methods, both of which are time-consuming.[433] Spectra of lyophilized sera from patients with viral hepatitis or jaundice have been compared with the i.r. spectra from normal serum or serum albumin, and qualitative conclusions drawn.[434] The spectra of adenosylcobalamin (vitamin B_{12} coenzyme) and B_{12r} (vitamin B_{12}–Co^{II}) have been found to be similar to each other but different from the spectra of cyanocobalamin (vitamin B_{12}) and aquo- and hydroxo-cobalamin. Methylcobalamin was also studied. Significant changes were observed in the i.r. spectra of acid solutions, in which the benzimidazole base is protonated.[435]

Analysis of the amide I band for a large number of globular proteins has been made using computational techniques. The relative amounts of α-helix, β-antiparallel chains, and random conformations were calculated. The percentage of β-conformation present in a sample either derived from *X*-ray analysis or computed from the i.r. spectra was in good agreement.[436] Multiple internal reflection techniques have also been used to determine the amount of protein in various wheats and soya beans, and a complete data analysis has been performed.[437] The amount of residual protein in extracted pectic substances has been determined by measuring the ratio of the intensities of the amide I or II bands to that of the CO band from the free carboxy-group. The pectic substances had to be thoroughly demethoxylated before their i.r. spectra could be recorded.[438] The mode of binding of carbon monoxide to iron in cytochromes P450 and P420 has been investigated using i.r. spectroscopy. Changes in the intensity of bands and bandwidths of bands in the amide I region are discussed in terms of the interaction between amino-acid residues in the haem pocket and carbon monoxide.[439] Near-i.r. (600—2000 nm) absorption spectra and magnetic circular dichroism spectra have been used to investigate the spin states of haem proteins, and conclusions are compared with model calculations.[440] Hydrogen-bonding

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[434] V. A. Mamontova, V. M. Barai, and V. M. Merezhinskii, *Biokhimiya*, 1973, **1**, 124.
[435] D. S. Rajaria and A. Nath, *J. Inorg. Nuclear Chem.*, 1977, **39**, 1291.
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[437] W. H. Hunt, D. W. Fulk, B. Elder, and K. Norris, *Cereal Foods World*, 1977, **22**, 534.
[438] M. P. Filippov, G. Kaldare, and O. V. Bondareva, *Zhur. priklad. Spectroskopii*, 1977, **26**, 938.
[439] H. Rein, S. Boehm, G. R. Jaenig, and K. Ruckpaul, *Croat. Chem. Acta*, 1977, **49**, 333.
[440] P. J. Stephens, J. C. Sutherland, J. C. Cheng, and W. A. Eaton, 'Excited States Biol. Mol. Proc. Int. Conf., Wiley, New York, 1976, p. 34.

between lecithins [$C_3H_5(RO_2{=}C)$ $(R{-}O_2C)$ (PO_4) $(CH_2)NMe_2$] and hydroxy solvents, *e.g.* H_2O, MeOH, EtOH, n-$C_{10}H_{21}OH$, or cyclohexanol or cholesterol has been studied between 3000 and 3800 cm^{-1} at temperatures between 10 and 60 °C. Thermodynamic parameters (ΔH, ΔS) and equilibrium constants are given.[441]

5 Circular Dichroism

Contributed by T. Brittain

During the period covered by this Report the literature predominantly shows the use of circular dichroism as a probe of structure and conformation. However, a number of theoretical papers have appeared together with those instrumental papers, which in particular describe the use of c.d. for the characterization of transients occurring during reactions.

General.—*Reviews.* A review of the basic theory of c.d. together with some examples of recent applications has appeared.[442] The use of c.d. as a probe of the conformations of amino-acids, peptides, and biopolymers has also been reviewed.[443, 444] Data have been gathered together concerning the theory and methodology of determining the absolute configuration of complexes and this is illustrated diagrammatically for a large number of examples.[445]

Instrumental. The application of small He–Ne lasers to rapid c.d. measurements using stopped flow and temperature jump has been described.[446] A new system, utilizing these lasers, with improved bandwidth, optical aperture, and source ratioing electronics, has been applied to temperature-jump studies on optically active complexes of ligands and biological molecules. Transient c.d. and optical transmission are measured simultaneously with an optical c.d. resolution of 2×10^{-6} (66×10^{-6} deg) at 300 μs time constant, when the optimal absorption resolution is $\pm 4 \times 10^{-5}$. The noise spectrum of the light source has been found to be important in determining the resolutions and it is in this area that low-noise lasers are most valuable. A theoretical treatment of c.d. resolution was given specifically as a function of the sample absorption and the amount of available light. The theoretical resolution for photon-noise-limited sources is approached in this system. Absorption spectroscopic features characterizing simple equilibria are discussed that may be derived from the observation of transient c.d. in temperature-jump experiments.[447] A machine has also been described in which light is modulated by a polarimeter and stress modulator for studying rapid c.d. changes occurring in stopped-flow and pH-jump experiments. Its capabilities have been determined by investigations on α-lactalbumin in the u.v. region, which showed first-order kinetics at 270 nm in reaction with 2M-guanidine hydrochloride. After addition of dithioerythritol at pH 8.0 two first-order processes

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[442] A. Galat and M. Kielozewski, *Wiad. Chem.*, 1976, **30**, 529.
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[445] Y. Saito and J. Fujita, *Kagaku Sosetsu*, 1976, **13**, 57.
[446] M. Anson, *Electro Opt./Laser Int. U.K., Conf. Proc.*, 1976, 117.
[447] M. Anson, S. R. Martin, and P. M. Bayley, *Rev. Sci. Instr.*, 1977, **48**, 953.

were observed indicating conformational changes caused by reduction of disulphide bonds.[448]

A c.d. modulation excitation spectrophotometer has been constructed using a 100 W tungsten iodide lamp to give more radiation in the 400—800 nm region than the Xe arc. A solid-state UDT PIN 10 u.v. photodiode operated in the photovoltaic mode was used in conjunction with an electronic analogue divider to study the c.d. of fragments of purple membrane from *Halobacterium halobium*. This apparatus, because of the low intensity of the excitation light compared with flash-photolysis, may be used to study many biological systems that either saturate or are subject to light blocking at high intensity.[449]

New methods of dispersion-induced c.d. have permitted direct observations of electronic transitions which are either magnetic or electric-dipole allowed. They involve measurements of c.d. of achiral complexes in solutions containing some non-associating chiral solute or in chiral solvents. In this way the *d–d* transitions have been assigned for Cu^{2+}.[450]

Instrumental calibration with (+)-camphor-10-sulphonic acid at 290 nm has yielded results for molecular ellipticity, $[\theta]$, with deviations of up to 30% at 220 and 490 nm. Older instruments give higher values of $[\theta]$ at 490 nm and lower values at 220 nm, probably owing to ageing of Pockel cells. A proposal is made to use D-pantolactone and (+)-tris(ethylenediamine)Co^{III} monohydrate as standards for use at 220 and 490 nm respectively, whilst using (+)-camphor-10-sulphonic acid for calibration only at 290 nm.[451]

Theory and Analysis. It has been shown that if the same set of reference spectra is used then the methods of analysis normally employed, *i.e.* linear least-squares curve fitting and reciprocal functions, are equivalent and so no new information is obtained by the use of either method.[452] The method proposed by Baker and Isenberg for the establishment of secondary structure of proteins has been shown to be a least-squares technique. Estimations of structural content obtained by this method for ribonuclease, myoglobin, lysozyme, lactate dehydrogenase, and papain are not substantially different from those obtained using an unconstrained linear least-squares procedure.[453]

The moment summation method has been applied to both natural and magnetic c.d., leading to evaluation of the quantities $\Omega_q = \Sigma(E_n - E_0)qR_{ON}$ (R_{ON} = rotational strength) for $q = 0$—6. These expressions contain terms which facilitate the analysis of optical activity using four-atom clusters. The relative contributions from relaxation and correlation effects for an arbitrary one-electron hole have been assessed. The relationships and quantum methods for distinguishing between the types of dissymmetry are outlined. The analogous moments for the Faraday effect derived are $F(1) = 3e\hbar cK$ (K = magnetic susceptibility) and

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[449] M. A. Slif'kin and T. I. Y. Allos, *J. Phys.* (*E*), 1977, **10**, 508.
[450] P. E. Schipper, *J. Amer. Chem. Soc.*, 1976, **98**, 7938.
[451] T. Tuzimaru, T. Komo, H. Meguro, M. Hatano, T. Murakami, K. Kashiwabara, K. Saito, Y. Kondo, and T. M. Suzsuki, *Analyt. Biochem.*, 1977, **81**, 167.
[452] G. Willick and M. Zucker, *Biophys. Chem.*, 1977, **7**, 223.
[453] R. G. Hammonds, *European J. Biochem.*, 1977, **74**, 421.

$F(2,3,4) = -(2,3,4)(e\hbar/2mc)S^{2,3,4}$, where $S(q) = \Sigma q$th moment for ordinary absorption.[454]

A number of papers have treated the theory of optical activity of helical polmers. The classical theory and calculations of c.d. for an infinite helical polymer [455] have been extended. A method for obtaining the optical activity of helical polymers has been derived, which applies helical symmetry and periodic boundary conditions to classical polarizability theory. A complex unit of symmetry containing many optical transitions is treated and gives the c.d. bandshape in terms of monomer transition bandshapes. However, no electron exchange or overlap is considered, static field effects are omitted, and internal interactions and solvent effects are not explicitly treated.[456, 457] Another treatment of this problem has used a linear response tensor for calculating the optical activity and has been extended to include detailed analysis of the contributions from light incident perpendicular to the major axis. Because of the helical symmetry and exciton selection rules for periodic boundary conditions the radial term resulting from the analysis assumes a simple form in terms of the components of the linear response tensor. Both radial and helical terms are expressed in closed form in terms of monomer interactions by use of a decorrelation approximation. The resulting expressions may be used for calculation and to evaluate the role of geometry in determining optical activity.[458]

A theoretical analysis of the appearance of optical activity in long-wavelength absorption bands of low molecular weight ligands adsorbed on to an asymmetric polymer template has appeared.[459]

For the first time a set of general equations has been derived from first principles to describe the phenomenon of vibrational c.d. In order to overcome a vanishing electronic contribution to rotational strength in the Born–Oppenheimer approximation, the equations include correction terms, which provide the necessary correlation of velocities of electrons and nuclei. The summation over all excited states is formally removed by introduction of vibronic gauge functions, which satisfy a known differential equation. The resulting vibrational c.d. spectra for fundamental transitions are identical to those previously derived in the localized molecular model of vibrational c.d.[460, 461]

A formalism has also been developed for the evaluation of rotational strength of vibrational transitions using first-order anharmonic wavefunctions. This formalism, in contrast to the previous harmonic treatments, admits c.d. in overtone and combination transitions. Explicit expressions are derived for rotational strength in two quantum vibrational transitions. Second-order anharmonic effects are shown to be of the same order of magnitude as the non-Born–Oppenheimer contributions to rotational strength.[462]

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[460] L. A. Nafie, *Chem. Phys. Letters*, 1977, **49**, 441.
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[462] T. R. Faulkner, C. Marcott, A. Mosowitz, and J. Overend, *J. Amer. Chem. Soc.*, 1977, **99**, 8160.

Green operator techniques have been used within the susceptibility framework to express the c.d. of a diamide model of an unordered polypeptide. The formulation containing all the common mechanisms giving rise to c.d. has the advantage that the bandshape functions can be included for each absorption band of each isolated non-overlapping chromophore. The rotational strength and bandshape are determined by solving a complex eigenfunction problem.[463]

The effect of including charge-transfer states in the optical activity of *d–d* transitions of Cu^{2+} α-amino-acid complexes has been analysed using a static field perturbation model and a simplified structural model.[464]

Chiroptic properties have been examined on a theoretical model in which electronic wavefunctions were obtained from semi-empirical MO calculations. The CNDO/S MO models used to perform SCF–MO calculations on the ground and excited states were constructed in the virtual orbital–configuration interaction approximation. Electronic rotational strength and dipole strength calculations were made directly from the complete molecular wavefunctions. Zwitterion, cationic, and anionic S-proline have been studied. Two types of conformational variable are represented in the calculation, (i) pyrrolidine ring conformation and (ii) rotation about Cα—COO^- bond. The rotational strength was found to be insensitive to the conformational changes within the pyrrolidine ring. C.d. spectra of the zwitterionic S-proline down to 160 nm are accounted for by the calculations if conformational preference with respect to rotation about the Cα-COO^- bond can be assumed to exist. Spectrum–structure correlations are offered for anionic and cationic forms.[465]

A theory has been presented for the c.d. of an achiral chromophore associated or bonded to some other chromophore, which may or may not itself be chiral. Second-order perturbation terms in the dipole approximation are at least of the same order as the first-order perturbation terms involving higher multipoles. These second-order terms are derived and the symmetry rules of the mechanism are discussed.[466]

Jahn–Teller effects in optical bands have led to the use of the method of moments for the determination of linear dichroism and c.d. of $^{2S+1}A \rightarrow {}^{2S+1}\Gamma$ transitions.[467]

It has been shown that an achiral molecule may exhibit c.d. in the presence of an intense beam of circularly polarized light. Differential absorption rates based on quantum electrodynamics calculations have been reported for (i) electric-dipole-allowed and (ii) electric-dipole-forbidden but two-photon-allowed transitions. In both cases the differential rate is shown to be linearly dependent on the intensity of the laser beam.[468]

Computer programs based on one-, two-, and three-site binding models have been prepared in order to facilitate analysis of controlled pH and temperature

[463] D. A. Rabenold, *J. Chem. Phys.*, 1976, **65**, 4840.
[464] S. Bunel, L. Gil, and H. Bobadilla, *J. Inorg. Nuclear Chem.*, 1977, **39**, 365.
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[467] Y. E. Perlin, L. S. Kharchenko, and B. S. Tsukerblat, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1976, 1770.
[468] T. Thirumamachandran, *Chem. Phys. Letters*, 1977, **49**, 536.

titrations of macromolecules followed by c.d. The binding of RO 5–0991 to human serum albumin was used as a test of the programs, which compare experimental data with theoretical predictions.[469]

A review of the general theory and experimental c.d. studies of the binding of bilirubin to serum albumin has been presented.[470]

A general non-perturbation theoretical analysis for two-photon magneto-optic effects, such as polarization rotation and c.d., has led to the proposal of a new optical analogue of the Hanle effect.[471]

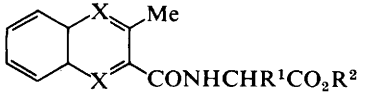
The chiroptic properties associated with the two lowest-energy S—S transitions have been calculated for *cyclo*-L-cystine using a model in which both dynamic and static coupling between the dioxopiperazine and S—S moieties were considered. The structure permits chirality at the S—S junctions and requires the dihedral angle to be $\pm 90°$. The vicinal contributions of the S—S group to optical activity arising from the dissymmetric interactions between the dioxopiperazine and S—S groups have been calculated and related to the observed c.d. spectra.[472]

Small Molecules, Model Compounds, and Synthetic Polymers.—*Amino-acids and Derivatives.* A new sensitive method for the determination of the absolute configuration of amino-acids utilizes the c.d. properties of *N*-2,4-dinitrophenyl-α-amino-acylamides.[473] Another approach has been reported which utilizes the sign of the Cotton effect near 255 and 315 nm in *N*-salicylidene derivatives of amino-acids formed *in situ*. It was found that the Cotton effects have the same sign as the chirality (right-hand screw for positive chirality).[474]

N-Acetoacetyl derivatives of aliphatic and aromatic (*S*)-α-amino-acids show enantiomorphic c.d. in ethanolic potassium hydroxide but similar spectra in acetonitrile, ethanol, and trifluoromethylethanol.[475]

The absolute configurations of biguanide (Hbg)–amino-acid complexes of the type $[Co(am)(Hbg)_2]^{2+}$, where am is an anion of glycine, L-alanine, L-valine, L-isoleucine, L-proline, or sarcosine, have been assigned using c.d.[476]

The quinoxaline derivatives of structure (1; X = N) of aliphatic and aromatic L-α-amino-acids exhibit enantiomorphic c.d. in ethanol as well as in ethanolic potassium hydroxide. However, the corresponding quinoxaline 1,4-dioxide (1; X = NO) of L-α-aliphatic and aromatic amino-acids show similar c.d. in organic solvents. This behaviour is attributed to the difference in conformational equilibria in both of the series.[477]



(1)

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[476] H. Kawaguchi and B. Douglas, *J. Co-ordination Chem.*, 1976, **5**, 115.
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In complexes of L-aspartic acid, L-isoaspartic acid, L-aspartic acid diamide, L-glutamic acid, L-isoglutamic acid, and L-glutamic acid diamide with Cu^{2+} (2 : 1, ligand : metal) the signs observed for Cotton effects were in agreement with predictions made by the hexadecant rule. There was no agreement in the case of the analogous Ni^{2+} complexes, presumably owing to octahedral or square-planar complexation, or else formation of polynuclear complexes.[478]

An induced Cotton effect in a distant ligand has been observed with mixed complexes of Cu^{2+} with L- and D-proline and 2,2'-bipyridyl (bipy) or 1,10-phenanthroline (phen) in the u.v. region. Both [Cu(bipy)L-Pro] and [Cu(phen)L-Pro] show strong positive c.d. at 310 and 275 nm respectively in the region of $^1A_1 \rightarrow {}^1B_1$ transitions of the heterocyclic diamines. [Cu(bipy)L-Pro] shows c.d. bands due to charge transfer $n \rightarrow \pi^*$ transitions, which are characteristic of aminoacidates. However, with $[Cu(phen)L\text{-}Pro]^+$ the negative c.d. at 275 nm is obscured by strong positive bands due to the $^1A_1 \rightarrow {}^1B_1$ transition of phen.[479]

Pd^{II} gives tetragonal complexes with L-cystine and L-homocystine in aqueous solution of two types, 1Pd : S—S and 2Pd : S—S. The use of c.d. titrations in the u.v. and visible region has led to the following suggestions: (i) the conformation is such that the dihedral angle has values of 0° and 60° in the case of L-cystine for the 1 : 1 and 2 : 1 complexes respectively and 70° for the 1 : 1 oxidized glutathione complex; (ii) there is a reversed chirality of the disulphide group (M → P) in neutral or basic solution for the L-homocystine case; and (iii) strong Pd—S bonding in the L-cystine case.[480]

The first ever reported case of the observation of vibrational c.d. of an amino-acid (alanine) has appeared. All four hydrogen atom stretch vibrations have been assigned. With the aid of these an interpretation based on the chiral perturbation of degenerate asymmetric methyl stretch vibrations is proposed.[481]

Mixed-ligand Co^{III} complexes with (*S*)-aspartic-*N*-monoacetic acid and various amino-acids have been characterized by the use of c.d.[482]

Dipeptides and Oligopeptides. Variable-temperature c.d. in the u.v. region shows that β-formation is induced in α-L-glutamic acid oligomers of the type MeCO—$(Glu)_n$—NHEt as temperature increases. The transition necessarily passes through an unordered form and the $\alpha \rightarrow \beta$ transition is reversible when the β content is lower than some critical value.[483]

Cyclo-(D-Leu-L-His) shows a large hydrolytic activity towards *p*-nitrophenyl laurate. The c.d. of this compound has been compared with that of *cyclo*-(L-Leu-L-His), which is almost inactive in the hydrolysis. It was demonstrated that the spatial arrangement of the hydrophobic isobutyl groups of D-leucine residues and the nucleophilic imidazoyl groups of L-histidine match well with the long acyl chain and active ester groups of *p*-nitrophenyl laurate. However, in the case of

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[480] S. Bunel, C. Ibarra, A. Urbana, and C. A. Bunton, *Inorg. Nuclear Chem. Letters*, 1977, **13**, 259.
[481] M. Diem, P. J. Gotkin, J. M. Kupfer, A. P. Tindall, and L. A. Nafie, *J. Amer. Chem. Soc.*, 1977, **99**, 8103.
[482] G. Colomb and K. Bernauer, *Helv. Chim. Acta*, 1977, **60**, 459.
[483] M. Rinaudo and A. Domard, *Macromolecules*, 1977, **10**, 720.

cyclo-(L-Leu-L-His) this match is not present and so explains the observed differences in hydrolytic activity.[484]

Somatostatin, H_2N-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-CO_2H, and its analogues have been studied by c.d. in the near- and far-u.v. region, in 6M-guanidine solution. Solvent-induced difference spectra show that structural variants D-Phe[6] and Leu[12] have similar conformations. D-Ala[8], D-Trp[8], and D-Phe[11] have altered reverse turns, whereas Gly[8], Ala[8], and D-Phe[7] have quite different spectra from the native material. Based on model studies, it was found that these results are consistent with the presence of a reverse turn and an average contribution from Phe of $+18\,000$ deg cm^2 dmol^{-1} at 219 nm with Trp[8] contributing $+27\,000$ deg cm^2 dmol^{-1} at 226 nm. This then leads to a contribution of the single S—S group and thirteen peptide chromophores of $-43\,400$ at 225 nm, making this the first example of an estimation of the average contribution of aromatic residues to the c.d. in the peptide region for a protein with more than two or three peptide chromophores.[485]

C.d. studies of Bu^t CO_2—$(Met)_n$—OMe (n = 2—7) in CF_3CH_2OH show that all the species exist in predominantly unordered conformations. The species with n = 7 does not form β-aggregates in CF_3CH_2OH, which is in contrast to heptamers of isoleucine and valine.[486]

The effect of ClO_4^- on solutions of poly-lysine (mol. wt. = 10^5) and poly-ornithine (mol. wt. = 1.2×10^5) over the temperature ranges 20—70 °C and 10—40 °C respectively has been followed by the use of c.d. ClO_4^- which normally diminishes the stability of ordered conformations, stabilizes the α-form of poly-lysine. A model is proposed in which negative charges on ClO_4^- compensate for the overall positive charge on the protonated amino side-chains.[487]

A Glu–Lys copolymer containing 8% lysine, modified by treatment with acetyldehydrophenylalanine azlactone (I) or acetyldehydrophenylalanyldehydrophenylalanine azlactone (II), at alkaline pH, showed c.d. spectra characteristic of random coil. However, at acid pH the c.d. spectra were consistent with an α-helical structure. The copolymer modified with (I) showed no dichroism due to the unsaturated chromophore, whereas the copolymer modified with (II) showed bands which varied widely with changes in pH.[488]

H-Pyro-Glu-His-Pro-NH_2 (thyrotropin releasing factor) and some of its analogues in dioxan-rich solutions showed intramolecular hydrogen-bonding between the amide H of proline and the CO_2^- groups of histidine, inducing strong *negative Cotton effects* at 226 nm. It was also found that the linkage between the N^{π}-side of the imidazole ring and another group in the molecule affects the stability of the proline amide conformation.[489]

The polydipeptides poly[Glu(OBzl)-Gly]$_n$ and poly(Glu-Gly)$_n$ have a 2_1

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helical structure, whose structural integrity is reduced in the debenzylate form, whereas the polytripeptides poly[Gly-{Glu(OBzl)-Gly}] and poly(Gly-Glu-Gly) show a 3_1 helical structure in addition to the 2_1 form, depending on the nature of the solvent used.[490]

Triblock copolypeptides of γ-benzyl-L-Glu and L-Leu, of high molecular weight, assume an α-helical conformation, whereas the copolymers containing L-valine assume a β-sheet conformation.[491]

C.d. studies on γ-methyl-D-GluNH$_2$ polymers in the form of films cast from dichloroethane, methylene chloride, dimethylformamide, or methyl chloride–dimethylformamide show the presence of the cholesteric twisted structure, which is stable up to 180 °C but which at 240 °C is gradually annealed after 1 h.[492]

β-Benzyl-L-Asp–β-*p*-phenylazobenzyl-L-Asp copolymer has been shown to be able to undergo a light-induced conformational change and a subsequent relaxation back to its original conformation.[493]

The structure-inducing properties of L-leucine, L-isoleucine, and L-norleucine incorporated into poly-L-Lys have been reported. Studies have been carried out on the coil → helix transition in H_2O–MeOH mixtures, the formation of ordered structures at high pH, and the kinetics of the $\alpha \rightarrow \beta$ transition of the leucine and norleucine copolymers induced by temperature changes at pH 10.5. The results confirm the strong α-inducing role of leucine and also the idea that isoleucine is one of the most powerful candidates for β-formation, together with the intermediate properties of norleucine. These data are discussed on the basis of steric and hydrophobic properties of the three side-chains.[494]

The molecular structure of $(Lys\text{-}X\text{-}Gly)_n$, (I) (X = Ala, Nva, Val, or Leu; mol. wt. 5×10^3—10^4), has been studied both in the presence and absence of DNA. (I) (X = Ala, Nva, or Val) is unordered in aqueous solution at neutral or basic pH. At high ionic strength it is aggregated and takes on a form similar to the conformation of collagen. In 90% Me_2CHOH (I) shows a c.d. spectrum typical of a mixture of ordered and α-helix. DNA complexes of (I) are characterized by c.d. spectra similar to those of unordered poly(Ala-Lys-Pro) DNA complexes, whereas DNA (I) complexes with X = Leu show a positive c.d. signal typical of polypeptides with a high degree of α-helix.[495]

Poly-(L-Pro), poly-(γ-OH-L-Pro), ionized poly-(L-Glu), ionized poly-(L-Lys), and poly-(N^5-ω-hydroxyethyl-L-Glu) show similar c.d. changes on addition of high concentrations of salts. In each case isothermal addition of 4M-$NaClO_4$ eliminates the positive c.d. signals. These studies emphasize the use of c.d. to detect conformations common to all polypeptides, independent of side-chains. However, the effects of sodium dodecyl sulphate (SDS) were shown to be side-chain dependent. Calculations using an assumed equilibrium constant for the

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propagation of helix predict an increased helical content for lysine side-chains in the presence of SDS and account well for the observed c.d. in 1% SDS.[496]

The different conformations possible for polypeptides have been characterized by c.d., out into the vacuum-u.v. region. The linear β-pleated sheet shows a broad band down to 165 nm. Co-polypeptides of the type poly-(Lys-Leu-Lys-Leu) show an intense band at 169 nm. Reverse turns were measured using poly-(Ala_2-Gly_2) and show a good correlation with theory, with bands at 191 and 169 nm. The c.d. of unordered alternating copolypeptides in salt-free solution were also obtained.[497]

Acetylbis(dehydrophenyalanine) attached to the ε-NH_2 group of lysine in the co-polymer (Glu^{92}-Lys^{8}) shows an induced c.d. between 250 and 330 nm due to the relative dissymmetric disposition of the two dehydro-phenylalanyl groups under the chiral field of the polypeptide chain.[498]

Poly-(L-Lys-L-Ala-α-L-Glu) (I) is zwitterionic at pH 7.0 and its conformation has been found to be determined by its state of ionization, molecular weight, temperature, and solvent, being almost entirely α-helical at low pH for mol. wt. >25 000. C.d. shows that the α content varies linearly with molecular weight in the range 3×10^3—3×10^4. At temperatures where (I) is in the random-coil form, addition of trifluoroethanol induces appreciable amounts of α-helix. A comparison of (I) in various ionization states with other polypeptides containing alanine and glutamic acid or lysine shows that the α-helical content is directly proportional to the number of each residue, the α-inducing character being in the order Ala > Glu > Lys.[499]

The co-polymers of L-Lys and L-Ile[poly-(L-Lys,L-Val)] show an increased β-content with increasing Ile content at high pH at room temperature. C.d. has also been used to evaluate the fractional β-content as a function of pH as well as to derive the thermodynamic parameters associated with the coil → β transition.[500]

Films of poly-(β-benzyl-L-Asp) show c.d. spectra of the benzyl chromophore, which are related in magnitude and sign to the conformation of the polymer. The aromatic c.d. of the left-hand α- and ω-helices are negative in sign, whereas that of the β-conformation is positive. The α-helical form shows a positive $n \rightarrow \pi^*$ peptide transition at 226 nm and the ω-form a smaller band at 224 nm. In the β-form this transition occurs at 223 nm, the absolute magnitude being $\beta < \omega < \alpha$.[501]

Vacuum-u.v. c.d. of films of Bu^tCO_2—$(Leu)_n$—OMe (n = 2—7) showed that for n = 6 or 7 a β-structure exists in which both parallel and antiparallel chains are present. For n = 4 or 5 there is a mainly disordered structure, and the c.d. of the compound with n = 3 resembles that of the contribution from an internal Leu-Leu peptide chromophore with randomly coiled chains.[502]

N-Acetyl-*N*′-ethylprolineamide, *N*-acetyl-*N*′-methylglycinamide, *N*-acetyl-*N*′-methylprolineamide, and *N*-acetyl-*N*′-methylprolyglycylamide all exist as an

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ensemble of conformation at 29 °C. There is no evidence for large amounts of β-structure in the Gly-Pro derivatives, and the temperature dependence of the c.d. of the blocked Gly-Pro derivatives, in water, is similar to that of poly-(Gly-Pro), indicating that the conformation of the polymer is due to local interaction.[503]

C.d. studies have shown that poly-(L-*p*-aminophenylanine), when charge free, can assume two ordered conformations depending on the temperature. At room temperature the polymer is a right-hand helix but at temperatures > 40 °C a new conformation is produced, whose rate of formation is typical of β-structures. Thermodynamic parameters for the coil → β-transition show that the stability of the β-form is due mainly to enthalpy factors, the formation being kinetically unfavourable.[504]

H-Gly-Phe-$(Gly)_n$-Trp-Gly-OH (I) (n = 0, 1, or 2), H-Phe-Gly-OH, H-Gly-Phe-OH, H-Gly-Phe-Gly-OH, H-Phe-Trp-OH, H-Phe-Trp-Gly-OH, and H-Gly-Phe-Trp-OH have been studied by use of c.d. in both water and trifluoroethanol solutions. Peptides containing one Phe residue show markedly different near-u.v. spectra depending on whether the Phe is *N*-terminal or not. The oligopeptides (I) (n = 0, 1, or 2) show no strong intramolecular interactions between the two aromatics. However, the dichroic properties of (I; n = 0) are anomalous and a comparison with H-Gly-Phe-Trp-OH, H-Phe-Trp-Gly-OH, and H-Phe-Trp-OH at different pH values has confirmed that the presence of two adjacent aromatics leads to a restriction of the conformational equilibria.[505]

C.d. pH titrations of His, His-Gly, Gly-His, and Gly-His-Gly show that the spatial orientation of the molecules depends on vicinal charges. For His-Gly and Gly-His-Gly a good correlation exists between the ionization of the Gly carboxy-group and the increase in rotamer III (g—g) as visualized by the enhancement of c.d. activity at 212 nm. In both peptides hydrogen-bonding between imidazoline and CO_2H appears to stabilize rotamer III at pH 4—5.[506]

Detailed conformational features and the helix–coil transition have been examined for poly-(*O*-carbobenzoxyl-L-Tyr) in pure and mixed solvents. In $CHCl_3$, dioxan, Me_3PO_4, MeCHCl(OH), pyridine, or $(CF_3)_2CHOH$ it has a right-handed α-helical conformation, but in $CHCl_2CO_2H$, CF_3CO_2H, $(Me_2N)_3P$ hexafluoroacetate sesquihydrate, or 1,3-dichlorotetrafluoroacetate-2,5-hydrate a random coil is adopted.[507]

The effects of pH on Trp-Trp, Trp-Trp-Gly, and Gly-Trp-Trp have been compared with those of systems containing only one Trp residue. The negative Cotton effect at 225 nm, observed in Trp-Trp and Trp-Trp-Gly when the terminal α-NH_2 is deprotonated, changes with a change in solvent or temperature, indicating that the two adjacent aromatic side-chains become conformationally more rigid on an increase in pH. This generates exciton coupling between B_b transitions of the two indole rings. It appears that hydrophobic forces, including stacking interactions, are not important in stabilizing this conformationally rigid structure.

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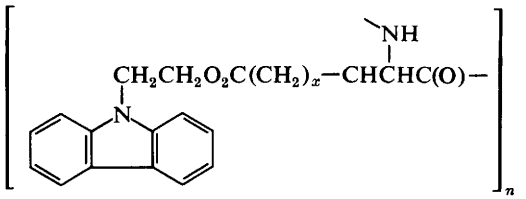
However, intramolecular electrostatic interactions as well as interactions with the OH groups of the solvent are salient features.[508]

The chiroptic properties of the cyclic dipeptides *cyclo*-[L-Ala-L-Tyr] and *cyclo*-[L-Tyr-L-Tyr] have been investigated as a function of molecular conformation. A good agreement is found between theoretical calculations, as a function of side-chain dihedral angles and angle of fold of the cyclic peptide, and the experimentally observed c.d. for *cyclo*-[L-Tyr-L-Tyr], but in the case of *cyclo*-[L-Ala-L-Tyr] such a good agreement was not found, although the sign of the observed Cotton effect was correctly predicted.[509]

The derivatives poly-{γ-[2-(9-carbazoyl)ethyl]L-Glu} (2) and poly-(β-{2-(9-carbazoyl)ethyl]-L-Asp} (3) have been shown to exist in a right hand α-helix in CH_2Cl_2 and in a left-hand α-helix, when in the dry state.[510]



(2) $x = 2$
(3) $x = 1$

A general study of the c.d. and kinetic properties of metal oligopeptides has appeared.[511]

The c.d. spectra of 19 different hexa-, nona-, deca- and dodeca-proline-containing peptide complexes with Cu^{2+} have been reported. In the cases where D-Phe was substituted for L-Phe a Cotton effect at 550 nm appeared or alternatively the spectrum between 490 and 520 nm was shifted. Proline-containing hexapeptides show optical activity at 490—520 and 580—600 nm.[512]

It has been shown that asymmetric centres, located one or two amino-acid residues beyond the planar centre of the ring structure, of Cu^{2+} and Ni^{2+} oligopeptide complexes can still contribute to the visible c.d. of the metal-ion chromophore. The c.d. of triply protonated tetrapeptide complexes of Gly_3Lys, Lys_4, Val_4, and $Gly_3Lys\text{-}OMe_3$ together with the pentapeptides Lys_5, Gly_4Lys, and $Gly_4Lys\text{-}OMe_3$ has been compared with the c.d. of tripeptide complexes. Intramolecular hydrogen-bonding and weak axial co-ordination probably account for the vicinal interactions between the metal and asymmetric centres in the fourth and fifth amino-acids. The c.d. of Cu^{3+} and Ni^{3+} oligopeptides was also reported.[513]

The binding of sodium poly-(L-Glu) to pseudo-octahedral iron complexes has been followed using *trans*-$[FeL^1(OH)_2]^+$ and *cis*-$[FeL^2(OH)_2]^+$ [L^1 = 2,2′,2″,2‴-tetrapyridyl: L^2 = *NN*′-bis-(2-methylpyridyl)ethylenediamine]. The binding

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isotherms for the reaction between sodium poly-(L-Glu) and *trans*-$[FeL(OH)_2]^+$ and the c.d. spectra of the polypeptide as a function of the ratio of bound complex to polymer residue have been treated by a two-state model for the polyelectrolyte. A preferred association between the complex ions and the helical conformation of the polymeric matrix has been found.[514]

At pH 7.5 sodium poly-(L-Glu) interacts with *NN'*-diethylpseudoisocyanine, leading to an extremely sharp c.d. signal in the region of the *J*-band. At pH 4.6, where the polypeptide has an α-helical structure, no band is seen in this region. C.d. studies in the u.v. region show that the occurrence of the polypeptide–dye complex, which gives rise to the *J*-band, is not accompanied by a conformational transition of the polypeptide towards the α-form, as might be expected.[515]

Cyclo-(Pro-Gly)$_4$ binds Li^+, Na^+, K^+, Cs^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} to form cation–peptide complexes in 1 : 1 and 1 : 2 ratios. The cyclic peptide binds Ca^{2+} and Ba^{2+} with affinities comparable to those of natural cyclic peptides. The conformations of the resulting complexes have been analysed by the use of c.d.[516]

Two papers have appeared which are concerned with the recombination of synthetic RNAase-S peptide analogues (1^{ϵ},7^{ϵ}-diguanidino-[Tyr^8]-, 4^{ϵ},7^{ϵ}-diguanidino-[Asn^{14}]-, [Phe(F)^8, Orn^{10}]-, and [Cha^8, Orn^{10}], (Cha = cyclohexylalanine). C.d. studies have revealed an environmental alteration of Phe^8 and Tyr^{25} in the S peptide on recombination, which is characterized by a strong optical signal, clearly defined by means of comparative analysis of the spectra. The aromatic chromophore contribution in the 220—250 nm region strongly exceeds the hyperchromism due to the random coil → right-hand α-helix transition accompanying the association. The contribution from the peptide constitutes a large portion of the total dichroism, but nevertheless substitution of non-aromatic Cha in position 8 with Phe, *p*-F-Phe, or Tyr leads to a negative c.d. signal at 215, 215, and 212.5 nm respectively, the strongest ellipticity increment being 28% for the substitution with Tyr. These data, together with those on RNAase A and S, permit the assignment of the positive c.d. at 240 nm in RNAase A to transitions of Phe and inaccessible Tyr.[517, 518]

A change in temperature between 6 and 24 °C for actinomycin D in aqueous solution produces little change in its c.d. signal, whereas in the dimer a large temperature dependence was observed. Dimerization studies were further complicated by the formation of higher aggregates at millimolar concentrations. The c.d. spectra of actinomycin D showed three bands at ~372, 415—445, and 490 nm, the latter probably associated with the quinoid ring present.[519]

The c.d. spectra of gramicidin A analogues shortened by two to four amino-acids were similar to those of the analogues shortened by only one amino-acid.[520] However, the gramicidin S analogues *cyclo*-[Val-Orn(R)-Leu-D-Phe-Pro]$_2$ (R =

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$PhCh_2O_2CAc$), *cyclo*-[Val-Orn(CO_2CH_2Ph)-Leu-D-Phe-Gly]$_2$, and *cyclo*-[Val-Orn-(Ac)-Leu-D-NHCH(CH_2R^1)CO-Pro]$_2$ (R^1 = cyclohexyl) all possessed β-sheet structures characteristic of gramicidin S, but *N*-acetylated analogues of all the gramicidin S and retro-gramicidin S did not. Thus substitution of Gly for Pro and the presence of Orn or Phe side-chains do not affect the conformation of the molecule.[521]

Proteins.—*Aromatic and Disulphide Chromophores.* Reduced RNAase A when reoxidized has been found to give a fully active enzyme, which, however, shows a c.d. spectrum different from that of the native or random-coil forms. S—S-reduced RNAase A and the fully cysteine-S-carboxamido-methylated RNAase A have been used as stable models for the c.d. analysis of the native molecule. Curve fitting has been used to determine the α-helical content.[522]

The u.v. c.d. of di-isopropylphosphoryl-subtilisins Carlsberg and Novo has been studied as a function of pH. The c.d. signals below 260 nm are pH dependent in the range 4—12. At pH values outside this range the protein is transformed into a random coil. Above pH 8.0 contributions due to the ionization of Tyr appear in the c.d. spectra above 260 nm, as bands shift to higher wavelengths. Three independently titrating components are obtained by matrix rank analysis; this accounts well for the observed c.d. above 260 nm for subtilisin Carlsberg in the range pH 8—12. By contrast two components are found for the Novo form. The identity of matrix rank components was surmised from the apparent p*K*s. In both cases exposed Tyr residues are evident, which are optically active only in the ionized form. In subtilisin Carlsberg two types of exposed tyrosine are apparent, which are accounted for by local pH-induced conformational changes.[523]

Two papers have appeared dealing with liver fructose diphosphatase (I). Marked differences were observed in the c.d. of (I) from rat and hamster, whereas rabbit (I) resembled that of hamster. Rat (I) showed a much lower ellipticity due to Tyr in a multiplicity of conformations with opposite signs. Hamster and rabbit (I) appeared rigid, with interior Tyr playing an important role in the stabilization of the protein. The higher α-content of the hamster protein leads to added stability, which may be important for hibernating animals.[524] The c.d. of (I) shows significant and specific changes in Tyr activity on binding fructose-1,6-diphosphate, AMP, EDTA, and activators. These effects are identical in the rat and hamster proteins. It was observed that the binding of AMP leads to a large increase in the rigidity of certain Phe residues, whilst the binding of substrate causes alterations in the asymmetric environment of certain Tyr residues. Activation by EDTA is associated with an overall decrease in the rigidity of (I).[525] The greater resistance of hamster (I) to conformational changes induced by these substances may well allow the animal to carry on a sufficient level of gluconeogenesis in conditions of starvation and cold exposure.

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'Non-chromophoric' Proteins. The c.d. spectra of homogeneous ATPase from bovine cardiac mitochondria indicate that ~80% of the amino-acids of the protein are in the α-helical form with only minimal β-structure and ~20% random coil.[526]

Purified wheat germ agglutinin (WGA) shows four homogeneous isolectins with similar c.d. spectra, with negative bands at 265—310 nm, positive bands at

Table 1 *Major protein c.d. studies in 1977*

Protein	*Nature of study*	*Ref.*
Fructose diphosphatase	Structure, stability, and substrate binding	524, 525
Wheat germ agglutunin	Structure and saccharide binding	527
Troponin	Subunit interactions, Ca^2 binding	530
Glucagon	Conformational transitions	533
Pancreatic Phospholipase	Structure and denaturation	534
Thyroglobulin and ovalbumin	Carbohydrate–protein interactions	538
Turnip peroxidase	Comparative	539
D-Amino-acid oxidase	Active site/substrate investigations	541
Glycogen Phosphorylase	Coenzyme binding	543
Haemoglobin	Allosteric transitions and carbamylation	544, 545 546
Cytochrome P450	Haem site, substrate binding	549
E. coli lac repressor	Saccharide binding	551
Carboxypeptidase A	Mapping topology	553
Serum albumin	Effects of detergent and ligand binding	554, 555
Histone H_4	Structure	557
Bradykinin	Intramolecular hydrogen-bonding	564, 565
Ovine lutropin	Subunit interactions	567
Human myeloma Jo (IgG1, κ)	Reassembly of immunoglobulins	569
MOPC-315 Immunoglobulin	Combination site with hapten	570
Complement factor, C1q	Structure	571
Bacteriorhodopsin	Structure and aggregation	574, 575

245—265 and 215—245 nm, and a further negative peak at 206 nm. The far-u.v. c.d. of isolectin I indicates the presence of 12% β- and no α-helical structure. The presence of 2-acetamido-2-deoxy-D-mannose or *N*-acetylchitodextrins induces conformational changes evidenced by changes in ellipticity at 250, 272, 285, and 290 nm. These changes are explained by interactions of the saccharides with binding sites composed of several subsites. The c.d. changes at 270—290 nm indicate the involvement of Tyr, probably Trp, and S—S bonds. However, the polypeptide backbone is not affected by any of the saccharides.[527]

The far-u.v. c.d. of bacterial spinae has been used to establish its secondary structure by three-component curve-fitting supplemented by rank and factor analysis of c.d. data matrices. Native spinae contain ~88% antiparallel β-sheet, 7% α-helix, and 5% random coil, based on poly-(L-Lys) as a model as it was found that basic c.d. data from globular proteins gave unreliable estimates.[528]

C.d. measurements on human prealbumin yield values of 4 ± 10%, 43 ± 20%,

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and variable amounts for the α-, β-, and coil contents respectively, which may be compared with the values of 30, 21, and 28% obtained by the predictive Chou and Fasman calculations and 6, 45, and 15% from *X*-ray data.[529]

The u.v. c.d. studies carried out on hybrids of bovine cardiac and rabbit skeletal troponin subunits are in good agreement with biological activity studies, which indicate the interactions occurring between troponin subunits of these tissues. They further imply that the regulatory proteins in each system are structurally similar. Differences do appear, however, in the c.d. signals on Ca^{2+} binding. The Ca^{2+}-induced conformational changes in tertiary complexes containing cardiac Ca-binding subunits of troponin and the smaller degree of inhibition by cardiac inhibitory subunits in the parent actomyosin system are seen as expressions of the unique differences between the two muscle regulatory systems.[530]

C.d. spectra and thermal denaturation curves indicate that low concentrations of bivalent cations or high concentrations of univalent cations cause an increase in the ordered structure of *E. coli* acyl carrier protein. However, only bivalent cations stabilize the protein towards thermal denaturation.[531]

Two electrophoretic variants of rat α-fetoprotein show identical, c.d. spectra typical of α-helical peptides. The stability of the variants towards denaturation is consistent with their high degree of α-helical content.[532]

Glucagon has been proved, by the use of c.d., to exist in two predicted conformational states in solution. The composition of the mixture depends on solvent and concentration, as the two forms represent predominantly α-helical and β-structural forms respectively. On prolonged standing the protein adopts a β-form regardless of the concentration or pH. The relevance of the conformation of the protein to receptor interactions has been discussed.[533]

Porcine, bovine, and equine pancreatic phospholipases A_2 all show moderate amounts of α-structure. However, on the basis of *X*-ray data and primary structures it has been concluded that the c.d. bands at 190—191, 208—210, and 222—225 nm arise not from α-structures but from asymmetric structures, probably rings containing S—S groups. The main chain conformations were found to be very resistant to acid but sensitive to SDS in acid and to a lesser extent base. The observed c.d. suggests that this stability is due to S—S and hydrophobic bonds. Near-u.v. c.d. spectra show differences between enzymes and zymogens with respect to tertiary structure.[534]

C.d. studies have revealed that the glycoprotein α_1 antitrypsin from human blood plasma contains 16—20% α-structure with the remainder being mainly β and aperiodic. This structure is stable at pH 4.7—8.8 but reversibly changes at pH 10.3 and denatures above pH 11.6. The side-chain environments are drastically altered at pH 2.5, whereas the main chain shows only slight changes. Guani-

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[534] B. Jirgensons, *Biochim. Biophys. Acta*, 1977, **494**, 285.

dine hydrochloride at 3.5 mol l^{-1}, SDS, and acid disorganize the protein and allow observation of phenylalanine fine structure.[535]

The principal intrinsic sialoglycoprotein of human erythrocyte membranes, glycophorin, interacts with various phospholipids. Di- and tri-phosphoinositides and lysophospholipids lead to an increase in α-helical content of the native and sialic acid-free glycophorin.[536]

Human urinary Tamm–Horsfall sialoglycoprotein has been characterized by use of c.d. The native protein as isolated consists of intertwining helical superstructures of mol. wt. $> 10^7$. If, however, the protein is denatured and then renatured no superstructure is produced, even though the c.d. spectra of the renatured and native proteins are very similar, suggesting an α-helical content of 10% and β-content of 33%.[537]

Pronase-digested glycopeptide fragments have been prepared from human α_1 acid glycoprotein, hen ovalbumin, and bovine thyroglobulin, two types of fragment being obtained from the latter. The fragments were characterized in terms of hexose, hexosamine, sialic acid, and peptide content. The c.d. spectra of the two complex fragments of thyroglobulin and ovalbumin were similar with negative bands at 207.5–211 nm with ellipticities of 6400—7200 deg cm^2 $dmol^{-1}$. Removal of sialic acid from the complex fragments greatly enhanced the observed ellipticity. The contributions from sialic acid were found to be nearly additive, thus implying that the covalent attachment of the terminal residues to the oligosaccharides does not lead to strong interactions with other chromophores, nor to positioning in an asymmetric environment.[538]

'*Chromophoric*' *Proteins*. Two turnip peroxidase isoenzymes, P_1 and P_7, together with their derivatives, have been investigated between 200 and 650 nm by the use of c.d. Although the Soret and visible bands occur at very similar wavelengths in the two isoenzymes, the associated ellipticities are quite different. The results suggest that the active sites are similar and these data have been compared with those for Japanese radish peroxidase and horseradish peroxidase. It appears that a common feature of plant peroxidase is a negative Soret c.d. signal for the reduced form, which inverts on addition of cyanide. C.d. in the far u.v. indicates an appreciable quantity of α-structure in the native peroxidase with an amount of unordered structure in excess of 50%, in agreement with results for other glycoproteins.[539]

In the presence of polygalacturonic acid, horseradish peroxidase loses its mainly α-structure and adopts a conformation similar to the β-chain form. This is apparently responsible for the change in the enzymic activity in the presence of pectin C.[540]

The visible-region c.d. of D-amino-acid oxidase in complex with quasi-substrates, ring-substituted benzoates, has been compared with those with the substrate benzoate. The gross modification of the c.d. signals is apparently due to the

[535] B. Jirgensons, *Biochim. Biophys. Acta*, 1977, **493**, 352.
[536] J. A. Verpoorte, and F. B. St. C. Palmer, *F.E.B.S. Letters*, 1977, **84**, 159.
[537] D. Puett, L. A. Holladay, and J. P. Robinson, *Mol. Cell. Biochem.*, 1977, **15**, 109.
[538] D. Puett, L. A. Holladay, J. D. Ford, and L. W. Cunningham, *Biochim. Biophys. Acta*, 1977, **491**, 129.
[539] D. Job and H. B. Dunford, *Cand. J. Biochem.*, 1977, **55**, 804.
[540] I. L. Gatfield and R. Stute, *Lebensm.-Wiss. Technol.*, 1976, **9**, 363.

substituent groups and is determined mainly by the type of substituent and to a lesser extent by the position of substitution. These findings suggest that each group interacts with the oxidase at the active site in a specific mode. The drastic changes in spectrum correspond to the lowest transition in the absorption spectrum.[541]

Comparative c.d. of native and cross-linked glucose oxidase, as well as of the apoenzyme, has shown that the FAD coenzyme is not a gross structural determinant.[542]

The optical activity of the pyridoxal-5′-phosphate site in glycogen phosphorylase has been monitored by c.d. as a probe of the binding mechanism. These data, together with those from circular polarization luminescence, have led to the proposal that the phosphate is covalently bound to the enzyme by an ε-NH_2 of a lysine residue, through a hydrogen-bonded Schiff base.[543]

A number of papers have appeared on the c.d. of haemoglobin (Hb) and its derivatives. The c.d. spectra at pH 6.5 of a number of haemoglobins and their derivatives have been recorded in the 280 nm region and interpreted in terms of an R $\rightleftharpoons$ T transition. Inositol hexaphosphate (IHP) converts aquo-met-A(S) into the T form, but the carbamylated derivatives are unaffected by such treatment. Fuoronitrobenzene and dimethyl adipimidate modification locks haemoglobins in an intermediate form and IHP has little or no effect.[544] The c.d. spectra of Mn^{II}- and Mn^{III}-Hb in the u.v. and Soret region, and particularly at 280 nm, indicate the presence of an R $\rightleftharpoons$ T allosteric transition. However, Mn^{III}-Hb in the u.v. region is distinctively different from aquo-met-Hb. Addition of IHP to Mn^{III}-Hb gives a u.v. c.d. characteristic of the T form. In contrast to the azide derivative of Fe^{III}-Hb, the azide derivative of Mn^{III} is converted into the T form by IHP.[545]

Fresh carbamyl phosphate when added anaerobically to haemoglobin has been shown by c.d. to stabilize the pure deoxyform, without detectable NH_2 carbamylation of terminal groups. Addition of preincubated carbamyl phosphate, however, results in carbamylation and formation of a different deoxy-form with enhanced oxygen affinity. Reversal of SS erythrocyte sickling by carbamyl phosphate is attributed to carbamylation by the hydrolysis product, cyanate.[546]

The c.d. spectra of oxy-, deoxy-, and apo-haemocyanin from the crayfish *Jasus Edwardsii* at pH 7.0, 8.6, 10.0, and 11.0 show slight differences in the magnitude of the ellipticities of bands, dependent upon whether Mg^{2+} is absent or present.[547]

Haemocyanins from *Octopus vulgaris*, *Carcinus maenas*, and *Limulus polyphemus* show similar c.d. spectra in the visible region. *d–d* Transitions have been assigned and a comparison of the native oxy- and deoxy-forms, together with the

[541] K. Shiga, K. Horicke, Y. Nishima, A. Isomoto, and T. Yamano, *J. Biochem.* (*Japan*), 1977, **81**, 1465.
[542] B. Solomon, N. Lotan, and E. Katchalski-Katzir, *Biopolymers*, 1977, **16**, 1837.
[543] S. Veinberg, I. Z. Steinberg, and S. Shaltiel, *Metabolic Interconversions Enzymes, Int. Symp.*, 1975, 44.
[544] C. F. Plese and E. L. Amma, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 691.
[545] C. F. Plese and E. L. Amma, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 837.
[546] C. E. Chapman and E. R. Simons, *Biochim. Biophys. Acta*, 1977, **494**, 408.
[547] H. D. Ellerton, L. B. Collins, J. S. Gale, and A. Y. P. Yung, *Biophys. Chem.*, 1976, **6**, 47.

Cu-free form, in 3M-urea with those in buffered solution suggest that tyrosine is involved in the reversible oxygenation process.[548]

Cytochrome P450 from rabbit liver microsomes shows a negative c.d. band in the Soret region, the wavelength and ellipticity of which depend on the axial ligand present and the oxidation state of Fe. The lack of any observed conformational change on substrate binding is rationalized as arising from the relatively weak non-polar interactions between the oxidized enzyme and the substrate. The conformational change on substrate binding to the reduced enzyme is due to the formation of a five-co-ordinate high-spin form. The negative Soret c.d. for P 450 is similar to those observed for *Chironomus* Hb, lamprey Hb, leg Hb, and catalase, whereas human Hb and myoglobin, together with cytochrome *c*, have a positive Soret c.d. These results are correlated with an open haem crevice for the former and a closed crevice for the latter. This is corroborated by calculations of rotational and dipole–dipole strengths with ensuing anisotropy.[549]

The potentiometric titration of spinach ferredoxin has been followed by the use of c.d. This method is generally useful as it is not affected by absorption of the redox mediators. The anaerobic apparatus and experimental conditions are described.[550]

Added Extrinsic Chromophores. The binding of *o*-nitrophenyl-β-D-fucoside and *o*-nitrophenyl-β-D-galactoside to *E. coli* lac repressor has been followed by c.d. in the range corresponding to the *o*-nitrophenyl chromophore, 300—400 nm. The c.d. of both ligands are drastically altered when bound to lac repressor protein, owing to asymmetrical interactions of the *o*-nitrophenyl ring with various chemical groups within the protein. The c.d. spectra indicate a close similarity in binding site for both ligands. These data have allowed evaluation of affinity constants and a number of binding sites. It has also been shown that limited proteolysis of the lac repressor gives a core protein in which the ligand environments are not charged.[551]

The NH_2 groups of peptides react with fluorescamine to form pyrrolinone-type chromophores with wavelength maxima at 380 nm. This has allowed the establishment of a test-tube procedure for the *in situ* determination of the absolute configuration of *N*-terminal amino-acids based on the chiroptic properties of chromophoric derivatives.[552]

Diazotized arsanilic acid reacts with Tyr-248 of carboxypeptidase A, leading to the production of two different c.d. probes, azo-Tyr-248 and the azo-Tyr-248 Zn chelate, both of which are environmentally sensitive. The dual probes have been used to map topological changes on substrate binding. The number and nature of the binding subsites of Zn-azo-Tyr-248 were elucidated by a method employing reversible binding of an effector pair, in order to reveal mutual competition of synergism. In addition the c.d. spectra of Zn^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+} complexes of carboxypeptidase A have been studied. The resulting maps of

[548] A. M. Tamburro, B. Salvato, and P. Zatta, *Comp. Biochem. Physiol.*, 1976, **55**, 347.
[549] K. Ruckpal, H. Rein, G. R. Jaenig, W. Winkler, and O. Ristau, *Croat. Chem. Acta*, 1977, **49**, 339.
[550] B. Ke, *Bioelectrochem. Bioenerg.*, 1975, **2**, 93.
[551] J. Maurizot and M. Charlier, *European J. Biochem.*, 1977, **79**, 395.
[552] V. Toome, B. Wegrzynski, and J. Dell, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 825.

subsites have allowed the analysis of several apparent kinetic anomalies, and are interpreted in terms of an earlier model based on the kinetics of dipeptide hydrolysis.[553]

The binding constants of the first two sites of serum albumin for salicylate have been evaluated at 1.05×10^3 and 5.1×10^3 l mol^{-1}. The fact that acetate diminishes the induced ellipticity of salicylate has led to the suggestions that hydrophobic and electrostatic forces may be involved in the binding process. Aspirin, indomethacin, and phenylbutazone may share the same primary site with salicylate.[554]

Aniline naphthalene sulphonate (ANS) has been used to study the effects of sodium dodecyl sulphate (SDS) and stearic acid on the conformations of bovine serum albumin (BSA) and human serum albumin (HSA). Both reagents displace ANS and also affect the extrinsic c.d. of ANS, in both proteins. Each of the four extrinsic bands of bound ANS are influenced differently. Using c.d. and fluorescence data it was possible to differentiate the effects of one ligand on both proteins.[555]

Protein–Nucleic Acid Complexes. A review of c.d. studies on histone–DNA interactions has been published.[556] C.d. spectra have been used to obtain conformational data on histones both in the presence and absence of anionic perturbants.[557] Four *N*-terminal peptides of histone H_4 have been shown to possess the poly-Pro II extended left-handed helix in acid and neutral solution at moderate ionic strength and also in 80% ethanol, 0.2M-SDS, or 8M-urea. However, the conformation was altered in 5M-$CaCl_2$ or when the temperature was raised in alkaline solution.[558] The c.d. and thermal denaturation of mono-penta-nucleosomes have been related to the stability of chromatin. The c.d. at 282 nm was the same for all the nucleosomes studied.[559]

A model complex of poly-(L-Cys-L-His) and DNA shows c.d. properties, which are dependent on solution pH, which determine the state of imidazole protonation. The binding of the polypeptide to DNA produces a red shift and allows the deduction of a positive c.d. signal at 275 nm arising from DNA.[560]

The c.d. spectrum of repressor–non-operator DNA complex has been found not to be equal to the sum of the individual contributions, the major difference appearing as an increase in activity at 275 nm. Titration studies have shown that the binding site for the repressor tetramer is 12 base pairs of DNA.[561]

Hormones.—The c.d. of *S*-carboxymethylated insulin A chains in H_2O–trifluoroethanol mixtures showed that a marked conformational transition occurs as the concentration is raised to 83%, leading to a polypeptide containing 43% α-helix. Several proposed methods of analysis, including linear and two non-linear least-

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[554] M. Otagiri and J. H. Perrin, *Biochem. Pharmacol.*, 1977, **26**, 283.
[555] W. E. Mueller, *Z. physiol. Chem.*, 1976, **357**, 1487.
[556] G. D. Fasman, *Chromatin Chromosome Structure*, 1975, 71.
[557] S. A. Berman, *Diss. Abs. Internat.*, 1977, **37**, 5632.
[558] E. I. Ramm, N. I. Koryakina, A. I. Pisachenko, V. M. Lobachev, N. G. Esipova, V. K. Burichenko, V. I. Vorobev, and Y. A. Lazarev, *Biofizika*, 1977, **22**, 32.
[559] R. Mandel and G. D. Fasman, *Organ. Expression Eukaryotic Genome*, 1977, 193.
[560] R. M. Santella and H. Jei Li, *Biopolymers*, 1977, **16**, 1879.
[561] A. P. Butler, A. Revzin, and P. H. von Hippel, *Biochemistry*, 1977, **16**, 4757.

squares procedures, were unable to quantify the β-content. Current models for far-u.v. c.d. were not adequate for analysis of the data obtained.[562]

Camel β-endorphin and ovine β-lipotropin show little if any secondary structure. In methanol or SDS the protein attains an α-structure. The conformation of the endorphin–SDS complex, it has been proposed, may be related to the opiate-like function of the peptide.[563]

Bradykinin has an ordered conformation in polar solution, which is stabilized by intramolecular hydrogen-bonding, which involves the participation of the NH group of Phe-8.[564] The proposed hydrogen-bonding has been probed by using c.d. to compare β-homo-Pro[7]-bradykinin (β-HPro B) and β-homo-Phe[8]-bradykinin (β-HPhe B) with native bradykinin. The extra CH_2 group in the analogues would be expected to facilitate the proposed bonding across Pro-3, Pro-7, and Phe-8, but in the case of β-HPro B the 234 nm trough is eliminated and it is reduced in size for β-HPhe B. These data are taken to strengthen the alternative proposal of a cyclic conformation stabilized by ionic interactions between Arg-11 and the CO_2^- terminal. Alternatively the data could arise from *cis–trans* isomerization around proline peptide bonds.[565]

Spontaneous activation of prothrombin upon reaction with citraconic anhydride leads to fragmentation, accompanied by a decrease in β- with respect to native structure, which contains 14% α, 36% β, and 50% random coil.[566]

At pH >7.0 ovine lutropin and its subunits give new c.d. bands at 250 nm without detectable changes in secondary structure, which has been correlated with the ionization of 2 to 3 exposed tyrosine residues in the hormone. The Tyr residues responsible are 21, 92, and 93 in the α-subunit. Nitration of the Tyr lowers their p*K*s. Evidence for the involvement of these residues was obtained by comparison of the alkaline-induced changes of refolded lutropin (α + β) and those of des-(92—96)-lutropin α (obtained by carboxypeptidase treatment of α-subunits) and lutropin β. Removal of Tyr-α-92 and -α-93 decreased the activity at 235 nm as well as at 250 nm at alkaline pH.[567]

Immunoproteins.—At wavelengths <250 nm the c.d. of Facb, $F(ab')_2$, Fc, pFc′, and tFc′ fragments of rabbit IgG are characterized by a negative band at 217 nm indicating the presence of β-structure. The 217 nm band of $F(a'b)_2$ is larger than that of the other fragments. A negative band present in IgG and Fc at 225 nm, but not in the other species, was destroyed on treatment with acid or plasmin digestion, reflecting the interactions of domains Cγ2 and Cγ3. Close similarities exist between human and rabbit Fc fragments, whilst the differences between the spectra of pFc′ fragments may be due to the human fragments undergoing structural changes on cleavage from IgG.[568] The mechanism of reassembly of immuno-

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[563] J. T. Yang, T. A. Bewley, G. C. Chen, and C. H. Li, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3235.
[564] M. P. Filatova, T. O. Reutova, V. S. Pashkov, S. L. Portanova, E. S. Efremov, and V. T. Ivanov, *Tezisy Doklady Vses. Simp. Khim. Pept. Belkov*, 1974, 153.
[565] D. Marlborough and J. W. Ryan, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 757.
[566] I. P. Baskova, M. S. Memon, V. V. Romanov, and A. F. Rusak, *Biokhimiya*, 1977, **42**, 95.
[567] M. Ascoli, D. N. Ward, and B. Jirgensons, *European J. Biochem.*, 1977, **72**, 157
[568] G. A. Stewart, P. M. Johnson, M. V. Barrett, P. M. Scopes, and D. R. Stanworth, *Immunochemistry*, 1977, **14**, 263.

globulins has been probed by following the non-covalent interactions between light chain (L) and heavy chain (H) or Fd isolated from human myeloma Jo-(IgG1, κ) by the use of c.d. At 293 nm c.d. shows that Jo-L exists as monomeric units under conditions used for H chains. The second-order rate constants derived for the interactions of H with L are in good agreement with those for Fd and L at various pHs. Fd and L show a single binding constant. Renaturation studies on L, Fd, Fab (SS), and Fab (RA) from 0.5- or 1-M-acetic acid on neutralization show that Fd renatures rapidly. L shows one fast and one slow first-order phase, and both Fab (SS) and Fab (RA) show one fast and one slow second-order phase.[569]

The extrinsic Cotton effect of the binding of haptens to native and reformed MOPC-315 protein, subunits, and Fv fragments has been used to allow the identification of the binding site. Trinitrophenyl aminocaproate complexed with L chains shows a different spectrum from that of the MOPC-315 complex, lacking the 495 nm peak. The negative 293 nm peak of trinitrophenylated MOPC-315 and Fv-315, which is absent from the spectra of L and H chains, may represent electronic interactions within the MOPC-315 combining site between Trp and chromophoric residues on different subunits.[570]

Human subcomponent C1q and the pepsin-resistant core show positive c.d. at 230 and 223 nm respectively, compared with a positive c.d. at 220 nm obtained from lathyritic rat skin collagen. The magnitude of the bands and the effect of collagenase treatment suggest that there may be some triple-helix structure in the pepsin-resistant core. After heat denaturation and cooling the pepsin-resistant core regains 60% of its collagen-like structure, but this is not the case for whole C1q or the reduced pepsin-resistant core.[571]

IgD myeloma protein has a unique digestion pattern amongst Ig molecules and is susceptible to conformational changes within the Fab_δ and Fc_δ regions. A definite quaternary structure is present, which is dependent upon interactions between the $C_\delta 1$ and C_λ domains. These features are consistent with the proposed function of the proteins as membrane-bound receptors, which on interaction with antigens provide the stimulus to small lymphocytes, determining further differentiation.[572]

Membrane Proteins.—The problem of scattered light distortion of c.d. signals from solubilized purple membranes of *Halobacterium halobium* has been treated in different ways. The asymmetry of the c.d. signal of purple membranes is pronounced in water solution but is not apparent in 60% (v/v) glycerol–H_2O solution and so is correlated with the refractive index of the solvent. The true spectrum has been calculated from the input data using a coated-sphere model of Rosenbeck and Schneider.[573] Another approach has been the application of a pseudo-reference state to the system, which from the signal at 224 nm with an ellipticity of $\sim 10\,000$ yields a value of α-helical content of $\sim 75\%$, which is in good agreement with diffraction data. In 80% trifluoroethanol the suspended mem-

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[571] B. Brodsky-Doyle, K. R. Leonard, and B. M. Kenworth, *Biochem. J.* 1976, **159**, 279.
[572] R. Jefferies, J. B. Matthews, and P. Bayley, *Biochem. Soc. Trans.*, 1977, **5**, 279.
[573] M. Brith-Lindner and K. Rosenheck, *F.E.B.S. Letters*, 1977, **76**, 41.

branes are solubilized but still give similar secondary structure, so validating the pseudo-reference approach.[574] The observed exciton coupling in the c.d. of reconstructed nicotine membranes indicates that the protein is not monomeric. However, the rapid rotational diffusion shows that large aggregates are not present. It is suggested that in brown membranes the protein exists as a trimer.[575]

6 Magnetic Circular Dichroism

Contributed by T. Brittain

During the past year the area of magnetic circular dichroism (m.c.d.) has continued to grow. A number of theoretical papers have appeared but once again the main area of growth has been in the study of haem proteins.

Theory and Analysis.—A general review of the m.c.d. of proteins has appeared,[576] together with a review of recent studies on myoglobin, its derivatives, and related haem proteins, which includes a description of the fundamental concepts of m.c.d.[577]

The m.c.d. intensities of local-symmetry-forbidden transitions generated by structural and vibrational perturbations, and their interactions, have been investigated. Such perturbations are additive and the lowest-order effect was found to be first order in static and second order in vibrational terms.[578]

Eigenfunctions have been obtained for all octahedral molecular states, in magnetic fields of arbitrary orientation, and have been used to calculate the relative transition probabilities of allowed Zeeman lines. Anisotropic Zeeman patterns which arise in absorption, emission m.c.d., and magnetic circular polarized emission are discussed for transitions between isotropic states. Mixing of octahedral states by the magnetic field is also discussed. The matrix required for the calculation of transition probabilities in anisotropic and/or magnetically mixed states in an arbitrary oriented magnetic field is given.[579]

Proteins and Models.—The m.c.d. of 1 : 1 and 2 : 1 complexes of Pd^{II} and L-cystine, in acid, allows the detection of ligand–metal charge-transfer bands at 230 and 290 nm and that of the metal-ion $^1A_{1g}$–$^1E_{1g}$ transition. The experimental assignment of n_a–σ^*_S—S and n_b–σ^*_S—S of glutathione in acid medium and in excess Pd^{II} agrees with theoretical calculations.[579]

The intensity of m.c.d. signals associated with the near-u.v. Soret region of ferric haem proteins is paramagnetic in origin, and the amount of low-spin ($S = \frac{1}{2}$) iron present is proposed to be proportional to the signal strength. For the characterization of a low-spin–high-spin ($S = \frac{5}{2}$) mixture m.c.d. offers advantages over other methods; the samples need not be pure as other components show only relatively weak magneto-optic effects, high sensitivity allows the use of micromolar concentrations, measurements may be made at physiological

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[576] Y. A. Sharonov, *Itogi Nauki Tekh. Mol. Biol.*, 1976, **8**, 70.
[577] M. Hatano and T. Nozawa, *Seibutsu Butsuri*, 1976, **16**, 159.
[578] L. Seamans, A. Moscowitz, R. E. Linder, K. Morill, J. S. Dixon, G. Barth, E. Bunnenberg, and C. Djerassi, *J. Amer. Chem. Soc.*, 1977, **99**, 724.
[579] W. C. Yenkel, *Mol. Phys.*, 1977, **33**, 1429.

or cryo-temperatures, and thermodynamic parameters may be evaluated, together with the possibility of rapid kinetic measurement. Results are described to illustrate the use of m.c.d. as a probe of spin state for myoglobin, haemoglobin, cytochrome *c* oxidase, haemopexin, and cytochromes *c*, *f*, b_5, and P450.[580]

Table 2 *Major protein m.c.d. studies in 1977*

Protein	*Nature of study*	*Ref.*
Horseradish peroxidase	Spin states and ligation	581
Carboxypeptidase	Active site topology	582
Myoglobin	Spin states	583, 584
Rubredoxins and ferredoxins	Charge transfer assignment	586, 587
Cytochrome *c* oxidase	Spin states, antiferromagnetic coupling, and effect of denaturants	588, 589
Pseudomonas aeruginosa cytochrome oxidase	Haem–haem interactions	590

The haem vicinities of the acid and alkaline forms of native Fe^{III} horseradish peroxidase have been probed by the use of m.c.d. The acid form is characterized by a spectrum of high-spin Fe^{III}. The similarity of this spectrum with that of aceto-Fe^{III}-protoporphyrin IX dimethyl ester suggests the presence of a five-co-ordinate haem. The m.c.d. of the peroxidase did not show any significant pH dependence in the range 5.2—9.0, but did indicate a p*K* for the alkaline to acid transition at pH 11.0. At pH 12.01 the alkaline form is low spin and near-i.r. m.c.d. shows that the sixth ligand is somewhat different from the normal N ligands of His or Lys. It is implied that the alkaline form has an overall ligand-field strength between that of the low-spin components of met-Mb-OH and met-Mb-N_3.[581]

Co^{2+} carboxypeptidase is characterized by a pronounced negative m.c.d. signal at 577 nm, a region devoid of optical activity in the absence of a magnetic field. The intensity of the signal is proportional to the magnetic field strength, from 0 to 47 kG. The addition of substrates such as glycyl-L-Tyr, benzyloxyl-carbonyl-Gly, β-Phe-propionate, and L-Phe-Ala alters neither the shape nor the sign of the m.c.d. signal. The spectrum of the enzyme therefore does not give evidence of any change in co-ordination on substrate or inhibitor binding. However, all the reagents affect the fine structure in a manner quite characteristic of each substrate, suggesting that the topology of the enzyme active site is complex.[582]

A paper has appeared concerning the m.c.d. signals of myoglobin (Mb) and its derivatives, suggesting a correlation between the Soret signal strength and the spin state of the haem iron. It is proposed that met-MbO_2^{2-} is low spin, as is met-MbCN.[583]

A near-i.r. (600—2000 nm) study of the m.c.d. of met-Mb derivatives has appeared in which the signals observed are very characteristic of the haem spin state. The m.c.d. spectra allow the distinction between the high-spin, low-spin,

[580] L. E. Vickery, *E.R.D.A. Energy Res. Abs.*, 1976, 1.
[581] N. Kobayashi, T. Nozawa, and M. Hatano, *Biochim. Biophys. Acta*, 1977, **493**, 340.
[582] A. A. Klesov and B. L. Vallee, *Bio-org. Khim.*, 1977, **3**, 958.
[583] M. Kajujashi and F. K. Anan, *J. Biochem. (Japan)*, 1977, **81**, 1327.

and intermediate-spin bands for met-MbOH and the origin of the m.c.d. is illustrated by model calculations.[584]

A group of papers has appeared concerned with the m.c.d. of iron–sulphur proteins. The rubredoxin model of Fe–S complexes shows bands at ~390 and 490 nm, which have been assigned to Faraday *A* terms by means of curve analysis, which indicated an effective T_d symmetry.[585] Variable-temperature m.c.d. of *Clostridium pasteurianum* rubredoxin shows evidence of two one-electron charge-transfer transitions $S \rightarrow Fe^{III}$ in the region 15 000—28 000 cm^{-1}. The first moment of the lower-energy band is consistent with an orbital transition from a t_1 non-bonding *S*-orbital to a two-electron Fe^{III} *d*-orbital. The magnitude of spin–orbit coupling in the lower excited state has been determined and is small relative to the axial distortion. The splitting of the lower-energy band observed in absorption spectra can therefore be equated with axial distortion of the lowest excited charge-transfer state.[586] A number of ferredoxins have also been studied by variable-temperature m.c.d. The spectra of fully oxidized spinach and *Spirulina maxima* ferredoxins are independent of temperature down to 18 K, showing no contributions from the small population of low-lying excited states originating from exchange coupling. Low-temperature spectra of the half-reduced ferredoxins and adrenodoxin are all reasonably intense and temperature dependent, suggesting the presence of charge-transfer transitions.[587]

A detailed study of the temperature-dependent m.c.d. of cytochrome *c* oxidase and its derivatives has allowed the assignment of haem spin states and the identification of antiferromagnetic coupling.[588] The effects of SDS on cytochrome *c* oxidase have been identified with a monomerization process leading to release of strain on the haem. In alkaline solution the disappearance of c.d. signals has been suggested as arising from Schiff-base formation.[589]

Pseudomonas aeruginosa cytochrome oxidase in the ferric form shows an m.c.d. spectrum similar in form to that of mammalian cytochrome *c*. However, in the ferrous state the intensity of the signals is much less than that expected unless the haem *d* is removed. These data have led to the suggestion of haem–haem interaction in the reduced enzyme.[590]

Near-i.r. m.c.d. spectra of *Rhodospirillium rubrum*, *Chromatin vinosum*, and *Rhodopseudomonas palustris* cytochrome *c* show that the reduced species are similar to deoxy-Mb. The spectra of the oxidized cytochromes in the pD range 1—13 have been analysed as arising from four species, A, B, C, and D. B is high spin and C and D are both low spin. The spectra of A are, however, close to those of high-spin species, so contradicting previous reports of a mixed-spin species.

[584] P. J. Stephens, J. C. Sutherland, J. C. Cheng, and W. A. Eaton, 'Excited States Biol. Mol. Proc. Int. Conf.', 1974, Wiley, New York, 1976, p. 434.
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[586] J. C. Rivoal, B. Briat, R. Cammack, D. O. Hall, K. K. Rao, I. N. Douglas, and A. J. Thomson, *Biochim. Biophys. Acta*, 1977, **493**, 122.
[587] A. J. Thomson, R. Cammack, D. O. Hall, K. K. Rao, B. Briat, and J. C. Rivoal, *Biochim. Biophys. Acta*, 1977, **493**, 132.
[588] A. J. Thomson, T. Brittain, C. Greenwood, and J. P. Springall, *Biochem. J.*, 1977, **165**, 327.
[589] Y. Orii, M. Manabe, and M. Yoneda, *J. Biochem.*, 1977, **81**, 505.
[590] Y. Orii, H. Shimada, T. Nozawa, and M. Hatano, *Biochem. Biophys. Res. Comm.*, 1977, **76**, 985.

The energies of the i.r. signals indicate that ligation of O_2 is opposite a His residue.[591]

7 Mössbauer Spectroscopy

Contributed by D. P. E. Dickson

The main sources of literature information for this Report are 'The Index of Publications in Mössbauer Spectroscopy of Biological Materials', by L. May, Department of Chemistry, The Catholic University of America, Washington, DC 20017, U.S.A., and 'The Mössbauer Effect Data Index', edited by J. G. Stevens, V. E. Stevens, and W. L. Gettys, Mössbauer Effect Data Center, Asheville, North Carolina, NC 28804, U.S.A.

During 1977 work has been reported on haem proteins, iron–sulphur proteins, and vitamins and on an amino-acid complex with tin. The previous work on the application of Mössbauer spectroscopy and magnetic resonance techniques to the study of the iron–sulphur proteins has been reviewed by Cammack *et al.*[592] This survey places particular emphasis on the way in which the data obtained using these probes have helped in building up an understanding of the different types of active centre found in this family of proteins. Oosterhuis and Spartalian [593] have reviewed the existing Mössbauer spectroscopic information on the iron-transport and -storage proteins. In the iron-transport proteins the iron atoms are isolated and the paramagnetic hyperfine structure observed in the Mössbauer spectra can give information on the environment of the iron atom. In the iron-storage proteins the iron is found in concentrated inorganic clusters. The size of these clusters can be determined from the temperature dependence of the Mössbauer spectra as a result of the superparamagnetic behaviour.

Although the majority of biological Mössbauer investigations are still concerned with the properties of isolated biomolecules it is now possible to use the existing data to observe the presence and changes of these molecules in a more complex situation, such as that within part or whole organisms. In a Mössbauer study of membrane fragments from the blue–green algae *Chlorogloea fritschii* and *Anacystis nidulans*, Evans *et al.*[594] observed two iron species. One gave spectra closely similar to those of an oxidized ferredoxin. This was interpreted as indicating the presence of an iron–sulphur centre in the photosynthetic system of the algae which is known to be within the membrane. The other spectral component had an intensity which was strongly dependent on the amount of iron in the growth medium, decreasing to zero for an iron-depleted growth. The authors concluded that this component arises from an iron transport or storage compound which was not previously known to be present in the membrane. This work shows how Mössbauer information on isolated biomolecules can be used in interpreting the spectrum of a complex system and identifying the various species present.

[591] J. Rawlings, P. J. Stephens, L. A. Nafie, and M. D. Kamen, *Biochemistry*, 1977, **16**, 1725.
[592] R. Cammack, D. P. E. Dickson, and C. E. Johnson, in 'Iron–Sulfur Proteins,' Vol. III, ed. W. Lovenberg, Academic Press, New York, 1977.
[593] W. T. Oosterhuis and K. Spartalian, in 'Applications of Mössbauer Spectroscopy,' Vol. I, ed. R. L. Cohen, Academic Press, New York, 1976.
[594] E. H. Evans, N. G. Carr, J. D. Rush, and C. E. Johnson, *Biochem. J.*, 1977, **166**, 547.

Haem Proteins.—The work on haem proteins reported in 1977 involves both experimental and theoretical investigations as well as studies of model compounds which emulate the more complex proteins but whose properties may be more readily understood. The aim of this research is to obtain data concerning the electronic structure and spatial arrangement of the iron atom in the haem group, often with a view to using this information to help in elucidating the biological function of these proteins. Although the majority of haem proteins contain iron in the ferrous or ferric form with either a high-spin or a low-spin configuration there has been considerable interest recently in haem proteins in which the iron is in an unusual spin or oxidation state.

Normal human haemoglobin is a tetramer made up of two α- and two β-subunits. Various physicochemical techniques have suggested that there may be differences between the haem environments in the two subunits and the whole protein. Tsai *et al.*[595] have obtained Mössbauer spectra from human oxyhaemoglobin (HbO_2) and its isolated subunits at temperatures between 4.2 and 200 K. The spectra are quadrupole-split doublets corresponding to iron in a low-spin ferrous state. The parameters for the α-subunit and for the whole protein are essentially identical. The β-subunit gives a quadrupole splitting which is 0.05 mm s^{-1} smaller over the whole temperature range and a spectral linewidth which is significantly greater below 40 K. The large temperature dependence of the quadrupole splitting of oxyhaemoglobin has been the subject of considerable discussion and these authors relate both this and the oxygen binding properties to an electron acceptor bond between oxygen and iron.

Carboxyhaemoglobin (HbCO) also contains iron in a low-spin ferrous state. Chow *et al.*[596] have obtained Mössbauer spectra from isolated subunits of human carboxyhaemoglobin between 85 and 185 K and have compared them with those of the complete protein. No temperature dependence of the chemical shift or quadrupole splitting was observed in the temperature range studied. The chemical shifts of the three substances were found to be the same. However, the quadrupole splittings show small but significant differences, being 0.39 ± 0.02 mm s^{-1} for the α-subunit, 0.33 ± 0.02 mm s^{-1} for the β-subunit, and 0.36 ± 0.02 mm s^{-1} for the complete protein. Thus the value for the carboxyhaemoglobin tetramer may be considered as the average of the splittings for the individual subunits.

The electronic structure of the high-spin ferrous ion in deoxymyoglobin (Mb) has been the subject of several Mössbauer investigations, including single-crystal measurements. One of the main aims has been to determine the orientation of the electric field gradient (EFG) principal axis system with respect to the haem plane and iron ligands. Until now all the models have contained the underlying assumption that the normal to the haem plane is an EFG principal axis. Kent *et al.*[597] have obtained spectra from horse deoxymyoglobin over a large range of temperatures and applied magnetic fields. Computer analysis of these spectra leads to a description of the quadrupole and magnetic hyperfine interactions which includes information on the orientations of the EFG and magnetic principal

[595] T. E. Tsai, J. L. Groves, and C. S. Wu, *Bull. Amer. Phys. Soc. Ser. II*, 1977, **22**, 625.
[596] Y. W. Chow, M. Fuchs, A. Mukerji, and Y. K. Yip, in 'Proceedings of the Nassau Mössbauer Conference 1977', Nassau Community College Press, Garden City, New York, 1977.
[597] T. Kent, K. Spartalian, G. Lang, and T. Yonetani, *Biochim. Biophys. Acta*, 1977, **490**, 331.

axis systems. Combining this information with the published single-crystal data leads to two possible solutions for the orientation of the EFG principal axes. In neither of these is an EFG principal axis near to the haem normal. In one solution an EFG principal plane is nearly coincident with the proximal imidazole plane, which makes this solution intuitively more attractive. These results lead to the suggestion that the force tending to pull the iron out of the haem plane must have a sideways component.

In methaemoglobin (HbH_2O) the iron is in a high-spin ferric state, although the spectra of slowly frozen samples usually show an additional low-spin component. Thomanek *et al.*[598] have shown that by fast freezing of methaemoglobin solutions at pH 7 the low-spin species can be largely eliminated. Mössbauer spectra of these samples were obtained at temperatures between 4.2 and 250 K and in small applied magnetic fields. The spectra show paramagnetic hyperfine structure and were computer-fitted using a spin Hamiltonian containing terms for the Coulomb repulsion of the five 3*d* electrons, a crystal field with C_{2v} symmetry, and spin–orbit coupling. The resulting crystal-field parameters were compared with those of other haem proteins and were interpreted in terms of differences in the spatial arrangement of the Fe^{3+} ion. The authors conclude that the iron atom lies closer to the haem plane in methaemoglobin than in metmyoglobin or deoxyhaemoglobin. This explains the presence of the low-spin species since in low-spin compounds the iron lies nearly within the haem plane, and it appears that slow freezing sufficiently alters the conformation in methaemoglobin to give a low-spin configuration.

Metmyoglobin (MbH_2O) and myoglobin fluoride (MbF) also contain iron in the high-spin ferric state. Thomanek *et al.*[599] have obtained Mössbauer spectra from these compounds at temperatures between 4.2 and 180 K and in small applied magnetic fields. The computer analysis of these spectra gives a value of 14.0 ± 1.5 cm^{-1} for the splitting of the two lowest Kramers levels of the Fe^{3+} ion, in good agreement with the value obtained by Lang.[600] The spectra obtained at temperatures above 10 K show paramagnetic relaxation effects.

Cytochrome *c'* is a haem protein that is thought to act as an electron carrier. Emptage *et al.*[601] have investigated the oxidized (ferric) form of the cytochrome *c'* from *Rhodospirillum rubrum* by Mössbauer spectroscopy and e.p.r. In samples prepared in the pH range 6—9.5 they observed three distinct spectroscopic species which belong to two pH-dependent equilibria with p*K* values near to 6 and 8.5. The $pK = 6$ transition is only resolved in Mössbauer spectra obtained in a large applied magnetic field. The Mössbauer parameters of this protein correlate with those of other high-spin ferric haem proteins. The spectra were computer-fitted using a spin Hamiltonian which included a weak mixing of an $S = \frac{3}{2}$ (intermediate-spin) excited state into the $S = \frac{5}{2}$ (high-spin) ground state of the Fe^{3+} ion.

[598] U. F. Thomanek, F. Parak, and B. Wintergerst, *Z. Naturforsch.*, 1977, **32c**, 11.
[599] U. F. Thomanek, F. Parak, S. Formanek, and G. M. Kalvius, *Biophys. Struct. Mechanism*, 1977, **3**, 207.
[600] G. Lang, *Quart. Rev. Biophys.*, 1970, **3**, 1.
[601] M. H. Emptage, R. Zimmermann, L. Que, jun., E. Münck, W. D. Hamilton, and W. H. Orme-Johnson, *Biochim. Biophys. Acta*, 1977, **495**, 12.

It has been previously suggested as a result of Mössbauer and other measurements that in hydrogen peroxide compounds of peroxidase (from Japanese radish) and metmyoglobin (from horse heart) iron exists in a quadrivalent form. Harami *et al.*[602] have obtained Mössbauer spectra from these compounds in the presence of applied magnetic fields. Computer analysis of these spectra enables the electronic configuration of the haem iron to be specified as Fe^{IV} $[(t_{2g})^4, S = 1]$ with a spin singlet $S_z = 0$ ground state. The axial and rhombic ligand-field parameters were determined to be 624 and $\leqslant 312$ cm^{-1} respectively. The high degree of covalency observed indicates that there is a large expansion of the radial charge distribution of the t_{2g} orbitals in these compounds.

An important feature of the reversible oxidation of haemoglobin is its co-operativity. This co-operativity has been associated with the tension along the histidine–iron linkage in deoxyhaemoglobin which results from the pull of the globin chain on the iron atom. Srivastava *et al.*[603] have examined the rigidity of the binding of the metal atom in a novel way. They have obtained emission Mössbauer spectra using oxy- and deoxy-^{57}Co-haemoglobin in which the iron had been replaced by radioactive ^{57}Co. The ^{57}Co decays by electron capture to the ^{57}Fe excited state which in turn emits a Mössbauer γ-ray in decaying to the ^{57}Fe ground state. In these experiments the sample under investigation is used as the source of γ-rays and a material such as stainless steel which has a monoenergetic absorption line is used as the absorber. This is the reverse of the procedure in the more usual absorption Mössbauer spectroscopy. The spectra obtained in this way are different from the absorption spectra of oxyhaemoglobin and deoxyhaemoglobin, and in particular the emission spectrum of deoxyhaemoglobin indicates that the iron atom is in an intermediate-spin state. These spectra seem to show that the daughter ^{57}Fe atom is 'frozen' in a configuration characteristic of the parent ^{57}Co atom. Thus the protein appears to hold the metal atom sufficiently rigidly that after the decay of the ^{57}Co atom it does not permit the ^{57}Fe atom to relax into its normal situation within the lifetime ($\sim 10^{-7}$ s) of the ^{57}Fe excited state.

Haptoglobin is a serum protein which binds haemoglobin. Alfsen *et al.*[604] have used Mössbauer spectroscopy to investigate haptoglobin–haemoglobin complexes with molar ratios of 1 : 1 and 2 : 1. The spectra obtained consist of single quadrupole-split doublets with the same parameters and temperature dependence, indicating that in both samples the iron environment is the same. A comparison with the known Mössbauer parameters of haemoglobin and myoglobin suggests that the electronic structure of the iron in these complexes is more closely similar to that in myoglobin than that in haemoglobin.

Despite considerable experimental and theoretical work there still remain questions to be answered concerning the interaction of molecular oxygen with the haem group in oxyhaemoglobin. The problem concerns the geometry of the dioxygen ligand and whether Fe^{II}–dioxygen or Fe^{III}–superoxide provides a better

[602] T. Harami, Y. Maeda, Y. Morita, A. Trautwein, and U. Gonser, *J. Chem. Phys.*, 1977, **67**, 1164.
[603] T. S. Srivastava, S. Tyagi, and A. Nath, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4996.
[604] A. Alfsen, D. Bade, U. van Bürck, H. Eicher, S. Formanek, G. M. Kalvius, F. Lavialle, A. Mayer, F. Parak, J. Tejada, and U. F. Thomanek, *Biophys. Struct. Mechanism*, 1977, **3**, 229.

description of the electronic configuration. In addition, the strong temperature dependence of the Mössbauer quadrupole splitting in oxyhaemoglobin has not yet been fully explained although it has been proposed by Lang [600] that it arises from rotation of the oxygen molecule about the iron–oxygen axis.

Kirchner and Loew [605] have made calculations of the Mössbauer quadrupole splittings in oxyhaemoglobin using the model of a nearly planar haem group with molecular oxygen and *N*-methylimidazole as the two axial ligands. This model is very similar to a recently synthesized oxyhaem analogue compound. Twenty-two conformations of the dioxygen ligand were considered and the EFG for each was calculated using an iterative extended Hückel theory. The resulting Mössbauer quadrupole splittings were compared with the experimental values for oxyhaemoglobin and oxyhaem model compounds and favour an Fe^{II}–dioxygen configuration with a bent end-on dioxygen ligand geometry.

Cianchi *et al.*[606] have calculated the temperature dependence of the Mössbauer quadrupole splitting in oxyhaemoglobin on the assumption that it arises from rotation of the oxygen molecule about the iron–oxygen axis. They consider two models for the orientation of the oxygen molecule, one with the oxygen–oxygen axis at 120° to the haem plane and the other with the oxygen–oxygen axis parallel to the haem plane. The first of these models leads to a good fit to the experimental values obtained from existing data and additional high-temperature measurements. The model with the oxygen–oxygen axis parallel to the haem plane gives no variation of the quadrupole splitting with temperature.

The variation of the Mössbauer chemical shift with temperature depends on the second-order Doppler shift and any temperature variation of the *s*-electron density at the nucleus. The chemical shift in oxyhaemoglobin shows very little temperature dependence which means that changes in the *s*-electron density at the nucleus must compensate for the effects of the second-order Doppler shift. Cianchi *et al.*[607] have calculated the variation of the *s*-electron density at the nucleus with temperature using the same models as in their quadrupole splitting calculations [606] and by considering the changes in the iron and oxygen orbitals which result from the rotation of the oxygen molecule. Combination of this with the calculated second-order Doppler shifts leads to a theoretical curve for the temperature dependence of the chemical shift. Using the model with the oxygen–oxygen axis at 120° to the haem plane, a good fit to the experimental values is obtained, but with the other model it is not possible to reproduce the observed temperature variation.

Tension in the binding of iron in deoxyhaemoglobin has been related to the oxidation properties of the protein. Loew [608] has made calculations of Mössbauer parameters for deoxyhaem units with both tense and relaxed structures and has compared these with the experimental values.

Loew *et al.*[609] have calculated Mössbauer parameters for various site models of horseradish peroxidase compound I (an intermediate formed by the action of

[605] R. F. Kirchner and G. H. Loew, *J. Amer. Chem. Soc.*, 1977, **99**, 4639.
[606] L. Cianchi, M. Mancini, G. Spina, and R. Cappelleti, *J. Theor. Biol.*, 1977, **67**, 757.
[607] L. Cianchi, M. Mancini, G. Spina, and R. Cappelletti, *J. Theor. Biol.*, 1977, **67**, 765.
[608] G. H. Loew, *Bull. Amer. Phys. Soc. Ser. II*, 1977, **22**, 336.
[609] G. H. Loew, C. J. Kert, L. M. Hjelmeland, and R. F. Kirchner, *J. Amer. Chem. Soc.*, 1977, **99**, 3534.

oxidants) and a cytochrome P450 analogue. The parameters are derived from electronic structures calculated using an iterative extended Hückel theory. Although magnetic susceptibility measurements indicate that these compounds contain iron in an intermediate-spin ferric state the measured Mössbauer parameters appear to be more consistent with an electronic configuration of [Fe^{IV} $S = 1$, porphyrin $S = \frac{1}{2}$] rather than [Fe^{III} $S = \frac{3}{2}$].

In order to investigate the intermediate-spin ferric state which is thought to exist in a number of haem proteins, Dolphin *et al.*[610] have obtained Mössbauer spectra from the model compounds octaethylporphyrinatoiron perchlorate and its bis(ethanol) solvate. The spectra obtained at temperatures between 4.2 and 295 K consist of quadrupole-split doublets with narrow lines. The quadrupole splittings are among the largest known in Fe^{III} compounds (3.57 mm s^{-1} in the case of the perchlorate at 4.2 K). The observation at all temperatures of a single spectrum with no line-broadening shows that there is no temperature-dependent spin equilibrium or spin cross-over and, taken with the magnetic susceptibility data, seems to give unequivocal evidence for an $S = \frac{3}{2}$ ground state. The authors conclude from the observed magnitude and positive sign of the quadrupole coupling constant, together with the asymmetry parameter of nearly zero, that the most likely ground-state electronic configuration is $(d_{xy})^2$ $(d_{xz}, d_{yz})^2(d_{z^2})^1$. From the observation of relaxation behaviour in the low-temperature applied magnetic field spectra it appears that spin–lattice relaxation is the dominant mechanism and that there are significant structural differences between the two complexes. No e.p.r. signals have yet been observed from these compounds.

The non-protein haemochromes provide simple models of known structure for several ferrous haem proteins. Connor and Straub[611] have made a Mössbauer study of haemochromes with phosphorus ligands. By obtaining Mössbauer data on a number of haemochromes having phosphine and phosphite ligands with varying π-bonding capabilities they have determined more precisely the effect of π-bonding axial ligands on the chemical shift and quadrupole splitting. The ligands were chosen to provide π-acceptor strengths ranging between those of the non-π-bonding amines such as piperidine and the strongly π-bonding carbon monoxide.

Iron–Sulphur Proteins.—In recent years a number of papers have reported the synthesis and physicochemical investigation of model compounds for the protein rubredoxin, which contains a single iron atom tetrahedrally co-ordinated to four cysteinyl sulphur atoms.

Lane *et al.*[612] give a complete discussion of the properties of the $[Fe(S_2\text{-}o\text{-}xyl)_2]^{-,2-}$ anions which provide good analogues for the oxidized and reduced forms of rubredoxin. The Mössbauer parameters derived from computer fits to spectra obtained at low temperatures and in applied magnetic fields demonstrate that the Fe—S_4 geometry, the ground-state electronic structure, and the Fe—S bond covalency are closely similar to those in the protein.

[610] D. H. Dolphin, J. R. Sams, and T. B. Tsin, *Inorg. Chem.*, 1977, **16**, 711.
[611] W. M. Connor and D. K. Straub, *Inorg. Chem.*, 1977, **16**, 491.
[612] R. W. Lane, J. A. Ibers, R. B. Frankel, G. C. Papaefthymiou, and R. H. Holm, *J. Amer. Chem. Soc.*, 1977, **99**, 84.

Eisenstein and Franceschetti [613] have made molecular orbital calculations to obtain the Mössbauer hyperfine parameters for the tetrakisthiophenolato-Fe^{II} anion, $[Fe(SPh)_4]^{2-}$, a reduced rubredoxin analogue. These show good agreement with the experimental values of Petrouleas *et al.*[614] The sensitivity of the calculated values to the geometry suggests that the comparison between calculated and measured parameters may provide useful information on the active site configuration. In particular, the sensitivity to the orientation of the sulphur–hydrogen bonds about the sulphur position suggests that the exact orientation of the cysteine ligands in the actual protein may play a decisive role in determining the reactivity of the active site.

A large group of iron–sulphur proteins have active centres containing four iron atoms and four labile sulphur atoms. The role of these proteins is in electron-transfer reactions in which the centre gains or loses one electron. The degree of non-equivalence of the iron atoms within the centre in the various redox states has been the subject of considerable interest in recent years. The $[Fe_4S_4(SR)_4]^{-,2-,3-}$ anions provide good analogues for the different oxidation states of these proteins, and in the latest of a series of papers Lane *et al.*[615] describe the isolation and spectroscopic investigation of the trianion form in the compounds $(Et_4N)_3[Fe_4S_4(SPh)_4]$, $(Me_4N)_3[Fe_4S_4(SPh)_4]$, and $(Et_4N)_3[Fe_4S_4(SCH_2Ph)_4]$. The Mössbauer spectra give chemical shifts and quadrupole splittings which are closely similar to those of the corresponding reduced form of the four-iron and eight-iron ferredoxins, and show evidence for a non-equivalence between the iron sites within the four-iron centre. In the spectra obtained in large applied magnetic fields there are lines which move out as the applied field is increased and other lines which move in as the applied field is increased. This indicates that there are two magnetically non-equivalent subsites within the four-iron centre with positive (parallel to the applied field) and negative (antiparallel to the applied field) hyperfine fields. In addition the magnitudes of the magnetic hyperfine interactions for the two subsites appear to be different. It has been established that there is a similar non-equivalence between the iron atoms within the active centre of the corresponding proteins.[616] The authors conclude that the close similarities between the properties of the $[Fe_4S_4(SR)_4]^{3-}$ analogues and the equivalent reduced ferredoxins indicate that iron-subsite non-equivalence is an intrinsic property and is only secondarily influenced by the protein structure which may serve to enhance or diminish it. In contrast the iron atoms in the oxidized ferredoxins are nearly equivalent and their $[Fe_4S_4(SR)_4]^{2-}$ analogues completely so.

Vitamins.—Inoue and Nath [617] have investigated the cobalamins cyanocob(III)-alamin (vitamin B_{12}), cob(II)alamin (vitamin B_{12r}), and cob(I)alamin (vitamin B_{12s}) using emission Mössbauer spectroscopy. In this technique the Mössbauer γ-ray is emitted from an ^{57}Fe atom on a cobalt site in the compound. The

[613] L. Eisenstein and D. R. Franceschetti, *Chem. Phys. Letters*, 1977, **50**, 167.
[614] V. Petrouleas, A. Simopoulos, A. Kostikas, and D. Coucouvanis, *J. Physique*, 1976, **37**, C6-159.
[615] R. W. Lane, A. G. Wedd, W. O. Gillum, E. J. Laskowski, R. H. Holm, R. B. Frankel, and G. C. Papaefthymiou, *J. Amer. Chem. Soc.*, 1977, **99**, 2350.
[616] D. P. E. Dickson, C. E. Johnson, P. Middleton, J. D. Rush, R. Cammack, D. O. Hall, R. N. Mullinger, and K. K. Rao, *J. Physique*, 1976, **37**, C6-171.
[617] K. Inoue and A. Nath, *Bioinorg. Chem.*, 1977, **7**, 159.

Mössbauer chemical shift is virtually identical in all three compounds which means that the *s*-electron density at the nucleus must change very little in going from vitamin $B_{12} \rightarrow B_{12r} \rightarrow B_{12s}$. This is despite the fact that the increasing population of the 3*d* subshell would be expected to enhance the shielding of the *s*-electrons and hence decrease the *s*-electron density at the nucleus. The authors rationalize these observations by postulating considerable d_π-electron delocalization on to the corrin ring and also hybridization of the d_{z^2} orbital which would lead to an increase in 4*s* electron density concomitantly with an increase in the population of the d_{z^2} orbital. The observed quadrupole splitting is discussed in terms of the anisotropy of the covalent bonding between the iron atom, and the corrin ring and axial ligands. By replacing the benzimidazole base with water both 'base on' and 'base off' forms of these compounds were investigated. Knowledge of the Mössbauer spectra of the various redox forms of vitamin B_{12} should be valuable in identifying the intermediates in enzymatic reactions involving vitamin B_{12}. For such applications emission Mössbauer spectroscopy is particularly useful because of its high sensitivity.

Wrobleski and Long [618] have studied 3*d* transition-metal complexes of pyridoxylideneamino-acid which are possible models for vitamin B_6. Mössbauer spectra of the iron complex gave parameters typical of high-spin Fe^{II} in a distorted octahedral ligand field.

Amino-acids.—The investigation by Mossbauer spectroscopy of metal complexes with amino-acids should be helpful in elucidating their ligand structure. It could possibly also be useful in providing 'fingerprints' for identification purposes although it is doubtful whether there would be sufficient variation in the Mössbauer parameters.

Pellerito *et al.*[619] have synthesized a complex of dimethyl tin(IV) and adenosine and have characterized it by Mössbauer and i.r. spectroscopies. Their measurements suggest a co-ordination number of five rather than four or six. The Mössbauer quadrupole splitting is typical of the trigonal-bipyramidal species Alk_2SnX_3, where X are electronegative ligand atoms.

8 Dissociation and Association of Proteins

Contributed by E. J. Wood

Analytical Ultracentrifuge.—*Techniques.* A new, simple, light-producing, -dispersing, and -collimating system for absorption scanning ultracentrifuges has been described.[620] It is claimed to have a number of advantages over commercial units, for which it can easily be substituted. In addition to the use of a faster monochromator and cylindrical lens, giving up to a 100-fold increase in light intensity, two pairs of mirrors give a pre-cell optical system focused in the radial direction at all wavelengths, and the entire unit is self-contained and may be, within limits, moved to any desired location without affecting the quality of the collimated light. Methods for fast, easy, and accurate alignment of the optical system are described and an explanation is given of the use of the system with a computer-

[618] J. T. Wrobleski and G. J. Long, *Inorg. Chem.*, 1977, **16**, 2752.
[619] L. Pellerito, G. Ruisi, R. Barbieri, and M. T. Lo Giudice, *Inorg. Chim. Acta*, 1977, **21**, L33.
[620] G. J. Wei and W. C. Deal, *Analyt. Biochem.*, 1977, **183**, 605.

controlled stepping motor scanner for the collection of data. The computer-centred scanner eliminates the commercial multiplexer and electronic controller for the scanner with savings in complexity and cost.

For sedimentation equilibrium experiments at low speeds where rotor precession is a problem, a modified system has been devised for Beckman model E ultracentrifuges.[621] In this system the bottom mercury cup is replaced by a lower bearing of PTFE.

The method of difference sedimentation has been much used to study the small changes in sedimentation coefficient produced when ligands bind to proteins and conformational changes ensue. However, the results are often equivocal for a number of reasons, the more so with proteins in the lower molecular weight range ($10—20 \times 10^3$), because of rapid diffusion which rapidly eliminates supernatant and plateau regions and broadens the difference curve, obscuring the peak maximum. Rees *et al.*[622] have modified the original Kirschner and Schachman technique [623] to eliminate the need for a supernatant region, making it more suitable for the investigation of changes in small proteins. The new technique employs a modified centrepiece adapted to enable the two menisci to be aligned exactly and a calculation routine for establishing the baseline height from initial and remixed interferograms, with correction for difference in radial dilution between the two sectors of the cell. The method was tested with lysozyme in D_2O and changes of 0.005 S were detected.

Theory. In order to interpret data on the interaction between isolated proteins from the 30S ribosomal subunit,[624, 625] Aune and Rohde [626] tried several approaches for the extraction of information from sedimentation equilibrium experiments. They eventually developed a procedure in which the concentration distribution at sedimentation equilibrium may be resolved into the contributing redistributed components in the system. Although the procedure was shown to be best suited to dealing with heterogeneous systems, it was possible, by making compositional constraints, to use it for systems in which the molecular weights of the interacting proteins were quite similar. The treatment was basically designed for data obtained using Raleigh interference optics, that is a method which does not necessarily provide absolute concentrations. The calculations were performed on a Hewlett–Packard 9810A programmable calculator having 2036 program steps, 111 storage registers, and a cassette storage device. For an ideal homogeneous solute, values of $f(a)$ representing the absolute fringe displacement at the radial position of the meniscus are related to y the fringe displacement, measured relative to the meniscus, by

$$y(r) = f(a)\{\exp[\sigma_i(r^2 - r_a^2)/2] - 1\} \quad (1)$$

where

$$\sigma_i = M_i(1 - v_i\rho)\omega^2/RT \quad (2)$$

[621] R. Rubenstein and A. C. H. Durham, *Analyt. Biochem.*, 1977, **81**, 447.
[622] A. W. Rees, M. S. DeBuysere, and E. A. Lewis, *Arch. Biochem. Biophys.*, 1977, **182**, 478.
[623] M. W. Kirschner and H. K. Schachman, *Biochemistry*, 1972, **10**, 1900.
[624] M. F. Rohde, S. O'Brien, S. Cooper, and K. C. Aune, *Biochemistry*, 1975, **14**, 1079.
[625] M. F. Rohde and K. C. Aune, *Biochemistry*, 1975, **14**, 4344.
[626] K. C. Aune and M. F. Rhode, *Analyt. Biochem.*, 1977, **79**, 110.

If N species are present, the total fringe displacement observed at radial position r is given by

$$y(r) = \sum_{i=1}^{N} f_i(a) \{\exp [\sigma_i(r^2 - r_a^2)/2] - 1\} \quad (3)$$

The iterative search scheme described starts from guess values for the meniscus concentrations and establishes the best set of $f_i(a)$ values which describe the data. Molecular weight values, M_i (and hence σ_i), have to be established from separate experiments on homogeneous systems.

Tang *et al.*[627] have extended the treatment of various indefinite self-associations, especially those in which the molar association constants are not all equal. Their procedures could be applied to non-ideal cases. Previous methods for analysing non-ideal self-associations have been restricted to those in which the molar association constants are equal (called Type I). They tested their procedure on data for sedimentation equilibrium with β-lactoglobulin A at 16 °C in ionic strength 0.15 acetate buffer, pH 4.65. By either of the methods they proposed, the self-association of this protein under these conditions was best described as a sequential indefinite self-association having two equilibrium constants and one second virial coefficient.

Chun and Yoon[628] have published a note in which they consider difference sedimentation equilibrium data presented by Springer in generating $\Delta\sigma$.[629] They suggest that the effects of thermodynamic non-ideality and molecular volume change contribute to a significant degree to the small difference in effective molecular weight computed from the data of Springer and should be taken into account in applying the technique to self-associating protein systems.

Proteins. The self-association characteristics of myosin at high ionic strength were shown by high-speed sedimentation equilibrium to be affected profoundly by the purification procedure employed.[630] Myosin purified by gel chromatography behaved as a single, non-ideal thermodynamic component with little reversible dimerization and a second virial coefficient of 0.64 dl g^{-1}, whereas myosin purified by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography dimerized reversibly to a significant extent and in addition higher polymers appeared to be present. The non-ideality of this latter system was also greater, $B_1 = 2.2$ dl g^{-1}. Possible explanations for this behaviour were considered.

Apolipoproteins from a number of sources have been receiving much attention, including those from human,[631] rhesus monkey,[632] and the dog.[633] Sedimentation equilibrium experiments showed that Apo-C-I from the human high-density lipoprotein complex self-associated in aqueous solution at neutral pH with concomitant changes in secondary structure. At acid pH values, in contrast, it was monomeric. Rhesus monkey apolipoprotein A-II also underwent a reversible

[627] L.-H. Tang, D. R. Powell, B. M. Escott, and E. T. Adams, *Biophys. Chem.*, 1977, **7**, 121.
[628] P. W. Chun and Y. T. Yoon, *Biopolymers*, 1977, **16**, 2579.
[629] M. S. Springer, M. W. Kirschner, and H. K. Schachman, *Biochemistry*, 1974, **13** 3718; E. J. Wood in 'Amino-acids, Peptides, and Proteins', ed R. C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1976, Vol. 7, p. 230.
[630] S. Szuchet, *Arch. Biochem. Biophys.*, 1977, **180**, 493.
[631] J. C. Osborne, T. J. Brouzert, and H. B. Brewer, *J. Biol. Chem.*, 1977, **252**, 5756.
[632] D. L. Barbeau, T.-L. Teng, and A. M. Scanu, *J. Biol. Chem.*, 1977, **252**, 6745.
[633] T.-L. Teng, C. Edelstein, D. L. Barbeau, and A. M. Scanu, *J. Biol. Chem.*, 1977, **252**, 8634.

self-association in aqueous solutions at pH 8.6 containing EDTA. It was not possible to choose between a monomer–dimer–tetramer equilibrium and a monomer–dimer–trimer–tetramer equilibrium although the former model was the more favoured. Like human apo-A-I,[634] canine apo-A-I, at pH 8.6 in the presence of EDTA, also appeared to exist in a monomer–dimer–tetramer–octomer equilibrium. This protein was also examined by the sedimentation velocity method.

Red deer β-lactoglobulin existed as a monomer–dimer system at pH 6.5,[635] and human luteinizing hormone appeared to self-associate,[636] although there was evidence that the isolated α- and β-subunits were not stable under certain storage conditions. Sedimentation equilibrium with bovine insulin containing two mol Zn^{II} per six base-mol insulin at pH 7.0 revealed the existence of a stable zinc–insulin hexamer together with linked polymerization reactions.[637] The possible biological significance of the linked polymerization pattern was considered, and it seemed likely that this hexamer is identical with the hexamer reported in *X*-ray crystallographic studies.

If oligomeric proteins dissociate readily at relatively high concentrations (compared with those at which enzyme activity typically occurs) it is possible to question the importance of polymerization in the functioning of the enzyme. Spragg *et al.*[638] used both sedimentation velocity and sedimentation equilibrium methods to investigate the association of yeast phosphoglycerate kinase. It was shown that a monomer–tetramer equilibrium existed, but values for the interaction coefficient at 20 °C ranging from 40 to 310 $l^3\ g^{-3}$ were obtained. The low temperature-sensitivity of the association reaction suggested that the interaction between subunits was largely hydrophobic and a low sensitivity to changes in ionic strength supported this notion. Another interesting system that has been studied by sedimentation equilibrium is ovine submaxillary mucin[639] whose aggregation behaviour depended on its carbohydrate content as well as on ionic strength and protein concentration.

Gel Chromatography.—As part of continuing studies on the thermodynamics of subunit assembly of human haemoglobins, Ackers and his co-workers have made extensive use of analytical molecular-sieve chromatography.[640] The aim of such studies is to provide a basis for comparing energetic properties associated with the various haemoglobin subsystems, *i.e.* ligand binding sites, inter-subunit contact sites, and α- and β-chains. Thus it was possible to analyse the homogeneous self-association of isolated α^{SH}- and β^{SH}-chains over the concentration range 0.004—15.2 mg ml^{-1}. The data for the α^{SH}-system were best interpreted in terms of a monomer–dimer system, whereas under the same conditions the β^{SH}-system was best described by a monomer–tetramer equilibrium. Increasing the NaCl concentration was found to increase the association constant for both the α^{SH}- and β^{SH}-chains. The equilibrium constants for the dimer–tetramer association for

[634] L. B. Vitello and A. M. Scanu, *J. Biol. Chem.*, 1975, **251**, 1131.
[635] E. I. McDougall and J. C. Stewart, *Biochem. J.*, 1977, **167**, 45.
[636] A. Nureddin and P. Johnson, *Biochemistry*, 1977, **16**, 1730.
[637] B. K. Milthorpe, L. W. Nichol, and P. D. Jeffrey, *Biochim. Biophys. Acta*, 1977, **495**, 195.
[638] S. P. Spragg, J. J. Roche, and J. Wilcox, *Biochem. J.*, 1977, **163**, 543.
[639] H. D. Hill, J. A. Reynolds, and R. L. Hill, *J. Biol. Chem.*, 1977, **252**, 3791.
[640] R. Valdes and G. K. Ackers, *J. Biol. Chem.*, 1977, **252**, 74; S. H. C. Ip and G. K. Ackers, *ibid.*, p. 82; R. Valdes and G. K. Ackers, *ibid.*, p. 88.

oxygenated and for unliganded human haemoglobin were also determined as a function of temperature. For the oxygenated form the van't Hoff enthalpy was 3.8 ± 1.6 kcal mol^{-1} and the unitary entropy 48.4 ± 5.7 cal K^{-1} mol^{-1}, and for the unliganded form under the same conditions the values were −28.9 ± 0.5 kcal mol^{-1} and −41.8 ± 1.7 cal K^{-1} mol^{-1}. The former values were obtained by gel chromatography and the latter from kinetic studies of the forward and reverse rate constants. The difference of the two enthalpy values is the oxygenation-linked enthalpy of subunit association, 32.7 kcal mol^{-1}, and the corresponding entropic coupling is 90.2 cal K^{-1} mol^{-1}. This is consistent with an increased role of hydrophobic interactions within the dimer–dimer contact regions upon oxygenation or a decreased role of H-bonds and ionic interactions. In further studies using isothermal heat-burst microcalorimetry it was possible to measure the contributions of measured heats arising from α-chain self-association, β-chain self-association, and association of dimers to form tetramers.

Although the polymerization of α-chymotrypsin is probably irrelevant in biological terms, the association reaction has been much studied in the past, especially by ultracentrifuge techniques, and has served as a useful model for the development of techniques for the study of rapid polymerization equilibria. Tellam and Winzor [641] have continued their studies with this enzyme and its di-isopropyl phosphoryl derivative, under conditions of comparatively low ionic strength at pH 7.9, by velocity sedimentation, equilibrium sedimentation, and difference gel chromatography. From the data obtained it was proposed that the polymerizing system involved indefinite association of a dimer which was formed in a discrete dimerization step with an equilibrium constant of 0.25 l g^{-1}. As this dimerization step was essentially unaffected by ionic strength, it was thought that higher polymer formation involved an entirely different mechanism involving electrostatic interactions between dimers.

Other protein systems that have been studied at least in part by gel chromatography are the dissociation of 7S-nerve growth factor,[642, 643] malate dehydrogenase from a variety of sources,[644] *E. coli* carbamyl phosphate synthetase,[645] and human and baboon apolipoproteins.[646] Certain protein–small molecule systems have also been investigated by gel chromatography, including the binding of zinc to rat liver fructose-1,6-bisphosphatase [647] and the binding of various combinations of labelled amino-acids, ^{14}C-labelled ATP, and γ-^{32}P-labelled ATP to monomeric and dimeric aminoacyl-tRNA synthetases.[648]

Light Scattering.—Lasers have been used a great deal to study the assembly of protein subunits, and the use of lasers in such studies has been reviewed.[649]

[641] R. Tellam and D. J. Winzor, *Biochem. J.*, 1977, **161**, 687.
[642] N. J. Pantazis, R. A. Murphy, J. D. Saide, M. H. Blanchard, and M. Young, *Biochemistry*, 1977, **16**, 1525.
[643] M. A. Bothwell and E. M. Shooter, *J. Biol. Chem.*, 1977, **252**, 8532.
[644] D. M. Bleile, R. A. Schulz, J. H. Harrison, and E. M. Gregory, *J. Biol. Chem.*, 1977, **252**, 755.
[645] P. M. Anderson, *Biochemistry*, 1977, **16**, 583.
[646] M. Rosseneu, V. Blaton, R. Vercaemst, F. Soetewey, and H. Peeters, *European J. Biochem.*, 1977, **74**, 83.
[647] F. O. Pedrosa, S. Pontreoli, and B. L. Horecker, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2742.
[648] R. S. Mulvey, and A. R. Fersht, *Biochemistry*, 1977, **16**, 4005.
[649] B. S. Hudson, *Ann. Rev. Biophys. Bioeng.*, 1977, **6**, 135.

Quasi-elastic light scattering is capable of giving very accurate values for the translational diffusion coefficient, D, and this technique was used to investigate the aggregation and activation of prothrombin.[650] Values of D_{20w}, at different protein concentrations were combined with sedimentation data to yield molecular weights which suggested that aggregation occurred under certain conditions. The evidence indicated the existence of end-to-end dimers, and in addition a method was presented for determining the percentage of converted prothrombin in an activated system containing aggregates.

In contrast to quasi-elastic scattering, the characteristics of laser beams make them well suited for use in stopped-flow systems for studying the kinetics of associating and dissociating systems by recording the total intensity of scattered light. Here the progress of a reaction is followed by the change in intensity of scattered light at 90° accompanying changes in the weight-average molecular weight of the reacting species. Thus it was possible to investigate the first-order dissociation of tetrameric carbonomonoxy haemoglobin to the dimer using a stopped-flow instrument in which the xenon arc source was replaced by an argon-ion laser (wavelength 488 nm).[651] Liddle *et al.*[652] have completely redesigned a stopped-flow instrument for use with a 50 mM He–Ne laser, and have considered the various factors which contribute to noise and how to minimize them. This is important as many interesting protein–protein interactions occur in the molecular weight range 30 000—300 000 and at relatively low concentrations. The sensitivity in the detection of reaction amplitudes of their instrument, working at low protein concentrations, was 5000 dalton mg ml^{-1} for the laser employed. The instrument was used to study muscle phosphofructokinase at concentrations down to 50 μg ml^{-1}, where the reaction amplitude was 6000 dalton mg ml^{-1}. The work with phosphofructokinase was extended using gel chromatography and also sedimentation velocity.[653] The results indicated that the enzyme existed as at least two 13S components differing in their phosphate contents and also in their self-association properties. It is important to note that the work was done with enzyme prepared from rabbit muscle by a new and more gentle technique which exploits the fact that at high ionic strength and pH 8 the molecular weight of the enzyme is 320 000 whereas at low ionic strength it is several millions.

Parker and Dalgleish[654] have employed stopped-flow light scattering and stopped-flow turbidity to investigate the Ca^{2+}-induced association of α_s-casein. As they point out, special problems arise in the use of light scattering to study the kinetics of aggregation in solutions where the solute particles eventually can have radii of $> \lambda/20$. Furthermore, in solutions containing even moderately large particles a correction must be applied for the diminution of the intensity of scattered light caused by the turbidity due to the aggregated particles. Since the turbidity increases as the reaction proceeds it is essential to make this measurement on the same time-scale as the kinetics of scattered light intensity. It was

[650] G. P. Agarawal, J. G. Gallagher, K. C. Aune, and C. D. Armeniades, *Biochemistry*, 1977, **16**, 1865.
[651] D. P. Flamig and L. J. Parkhurst, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3814.
[652] P. F. Liddle, D. J. Jacobs, and G. L. Kellett, *Analyt. Biochem.*, 1977, **79**, 276.
[653] C. R. Hussey, P. F. Liddle, D. Ardron, and G. L. Kellett, *European J. Biochem.*, 1977, **80**, 497.
[654] T. G. Parker and D. G. Dalgleish, *Biopolymers*, 1977, **16**, 2533.

therefore necessary to construct two stopped-flow reaction systems, one for light scattering and one for turbidity measurements. In the first system the light source was a 25 mW He–Ne laser, and the detection system was the optical unit of a Malvern 4300 digital autocorrelator. In the second, light of appropriate wavelength was selected by a monochromator from a Unicam SP500 spectrophotometer and the transmitted light detected by means of a photodiode and amplifying system. The reaction that they studied proceeded far beyond the molecular sizes which it is normally possible to investigate by light scattering. They explain their results in terms of a reaction in which Ca^{2+} associates with the α_s-casein, followed by association of the monomers and subsequent precipitation of caseinate.

Transport Studies.—In order to analyse the reaction boundaries observed in sedimentation studies with rapidly and reversibly associating solutes, Winzor *et al.*[655] have devised a simple method for the determination of the asymptotic boundary shape from a series of schlieren traces. Additional information, besides a value for the weight-average sedimentation coefficient, is available from the shape of the boundaries, but the theoretical description requires integration of second-order differential equations with terms to account for sedimentation, chemical reaction, and spreading due to diffusion. Winzor *et al.* have attempted to obtain the asymptotic boundary shapes for such solutes by adapting procedures developed for analysis of heterogeneous non-interacting systems. This approach is empirical but simple, but nevertheless yielded interaction parameters in agreement with those obtained by other procedures for DIP-chymotrypsin at ionic strength 0.29 in phosphate and pH 7.9 (monomer–dimer: see also ref. 641) and for β-lactoglobulin A at ionic strength 0.1 in acetate and pH 4.65 (monomer–dimer–trimer–tetramer: see also ref. 627). The asymptotic patterns so determined corresponded closely with those predicted by Gilbert theory.

Two types of phosphofructokinase have been investigated by sedimentation velocity methods. The enzyme from yeast, an octamer of molecular weight 835 000, had a sedimentation coefficient, $s^0_{20,w}$ of 20.81 S, which showed a linear dependence upon protein concentration down to 0.01 mg ml^{-1}.[656] Pig liver phosphofructokinase, in contrast, appeared to be larger than any previously studied phosphofructokinase, having a molecular weight at 4 °C and 5 mg ml^{-1} of greater than 1×10^7. At higher temperatures or lower protein concentrations partial dissociation occurred. Evidence was also obtained from viscosity experiments that the enzyme was highly asymmetric.[657] Transcarboxylase is another complex and interesting enzyme.[658] A new form was isolated with molecular weight 1 200 000 and $s_{20,w}$ of 26S which contained 12 biotinyl groups. It appeared to consist of a central hexameric subunit with six dimeric outer subunits, these at opposite faces of the central subunit. This larger form dissociated to the previously described 18S form at neutral pH, which has only three attached subunits. There was an equilibrium at neutral pH between active forms of the enzyme with

[655] D. J. Winzor, R. Tellam, and L. W. Nichol, *Arch. Biochem. Biophys.*, 1977, **178**, 327.
[656] G. Kopperschläger, J. Bär, K. Nissler, and E. Hofmann, *European J. Biochem.*, 1977, **81**, 317.
[657] J. L. Trujillo and W. C. Deal, *Biochemistry*, 1977, **16**, 3098.
[658] H. G. Wood, J.-P. Chiao, and E. M. Poto, *J. Biol. Chem.*, 1977, **252**, 1490; E. M. Poto and H. G. Wood, *Biochemistry*, 1977, **16**, 1949; N. G. Wrigley, J.-P. Chiao, and H. G. Wood, *J. Biol. Chem.*, 1977, **252**, 1500.

six, five, four, three, two, and one subunits attached to the central subunit. Multiple schlieren peaks were observed at 60 000 rev min^{-1} because the sedimentation rate outstripped the equilibration rate for the different forms, whereas at 30 000 rev min^{-1} a single peak of the multiple form was observed because equilibration kept abreast of sedimentation. Another complex system is muscle troponin which, under conditions approaching physiological ones, not only dissociated but also showed some tendency to polymerize well beyond the 85 000 molecular weight stage.[659]

Many sedimentation velocity studies have appeared on the effects of small molecules on the association properties and conformations of proteins. Thus rat kidney glutaminase (mol. wt. 160 000) dimerized in the presence of its activator, phosphate,[660] and *E. coli* aspartate transcarbamoylase underwent up to a 3.6% decrease in sedimentation coefficient upon addition of the substrate carbamoyl phosphate as well as of certain substrate analogues.[661] The predominantly hydrophobic interactions which are responsible for the maintenance of the multisubunit structure of sesame seed α-globulin were studied by sedimentation velocity in the presence of electrolytes. In terms of the Hofmeister series, SO_4^{2-} and Cl^- caused association, whereas other anions caused dissociation in the order, $Br^- < ClO_4^- < SCN^- \ll I^- < CCl_3CO_2^-$. Such reagents have an advantage over urea, sodium dodecyl sulphate, *etc.*, in that they can be effective at comparatively low concentrations at which conformational changes or dissociation do not occur.[662]

Finally, in this section it must be mentioned that Cann and Stimpson [663] have given some consideration to the behaviour of interacting systems during isoelectric focusing. They have described the theory both for the equilibrium distribution of macromolecule along the focusing column and for the time-course of approach to that distribution. These studies are important because they show that a single amphoteric macromolecule (*e.g.* a protein) can give a focusing pattern consisting of two peaks due to reversible binding of carrier ampholyte with accompanying macromolecular isomerization. They also consider pH-dependent conformational transitions and show that binding of H^+ by a macromolecule may give rise to well resolved equilibrium patterns. Whether the corresponding transient patterns are bimodal or virtually unimodal can depend upon the point of insertion of the sample into the pH gradient and the stoicheiometry of the interactions.

Electron Microscopy.—Electron microscopy with sodium phosphotungstate as negative stain was used to investigate the quaternary structure of the enzyme urease from jackbean.[664] It appears that the so-called A_1 urease (M_r 240 000) exists as cyclic trimers which pair up to form the hexameric α-urease (M_r 480 000) which displays D_3 symmetry of a trigonal prism. Higher polymers also form in

[659] S. J. Lovell and D. J. Winzor, *Biochem. J.*, 1977, **167**, 131.
[660] S. Godfrey, T. Kuhlenschmidt, and N. P. Curthoys, *J. Biol. Chem.*, 1977, **252**, 1927.
[661] G. J. Howlett and H. K. Schachman, *Biochemistry*, 1977, **16**, 5077.
[662] V. Prakash and P. K. Nandi, *J. Biol. Chem.*, 1977, **252**, 240.
[663] J. R. Cann and D. I. Stimpson, *Biophys. Chem.*, 1977, **7**, 103, 115.
[664] W. N. Fishbein, W. F. Engler, J. L. Griffin, W. Scurzi, and G. F. Bahr, *European J. Biochem.*, 1977, **73**, 185.

which the molecules align with their clefts coplanar and an angle of 120° between each triplet of three-fold axes.

When it is possible to observe repeating structures in electron micrographs, optical and digital methods of image reconstruction may be used.[665] Polymers of contracted sheath particles of bacteriophage Mu were examined by this technique.[666] It is known that the sheath is composed of a single type of protein of molecular weight 52 000, and the averaged density map at a resolution of 2 nm indicated that the structure may be described as a set of annular rings of height about 1.8 nm with six-fold symmetry. Successive rings in the structure changed by about 33° in azimuth, and the data obtained were discussed in relation to changes in the sheath molecules upon contraction. Another bacteriophage to be studied by electron microscopy was T4.[667] The method used in this instance was to label the individual proteins within the supramolecular structure with specific antibodies, or rather the Fab fragments derived from them by proteolytic digestion. Optically filtered electron micrographs of the labelled capsids revealed both the location of the specific proteins within the capsomers and also differing conformational states of the protein subunits.

Kinetic Studies.—*Protein–Protein Interactions.* Neurophysins exist in the posterior pituitary gland as non-covalent molecular complexes with the peptide hormones oxytocin and vasopressin. When either of these latter hormones is secreted, the corresponding neurophysin is also released into the blood. The kinetics of the interaction between oxytocin and vasopressin and neurophysin have been studied by a temperature-jump technique.[668] The fact that interaction is coupled to a proton transfer makes it possible to monitor complex formation by adding a pH indicator, phenol red. The formation rate constants using neurophysin I dimer at pH 7.4 at 25 °C were 2.8×10^6 and 2.3×10^6 l mol^{-1} s^{-1} for oxytocin and vasopressin respectively and the corresponding dissociation rate constants were 11 and 15 s^{-1}. For neurophysin II dimer, the values were 6.0 and 2.4×10^6 l mol^{-1} s^{-1} and 24 and 16 s^{-1}. Formation rate constants for interaction with neurophysin monomer were an order of magnitude lower than for the dimer.

An interesting protein–protein interaction occurs in the bioluminescent system of the boring mollusc, *Pholas dactylus*.[669] Both the luciferase and the luciferin are glycoproteins, have molecular weights of 34 000, and form complexes with a stoicheiometry of two mols luciferin (or oxyluciferin) per mol of enzyme (luciferase). The association between oxyluciferin and luciferase has a low rate constant, k_a, of 1.6×10^7 l mol^{-1} s^{-1}, with K_d 1.7×10^{-8} mol^{-1}. However, the dissociation rates differ in that the rate of dissociation of luciferin, as opposed to oxyluciferin, from the complex was too slow to be measured, K_d being estimated to be about 10^{-11} mol l^{-1}. These interactions thus control product as well as substrate associations with the enzyme.

[665] D. J. DeRosier and A. Klug, *Nature*, 1968, **217**, 130.
[666] A. F. M. Cremers, M. H. Schepman, M. P. Visser, and J. E. Mellema, *European J. Biochem.* 1977, **80**, 393.
[667] U. Aebi, B. ten Heggeler, L. Onorato, J. Kistler, and M. K. Showe, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 5514.
[668] A. F. Pearlmutter and C. McMains, *Biochemistry*, 1977, **16**, 628.
[669] J. P. Henry and C. Monny, *Biochemistry*, 1977, **16**, 2517.

Jaenicke and his group have made a number of studies of the refolding and reactivation of multisubunit dehydrogenases after dissociation in, for example, 6M-guanidinium chloride. The tetrameric heart lactate dehydrogenase (H_4) is inactive when dissociated to the monomer, but removal of denaturants leads to the regaining both of activity and of oligomeric structure.[670] The kinetics of reactivation were analysed in terms of a first-order transconformation and a second-order association reaction. The re-association of the corresponding isoenzyme from muscle (M_4) could be described by a second-order rate equation.[671] The presence of NAD^+ or NADH did not affect the kinetics of reactivation. Similar studies have been performed with yeast glyceraldehyde-3-phosphate dehydrogenase,[672] bovine pyruvate kinase,[673] and *B. megaterium* glucose dehydrogenase.[674]

Protein–Small Molecule Interactions. Certain of the plasma proteins bind small molecules (metabolites, hormones, drugs), but to date most studies have been on systems at equilibrium. However, two recent kinetic studies have appeared. Reed,[675] using stopped-flow spectrophotometry, found the binding of bilirubin to bovine serum albumin to be a second-order process, and also that palmitate had an effect on the binding process. At low molar ratios, palmitate had no effect on the association rate but decreased the dissociation rate, whereas at high molar ratios (palmitate : albumin > 5) it decreased the association rate and increased the dissociation rate. Such effects on the binding of one ligand by another are of great practical importance, and it is suggested that palmitate affects the ability of the albumin to undergo the conformational changes needed to accommodate bilirubin. Stroupe *et al.*[676] studied the kinetics of binding of progesterone to the progesterone-binding protein from pregnant guinea pig by stopped-flow fluorometry. The association rate constant was independent of the pH from pH 5 to 10, but the dissociation rate constant was strongly pH-dependent. It was concluded that at least three ionizing residues were responsible for the stability of the complex. Temperature-jump fluorometry was used to investigate the kinetics of binding of 4-methylumbelliferyl α-D-mannopyranoside to tetrameric concanavalin A.[677] There was no evidence for any interaction between the saccharide binding sites. Fluorescence was also employed to obtain the association and dissociation rate constants for the binding of nucleotides to actin.[678]

Many studies of the kinetics of binding of O_2 and other ligands to respiratory proteins have appeared. Stopped-flow and temperature-jump spectrophotometry were employed to show that the association rate constant for the combination of O_2 with a marine mollusc haemocyanin was largely independent of the pH.[679] In contrast the dissociation rate constant was strongly pH-dependent. This gives

[670] R. Rudolph, I. Heider, E. Westhof, and R. Jaenicke, *Biochemistry*, 1977, **16**, 3384.
[671] R. Rudoph, I. Heider, and R. Jaenicke, *Biochemistry*, 1977, **16**, 5527.
[672] R. Rudolph, I. Heider, and R. Jaenicke, *European J. Biochem.*, 1977, **81**, 563.
[673] J. M. Cardenas, D. R. Hubbard, and S. Anderson, *Biochemistry*, 1977, **16**, 191.
[674] H. E. Pauly and G. Pfleiderer, *Biochemistry*, 1977, **16**, 4599.
[675] R. G. Reed, *J. Biol. Chem.*, 1977, **252**, 7483.
[676] S. D. Stroupe, K. J. Acree, and U. Westphal, *Biochemistry*, 1977, **16**, 1350.
[677] F. G. Loontiens, R. M. Clegg, A. van Landschoot, and T. M. Jovin, *European J. Biochem.*, 1977, **78**, 465.
[678] F. Waechter and J. Engel, *European J. Biochem.*, 1977, **74**, 227.
[679] E. J. Wood, G. R. Cayley, and J. S. Pearson, *J. Mol. Biol.*, 1977, **109**, 1.

a kinetic explanation for the origin of the Bohr effect, which in this haemocyanin is in fact a reverse Bohr effect. Similar studies with the haemocyanin from *Palunirus interruptus* lead to similar conclusions, namely that the stability of the O_2–protein complex is largely determined by the value of the dissociation rate constant.[680] Photodissociation techniques cannot be used to obtain information on haemocyanin–oxygen kinetics, but continue to be used very effectively for haemoglobin systems. Saffran and Gibson[681] have investigated the effect of temperature on the quantum yield for photodissociation of O_2 and other ligands from haemoglobin and myoglobin, in the presence and absence of the allosteric effector inositol hexaphosphate. The quantum yield was greater at 40 °C than at 0 °C and inositol hexaphosphate increased the quantum yield of ligand photolysis from haemoglobin. Gibson's group have also used a dye laser pulse with an energy of up to 1.2 J at 540 nm to remove O_2 from oxyhaemoglobin. This technique was used to provide a direct measure of the rate of conformational change between the rapidly reacting intermediate product of full photolysis and 'normal' deoxyhaemoglobin. In borate buffer at pH 9 it appeared that the rapidly reacting deoxy products formed by laser photolysis of both HbO_2 and HbCO were similar, suggesting that the quaternary conformations of these derivatives are also similar.[682] The same equipment was used to provide both kinetic and equilibrium data for the T state of haemoglobin.[683]

Two other new techniques have been used for investigating haemoglobin kinetics. Changes in magnetic susceptibility during the recombination of haemoglobin with CO after flash photolysis were measured with a new instrument using superconducting technology.[684] Pulse radiolysis was used to obtain rapid reduction (in a few μs) of one haem group in a methaemoglobin tetramer.[685] The kinetics of the binding of O_2 to this intermediate (Hb^{3+}) were studied, and the results suggested that Hb^{3+} was in the relaxed quaternary conformation.

Protein–Small Molecule Equilibria.—Several theoretical treatments have been published for analysing data on the binding of small molecules to macromolecules. Nimmo *et al.*[686] have evaluated ways of using equilibrium dialysis to quantify the binding of ligands to macromolecules, and Nimmo and Bauermeister[687] have described a modified least-squares method for fitting the Hill equation to sets of data with common values for n and K but different values of V_{max}. Johnson and Ackers[688] have discussed the problems of analysing oxygenation curves obtained with dilute haemoglobin solutions where appreciable amounts of dimer may be present, and Otsuka and Kunisawa[689] have analysed recent experimental data of oxygen equilibrium constants for haemoglobin, measured over a wide range of O_2 pressures, whereby the change in the molecular structure of haemo-

[680] H. A. Kuiper, E. Antonini, and M. Brunori, *J. Mol. Biol.*, 1977, **116**, 569.
[681] W. A. Saffran and Q. H. Gibson, *J. Biol. Chem.*, 1977, **252**, 7955.
[682] C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.*, 1977, **252**, 5783.
[683] C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.*, 1977, **252**, 7538.
[684] J. S. Philo, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2620.
[685] A. Raap, J. W. van Leeuwen, T. van Eck-Schouten, H. S. Rollema, and S. H. de Bruin, *European J. Biochem.*, 1977, **81**, 619.
[686] I. A. Nimmo, G. L. Atkins, R. C. Strange, and I. W. Percy-Robb, *Biochem. J.*, 1977, **165**, 107.
[687] I. A. Nimmo, and A. Bauermeister, *Analyt. Biochem.*, 1977, **82**, 468.
[688] M. L. Johnson and G. K. Ackers, *Biophys. Chem.*, 1977, **7**, 77.
[689] J. Otsuka and T. Kunisawa, *Arch. Biochem. Biophys.*, 1977, **179**, 706.

globin induced by oxygenation is considered individually at each stage of oxygenation. Gelin and Karplus [690] present a reaction path by which the effects of O_2 binding to haemoglobin are transmitted from a haem group to its subunit. Klotz and Hunston [691] considered the various possible situations that can occur during the multiple binding of ligands to proteins where there is 'half-of-sites binding'. Three different situations are analysed: (*a*) where two classes of sites exist in the ligand-free macromolecule; (*b*) where all the sites are initially identical, but where after half of them are occupied the affinity of the residual ones is changed; and (*c*) where all the sites are identical at the outset but interact in a pair-wise manner. Plots of normalized binding constants (iK_i) against stoicheiometric step number (i) ('affinity profiles') are examined for each situation in order to provide a basis for discriminating among them.

Brodersen *et al.*[692] have described what they believe to be the first rigorous demonstration of the *independent* binding of two ligands to one protein molecule, namely the binding of a benzodiazepine and bilirubin to human serum albumin, by c.d. measurements. The free-energy coupling, $\Delta F_{x,y}$, was small, being less than 50 cal mol^{-1}.

In contrast to this independent binding, numerous studies have appeared on the linkage between binding sites for small molecules. The binding of Zn^{2+} to myoglobin, for example, increased the affinity for O_2 and CO [693] and had a similar effect on haemoglobin.[694] A strong co-operative effect existed between the binding of substrate ligands and metal ions to the enzyme pyruvate kinase,[695] but the tetrameric glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle showed negative co-operativity in NAD^+ binding.[696] Klarman and Daniel [697] studied the binding of O_2 by haemocyanins from a variety of arthropod species, in the absence of Ca^{2+} or Mg^{2+} ions. The binding was co-operative in all cases. This is in marked contrast to the behaviour of mollusc haemocyanins, which only display co-operative binding of O_2 in the presence of these bivalent metal ions. It is interesting that the radular muscle myoglobin from a sea snail, *Nassa mutabilis*, was dimeric, and bound O_2 co-operatively with a Hill coefficient, n, of 1.5.[698] The extracellular blood haemoglobin of another snail, *Heliosoma trivolvis*, is a very large protein (mol. wt. 1.75×10^6) with multiple binding domains, which binds O_2 co-operatively.[699] However, when individual domains (mol. wt. 17 000) were separated by gentle proteolysis, both co-operativity and the Bohr effect were lost. Hence the intact polypeptide chain seems to be necessary for the full expression of homotropic and heterotropic interactions.

Of the many papers that have appeared dealing with the binding of O_2 and other ligands by vertebrate (tetrameric) haemoglobins it is only possible to mention a

[690] B. R. Gelin and M. Karplus, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 801.
[691] I. M. Klotz and D. L. Hunston, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4959.
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[693] J. M. Rifkind, M. H. Keyes, and R. Lumry, *Biochemistry*, 1977, **16**, 5564.
[694] J. M. Rifkind and J. M. Heim, *Biochemistry*, 1977, **16**, 4438.
[695] T. Nowak and M. J. Lee, *Biochemistry*, 1977, **16**, 1343.
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[697] A. Klarman and E. Daniel, *J. Mol. Biol.*, 1977, **115**, 257.
[698] G. Geraci, A. Sada, and C. Cirotto, *European J. Biochem.*, 1977, **77**, 555.
[699] R. C. Terwilliger, N. B. Terwilliger, C. Bonaventura, and J. Bonaventura, *Biochim. Biophys. Acta*, 1977, **494**, 416.

few. A variety of attempts have been made to describe the mechanism of O_2 bonding and its control.[700] and Carey *et al.*[701] have shown that increasing the hydrostatic pressure to 1000 atm increases the O_2 affinity of human haemoglobin about two-fold. The R to T transition, however, was not affected by pressure. Various mutant or modified haemoglobins were used in order to obtain information on the mechanism of co-operative oxygenation, including haemoglobin Tacoma [Arg B12(30)$\beta \rightarrow$ Ser] which has a diminished O_2 affinity and Hill coefficient,[702] haemoglobin M Milwaukee (β67 E11 Val $\rightarrow$ Glu) which has two permanently oxidized haems on the β chains,[703] haemoglobins Austin and Waco, both of which have substitutions in the $\alpha_1\beta_2$ contact region,[704] and valency hybrids (*e.g.* $\alpha_2^{met}\ \beta_2$) derived from both normal haemoglobin (HbA) and HbS.[705] The Bohr effect has also been studied from a number of aspects. There is evidence for linkage between the alkaline Bohr effect and the differential binding of chloride to liganded and unliganded haemoglobin, and Haire and Hedlung[706] have provided a thermodynamic interpretation of this effect. Most of the oxygen-linked carbamate which is formed in normal human haemoglobin is confined to the β-subunits (Val-1β), but the relationship of CO_2 with the α-chains has been investigated in haemoglobin and derivatives in which the terminal α-amino-group of the β-chain was blocked with pyridoxal phosphate.[707] It has also been shown that 2,3-diphosphoglycerate (DPG) can effect the carbamino adducts in both the liganded and unliganded states.[708]

Benesch *et al.*[709] studied the interaction of inositol esters with haemoglobin. They used the proton uptake method to obtain the six binding constants for deoxy- and oxy-haemoglobin. The Bohr coefficient ($\Delta \log p_{50}/\Delta$pH) and the Haldane coefficient (ΔH^+, the difference per O_2 in the number of protons bound by the oxy- and deoxy-forms) are equal in the absence of allosteric cofactors.[710] However, in the presence of the inositol esters (the hexaphosphate, the pentaphosphate, and the hexasulphate) the two coefficients were unequal at low, but not at high, cofactor concentrations. The behaviour of DPG was quite different in that even at high concentrations the Haldane coefficient remained elevated, a reflection of the negligible affinity of DPG for the fully oxygenated form of haemoglobin.

Subunit Structures of Proteins.—Several attempts have been made to clarify some basic points relating to the behaviour of proteins upon treatment with guanidinium chloride and other compounds commonly used to dissociate multisubunit

[700] C. A. Reed and S. K. Cheung, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1780; B. D. Olafson and W. A. Goddard, *ibid.*, p. 1315; A. Warshel, *ibid.*, p. 1789.
[701] F. G. Carey, F. Knowles, and Q. H. Gibson, *J. Biol. Chem.*, 1977, **252**, 4102.
[702] P. W. Tucker, and M. F. Perutz, *J. Mol. Biol.*, 1977, **114**, 415.
[703] L. W. M. Fung, A. P. Minton, T. R. Lindstrom, A. V. Pisciotta, and C. Ho, *Biochemistry*, 1977, **16**, 1452.
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[705] J. Murari, *Arch. Biochem. Biophys.*, 1977, **181**, 1.
[706] R. N. Haire and B. E. Hedlund, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4135.
[707] C. Bauer and A. Kurtz, *J. Biol. Chem.*, 1977, **252**, 2952.
[708] J. B. Matthew, J. S. Morrow, R. J. Wittebort, and F. R. N. Gurd, *J. Biol. Chem.*, 1977, **252**, 2234.
[709] R. E. Benesch, R. Edalji, and R. Benesch, *Biochemistry*, 1977, **16**, 2594.
[710] I. Tyuma and Y. Ueda, *Biochem. Biophys. Res. Comm.*, 1975, **65**, 1278.

proteins. Work by Eisenberg's group on bovine serum albumin in guanidinium chloride [711] suggests that, over the range of concentrations of the latter investigated, about 0.2 g water as well as 0.28 g of denaturant were bound per gram of protein. This corresponds on the average to 1.3 molecules of water and 0.35 molecules of guanidinium chloride per amino-acid residue. Although albumin, of course, contains no subunits, similar values were obtained by recalculating previous data for the multisubunit enzyme aldolase. Crouch and Kupke [712] investigated volume changes (ΔV) accompanying interactions of proteins with guanidinium chloride. They studied ribonuclease (again a protein not composed of subunits) in aqueous 0—8M-guanidinium chloride and found a transition between about 2 and 4M. They give methods for calculating the ΔV of mixing, the ΔV in transporting protein from one concentration of perturbant to any other, and the ΔV in transporting perturbant from solvent to protein solution. The late J. W. Beams and Kupke have published details of a method for the simultaneous measurement of viscosity and density in solutions undergoing change, by means of a magnetic suspension device.[713]

Lapanje *et al.*[714] studied the isothermal interaction of β-lactoglobulin with guanidinium chloride and urea. In addition to obtaining enthalpies of interaction by calorimetric methods, they also used dilatometric methods to determine volume changes accompanying the interaction of guanidinium chloride with β-lactoglobulin. High concentrations of urea were used to dissociate human erythrocyte phosphoribosyl pyrophosphate synthetase, subunit molecular weight 33 200, which exists as a variety of aggregated forms with molecular weights of from 65 000 to 1 040 000.[715] Moderate concentrations of urea (0.9—3.0 mol l^{-1}) were used to study the influence of subunit interactions on the stability of bovine lactate dehydrogenase isozymes.[716] The dependence of inactivation rate on urea concentration followed the relationship $k = a[\text{urea}]^n$, where a and n are constants for each isozyme. Values of n were similar for all isozymes but values of a differed by up to several hundred-fold. The isozymes could be arranged in order of decreasing urea sensitivity thus: H_4, M_4, H_3M, HM_3, and H_2M_2. It was possible to estimate the contribution of various subunit interactions to the stability of the enzyme. Gurne *et al.*[717] used immobilized porphobilinogen synthase subunits for the investigation of intra- and inter-species hybridization, as well as for the preparation of purified enzyme from different species. Subunits attached to the affinity absorbent could be removed with 4M-urea and these could then be re-associated into soluble octameric enzyme.

Many studies on the interactions of sodium dodecyl sulphate (SDS) with multisubunit proteins have appeared and only a few examples can be mentioned. From experiments with immunoglobulin G and chymotrypsin it was concluded that both free SH groups and denaturing conditions were required for thermal

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[712] T. H. Crouch and D. W. Kupke, *Biochemistry*, 1977, **16**, 2586.
[713] J. W. Beams and D. W. Kupke, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4430.
[714] S. Lapanje, M. Lunder, V. Vlachy, and J. Skerjanc, *Biochim. Biophys. Acta*, 1977, **491**, 482.
[715] M. A. Becker, L. J. Meyer, W. H. Huisman, C. Lazar, and W. B. Adams, *J. Biol. Chem.*, 1977, **252**, 3911.
[716] W. W.-C. Chan and K. E. Shanks, *J. Biol. Chem.*, 1977, **252**, 6163.
[717] D. Gurne, J. Chen, and D. Shemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1383.

disassembly of disulphide-linked polypeptide chains to occur.[718] IgG disassembly was inhibited by the presence of iodoacetamide unless either 6M-urea or 1% SDS was present. Steinhardt's group have reported the reversible binding isotherms of SDS with 13 different, initially native proteins.[719] Extreme variations between certain classes of proteins were found. Thus haemoglobin and myoglobin had high affinities and high binding capacities whereas IgG, apoferritin, and transferrin had low initial affinities which changed markedly at higher concentrations. No certain correlations with amino-acid content, subunit structure, hydrophobicity, or solubility were revealed, but there was a weak inverse dependence on solubilizing effectiveness on molecular size and a suggestion of a strong dependence on content of cationic groups. The presence of sugar residues is always a problem when trying to estimate subunit molecular weights. Groome *et al.*,[720] studying influenza virus neuraminidase, a dimer of two disulphide-linked units of molecular weight 33 500, considered that the high carbohydrate content (46%, w/w) explained the discrepancy between the molecular weight of the polypeptide chains obtained by different methods.

Membrane proteins also present problems of molecular weight determination. A molecular weight of *ca.* 26 000 had been suggested for bacteriorhodopsin based on composition and sedimentation equilibrium in SDS. Bacteriorhodopsin solubilized with the non-ionic detergent Triton X-100 appeared to be monomeric with a molecular weight of 24 250 ± 2000 and bound approximately one micelle of Triton X-100.[721] The binding of ionic and non-ionic detergents to cytochrome *c* oxidase has also been investigated.[722] All bind and displace part, but not all, of the phospholipid associated with the enzyme: 6—10 phospholipid molecules did not exchange. Both Triton X-100 and deoxycholate bound to the cytochrome *c* oxidase complex above their critical micelle concentration. The amount of Triton X-100 bound per complex was 180 ± 10 molecules, and of deoxycholate 80 ± 4 molecules. Bovine heart cytochrome oxidase was studied by Yu and Yu.[723] This enzyme, at a purity of 12—14 nmol haem *a* per mg protein, was shown by SDS-electrophoresis to contain seven non-identical subunits in the ratio of unity, with molecular weights 40 000, 21 000, 14 800, 13 500, 11 600, 9500, and 7600. The cytochrome b–c_1 complex was also studied by similar methods, including the analysis of the amino-acid composition of the subunits directly from Coomassie Blue-stained bands after SDS-electrophoresis.[724] The subunit molar ratios of the seven bands were 2, 2, 2, 3, 2, 2, and 5 with corresponding molecular weights 53 000, 50 000, 37 000, 30 000, 28 000, 17 000, and 15 000. Nearest-neighbour relationships of the subunits of cytochrome *c* oxidase were investigated by cross-linking with reversible cross-linking reagents followed by analysis by two-dimensional gel electrophoresis.[725] Cross-linking methods have also been

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[719] J. Steinhardt, J. R. Scott, and K. S. Birdi, *Biochemistry*, 1977, **16**, 718.
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[725] M. M. Briggs and R. A. Capaldi, *Biochemistry*, 1977, **16**, 73.

used to study the quaternary structure of *E. coli* RNA polymerase [726] and of beef liver glutamate dehydrogenase [727] and the structural arrangement of the subunits of mitochondrial ATPase.[728] Aspartate carbamylase is 'cross-linked' naturally in the sense that the two catalytic trimers are cross-linked by three regulatory dimers.[729]

Associating–Dissociating Systems.—Four systems involving the assembly of small subunits to produce macro-structures will be considered: microtubule formation, sickle-cell haemoglobin polymerization, virus assembly, and ribosome assembly. All are of great biological importance and all have been the subject of intensive research effort in recent years.

Microtubule Assembly. The protein tubulin polymerizes to form microtubules, has been found in virtually every eukaryotic cell examined for its presence, and is associated with mitosis, flagellar locomotion, and the maintenance of cell shape. Some of the recent work has been reviewed,[730, 731] but a profusion of papers continues to appear. Lee and Timasheff [732] have investigated systematically the effect of solution composition on the re-assembly of microtubules from tubulin preparations essentially devoid of other proteins. An analysis of the dependence of the apparent association constant for microtubule formation on ligand concentration by the linked-function theory of Wyman [733] suggested that the formation of a tubulin–tubulin contact involved the binding of one additional Mg^{2+} ion per tubulin dimer. Formation of microtubules was also accompanied by the apparent binding of one additional proton and the release of water molecules. The effect of temperature on polymerization has been re-investigated.[734] Temperature change is often used to initiate both microtubule formation and HbS polymerization (see below) but tubulin polymerization appears to be the more sensitive to temperature. Sutherland [734] has shown that the effect of temperature on such systems may be expressed in terms of the van't Hoff enthalpy of polymerization, but that the two ways of defining this quantity experimentally (from the critical polymerization concentration or from the fractional conversion of protein into polymer) are not identical except in certain limits. Herzog and Weber [735] have confirmed earlier reports that pure tubulin, free from the so-called microtubule-associated proteins, will indeed polymerize, but only at higher Mg^{2+} concentrations and higher protein concentrations than in the absence of the associated proteins. The basic dimer which polymerizes is a 6S, 110 000-molecular weight unit, but it is known that this itself is composed of an α- and a β-subunit, each of molecular weight 55 000.[736, 737] An investigation of the kinetics of

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[737] Y. Kobayashi and H. Mohri, *J. Mol. Biol.*, 1977, **116**, 613.

microtubule self-assembly, using a variety of techniques,[738] revealed that in the elongation reaction in mixtures of 6S tubulin and microtubule fragments, which follows the initial nucleation phase, the net rate of assembly was the sum of the rates of polymerization and depolymerization, the rate of polymerization was proportional to the product of the microtubule number concentration and the 6S tubulin concentration, and the rate of depolymerization was proportional to the number concentration of microtubules. Elongation proceeded by the consecutive association of 6S units on to the ends of existing microtubules.

In addition to studies with pure tubulin, much work has been done on the microtubule-associated proteins. Their function would seem to be to initiate polymerization and to stabilize the formed microtubules: they do not appear to be required for elongation.[739] Several purification procedures have been proposed, but much remains to be done as the amount and type of non-tubulin protein detected depends largely on the purification methods used.[740] However, it is suggested [741] that the protein 'tau' is the only protein required to define a complete assembly system. The projections seen on cytoplasmic microtubules may be removed by trypsin treatment and this results in the disappearance of the high molecular weight microtubule-associated proteins.[742]

The other broad aspect of microtubule formation is the interaction of tubulin and microtubules with small molecules. The tubulin dimer possesses two high-affinity sites for guanine nucleotides: on one, the E-site, the nucleotide is readily exchangeable but on the other, the N-site, exchange is either very slow or non-existent. Tubulin may itself possess GTPase activity,[743] and a scheme has been presented in which GTP acts as an allosteric effector at the E-site during assembly.[744] The effect of non-hydrolysable analogues of GTP has also been studied.[745] In addition to the effect of Mg^{2+} ions on assembly already mentioned, Ca^{2+} ions in contrast can inhibit the assembly of tubulin and may play a role in the regulation of microtubule formation,[746] while Zn^{2+} ions induce the formation of sheets rather than of tubules.[747] Finally, a number of alkaloids affect microtubule formation or stability. Thus griseofulvin induces aggregation of tubulin in the cold [748] and colchicine interferes with assembly by binding to a 6S dimer which then adds to the growing microtubule, aborting further polymerization.[749]

Sickle-cell Haemoglobin. Sickle-cell anaemia is associated with the aggregation of the deoxy-form of haemoglobin S (HbS) into linear arrays or fibres, causing de-

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[749] R. L. Margolis and L. Wilson, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3466.

formation of the red cells with the consequent physiological manifestations of the disease. Much effort continues to be expended in studying the mechanism of aggregation and in trying to discover anti-sickling agents of potential therapeutic value. The kinetics of deoxy-HbS polymerization were studied by transverse water proton relaxation time measurements.[750] It was shown that at concentrations of about 300 mg ml^{-1} there was a dramatic decrease in the rate of polymerization in samples consisting of 50% HbA and 50% HbS compared with samples containing only HbS. Similar studies have been performed with erythrocytes,[751] and the ligand kinetics of HbS-containing erythrocytes have also been investigated.[752] The kinetics of HbS gelation as a function of temperature, haemoglobin concentration, and solvent composition have been re-investigated by a viscometric technique,[753] and a new crystalline state of HbS has been described.[754] Minton has extended his treatment of non-ideality and the thermodynamics of HbS gelation.[755] Aggregated forms of HbS have been examined by field emission transmission electron microscopy.[756] The solubilities of HbS, HbS–HbA, and HbS–HbF (foetal) were studied by ultracentrifugation,[757] and Benesch *et al.*[758] examined the solubility of 14 hybrid haemoglobins, composed of α-chains with a single substitution and β-chains from HbS, compared with HbS itself. The results enabled regions on the surface of the molecule to be defined at which significant bonding between neighbouring tetramers takes place in the polymerization of deoxy-HbS. HbS does not form *X*-ray-quality crystals in high salt solutions, but has been shown to form high-quality crystals in polyethylene glycol.[759]

Of the numerous potential anti-sickling reagents under study may be mentioned a range of carbonyl compounds that form Schiff bases with amino-groups of intracellular haemoglobin,[760] and dibromo-aspirin,[761] all of which increase the oxygen affinity of the erythrocytes. Carbamyl phosphate also inhibits the sickling phenomenon.[762] Treatment of red blood cells with acetyl-acetimidate prevents the anoxia-induced sickling *in vivo*.[763] Finally, oligopeptides that mimic segments of the amino-acid sequence of HbS at potential contact sites can also be used to

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inhibit aggregation.[764] The effect is to raise the minimum gelling concentration of the HbS.

Virus Assembly. Information about the pathways of assembly of virus particles is of great interest in a number of ways and is being studied by many groups using a wide variety of techniques.[765] Thus it has been shown that the unit which polymerizes to form the coat of alfalfa mosaic virus is a very stable dimer of molecular weight 48 400.[766] This dimer associates to form a 30S product of molecular weight 1.48×10^6 composed of 30 dimers. This larger unit then associates within an icosahedral lattice. It has been shown that the association of dimers into the icosahedral particle is an entropy-driven process. Electron microscopy was used to study the polymerization of bacteriophage T4 core protein subunits on to baseplates.[767] When core protein subunits were allowed to react with baseplates over a wide range of initial concentration ratios the most probable core length was about 1000 Å, which is the length found *in vivo*. The baseplate is a complex structure containing at least 14 different structural proteins.[768] An osmotic shock procedure was also used to study T4 assembly.[769] Many papers have appeared dealing with the assembly of tobacco mosaic virus (TMV). During all but the last stages of assembly the virus appeared to build itself from the inside out: thus the RNA appears to insert itself beneath layers of incoming protein from within the central channel.[770] The 4S protein of TMV can polymerize to form either double chains or two-turn helices, both with a sedimentation coefficient of *ca.* 20S. Titration studies indicated that which form results at pH 7 depends upon the availability of H^+ ions.[771] The interaction of RNA with the coat protein has also been studied.[772, 773]

Ribosome Assembly. The assembly of the numerous protein subunits and RNA to form the complicated functional entities known as ribosomes continues to intrigue many workers. The assembly of at least some ribosomes is a co-operative process: for example, mitochondrial ribosome assembly requires the participation of both nuclear and mitochondrial genetic systems.[774] Work continues on the isolation and characterization of the individual ribosome proteins by standard techniques[775] and on their proximity relationships by cross-linking and other methods, such as their accessibility to iodine.[776] The role of Mg^{2+} and a number of other bivalent metal ions in the association of *E. coli* 30S and 50S ribosomal subunits was studied by kinetic and equilibrium methods, including light-

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scattering[777] and turbidity–stopped-flow.[778] In addition, the assembly of individual proteins was studied.[779, 780]

9 Fluorescence

Contributed by J. G. Hoggett

The format of this article is similar to that used in previous years. Although much of the very extensive literature on membranes falls outside the scope of this review, coverage of this area has been increased over last year, reflecting the increasing use of fluorescence in studying protein–lipid interactions, and the conformations and structures of simple proteins and multi-subunit complexes both in their solubilized and membrane-bound states.

Books and Reviews.—A book covering the physico-chemical principles and practice of fluorescence and phosphorescence spectroscopy was published in 1977.[781] A review of the use of fluorescence probes in enzymes and proteins, concentrating on covalent probes, deals with basic principles and strategy of probe design; although not intended to be comprehensive, it provides a useful compendium of many probes which have been used and presents preliminary information on some probes recently developed in Japan, details of which have not yet been published.[782] Reviews have appeared on solute quenching of protein fluorescence[783] and the application of fluorescent techniques to the study of peptides.[784] Among the newer techniques, a very timely article on circularly polarized luminescence (CPL) spectroscopy has been published, dealing with the theory, experimental procedures, and application of CPL, together with a comprehensive review of biomolecular systems covering the period since the technique was developed.[785] General articles on chlorophyll fluorescence,[786] and fluorescence relaxation and correlation spectroscopy[787] have appeared. Reviews have been published of the 1976 literature on photon counting,[788] optically detected magnetic resonance in biomolecules,[789] and fluorescence probes of membranes.[790] A collection of articles on ultrashort light pulses deals with the optics and electronics of picosecond techniques, and the applications of picosecond light pulses, some of them biological.[791] Articles or reports of symposia on related areas outside of the scope of this review cover primary processes in

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[790] W. W. Mantulin and H. J. Pownall, *Photochem. Photobiol.*, 1977, **26**, 69.
[791] 'Ultrashort Light Pulses: Picosecond Techniques and Applications', ed. S. L. Shapiro, Springer, New York, 1977.

photosynthesis,[792] photochemistry and photobiology of nucleic acids,[793] molecular mechanisms of photoreactivation,[794] solid surface fluorescence methods,[795] and microfluorimetry of product formation in single enzyme sepharose beads.[796]

Theory, Methods and Techniques.—*Technical Developments.* The two principal methods of measuring fluorescence lifetimes using pulse techniques (*i.e.* sampling and single-photon time correlation) can be combined to yield a hybrid method which combines the simplicity and cheapness of sampling techniques with the sensitivity of time correlation measurements. An apparatus has been recently described[797] which extends the time resolution to the sub-nanosecond range from the microsecond[798] and nanosecond[799] ranges of earlier instruments; the accuracy claimed for the range 4 ns—50 ps was ± 6% or 10 ps whichever was greater. A new approach to measurements on the picosecond time scale employs a high speed streak camera coupled with image convertors and intensifiers;[800] data can be accumulated by digitizing the streaked image with an optical multi-channel analyser. An application of the method and comparison with alternative methods of measuring fluorescence lifetimes have been reported.[801] Fluorescence lifetime measurements can be made either by pulse-decay or by phase-shift techniques; the two methods are formally equivalent if the dependence of the phase-shift on the modulation frequency is determined (the amplitude time data being related to the phase-frequency by Fourier transformation). Measurement of sub-nanosecond decay times involves the use of modulation frequencies in the RF range; however, the main problem has been that the modulators either have involved the use of very high voltages which caused interference in the detection system, or have been insufficiently flexible in their frequency responses. These problems have been overcome using a laser light source with crystal electro-optic modulators;[802] the technique was tested measuring the lifetime of fluorescein quenched with increasing concentrations of KI. An apparatus has been described for rapid scanning of the excitation and emission wavelengths using a video fluorometer.[803] Quantitative analysis of the data (acquired as an excitation–emission matrix) from samples containing multiple components shows that it is possible to determine both the number of independent components contributing to the emission and their spectra, provided that only a small number of components are present.[804] Accounts have appeared of instruments for

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measuring reaction rates,[805] fluorescence polarization anisotropy by single-photon counting,[806] and differential fluorescence spectra;[807] the particular virtue of the latter is that it involves a single beam optical system with special hemi-cylindrical cells rotated at 25 Hz, so that the system can be fitted to conventional spectrofluorimeters with relatively little modification. A useful summary of the well-known problem of correcting fluorescence measurements for the absorption of excitation radiation has appeared, together with suggestions for using primary and secondary optical masks to facilitate this.[808] Designs for fibre optic fluorescence flow cells[809] and rapidly thermostatable ($\sim$0.5 s) fluorescence microcells (0.1—0.15 cm^3) using front face emission have been published.[810]

Newer Methods. The general principle of optoacoustic spectroscopy (OAS) is that if a sample absorbs intensity-modulated e.m. radiation, then upon de-excitation, the absorbed energy appears as heat which causes a periodic pressure rise in the gas surrounding the sample, which can be detected by a sensitive microphone transducer in the cell. Although OAS is likely to be particularly useful in studying optically dense biological material whose properties preclude easy measurement of absorption by conventional methods, it has also been used to obtain values of the absolute fluorescence quantum yields of quinine[811] and rhodamine *b*.[812] For quinine (10^{-3}—10^{-2} mol dm^{-3} in 0.1 N-H_2SO_4) a value of 0.53 $\pm$ 0.02 was obtained, in good agreement with the accepted value of 0.51.[813] Fluorescence and CPL were two of a range of physico-chemical techniques used to investigate the Ca binding site of trypsin probed by lanthanides (particularly Tb^{III}).[814] The CPL spectrum, which is very sensitive to the precise environment of the metal ion binding-site, was consistent with the idea that the metal binding-site comprised two carboxylate residues (Glu 70 and Glu 80) acting as bidentate ligands. A tryptophan residue (Trp 141) in the vicinity (7 Å) is involved in energy transfer with the bound Tb^{III}. Experiments in which luminescent lanthanides have been used as probes replacing Ca^{II} in proteins have usually depended on the enhancement of luminescence, primarily by energy transfer from tryptophan. A new approach to the use of lanthanides, which does not depend on the occurrence of energy transfer, involves use of laser excitation of the metal ion and measurement of the luminescence decay, which is very sensitive to the co-ordination state of the metal.[815] The applicability of the method has been demonstrated in a study of the binding of Eu^{III} and Tb^{III} at a Ca^{II} site on thermolysin. Results suggest that about two water molecules co-ordinate to the metal when bound.

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Fluorescence Lifetimes. A well-established difficulty facing the determination of fluorescence lifetimes from pulse measurements, is that certain instrumental factors must be taken into account in the deconvolution procedure. A particular difficulty arises from the fact that the time response of the photomultiplier tube varies with λ, hence the instrumental response functions at λ_{ex} and λ_{em} are not equal. Last year's report [816] referred to a method [817] of determining the response functions from the fluorescence decay profiles of a correction compound at λ_{ex} and λ_{em} when excited at $\lambda_a < \lambda_{ex}$. The problem in the u.v. region is that there are few suitable correction compounds with a broad fluorescence spectrum. An alternative procedure has been described in which correction compounds whose absorbance spans λ_{ex} and λ_{em} are used, and the fluorescence is followed at $\lambda_a > \lambda_{em}$.[818] The importance of taking instrumental factors into account is illustrated by the case of human serum albumin: the corrected decay curves yield a best fit to a bi-exponential decay with $\tau_1 = 5.26$ ns and $\tau_2 = 1.64$ ns; corresponding values for the uncorrected curve were $\tau_1 = 8.44$ ns and $\tau_2 = 2.22$ ns, and in addition the non-random residuals to this fit would suggest that the decay could not adequately be described by two exponential functions. Various methods have been used to judge the adequacy of fit of particular functions to the decay data; it has recently been suggested that use of a covariance ellipsoid is the most satisfactory approach.[819]

In an important paper, Brand and his co-workers have investigated the excited state interactions of *N*-(*p*-tolyl)-2-aminonaphthalene-6-sulphonate (TNS) bound to apomyoglobin, using nanosecond time-resolved emission spectroscopy (TRES).[820] This is apparently the first time that TRE spectra have been obtained for a protein–dye complex. The study was particularly concerned with excited state interactions taking place on the nanosecond time scale, in an attempt to understand better the environmental sensitivity of the fluorescence of *N*-acylaminonaphthalenes. The most significant finding was of a substantial red-shift in the emission maximum with time, the most likely explanation of which was that excited state reactions resulted in emission from a state of lower energy. Although these phenomena were qualitatively similar to those shown by TNS in a viscous solvent like glycerol, the authors conclude that the characteristics of a biological binding site cannot be adequately characterized in terms of a single parameter. TRES does, however, provide a kinetic description of the interactions occurring between the fluorescent group and the protein residues and water molecules comprising its immediate environment.

The decay of fluorescence anisotropy has been used to investigate the flexibility of myosin either covalently labelled with dansyl chloride or *N*-iodoacetyl-*N'*-(1-sulphonyl-5-naphthyl)ethylenediamine (1,5-AEDANS), or bound non-covalently to ANS.[821] The unusual feature of the results was the observation of an increasing anisotropy with time, corresponding to negative rotational

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[820] A. Gafni, R. P. DeToma, R. E. Marrow, and L. Brand, *Biophys. J.*, 1977, **17**, 155.
[821] S. C. Harvey and H. C. Cheung, *Biochemistry*, 1977, **16**, 5181.

correlation times for the dansyl- and ANS-labelled myosin. This observed behaviour is consistent with that expected from a rigid (or nearly rigid) rod with the fluorescent group oriented so that the absorption and emission dipoles are on opposite sides of the long axis of the molecule. Since the rotational diffusion of myosin about its axis is much more rapid (~few hundred ns) than end-over-end diffusion (~30 μsec), fluorescence decay measurements with short lifetime dyes (< *ca.* 20 nsec) respond only to the former rotational diffusion which effectively exchanges the positions of the emission and absorption dipoles causing an increased alignment of the emission dipoles along the axis of polarization. The overall picture emerging from these results is of myosin having a hinge region which is freely flexible at pH 4 but which shows considerable resistance to bending at pH 8. Although the decay of fluorescence anisotropy can yield valuable information about the motions of macromolecules in solution, approximate equalities and linear relationships between the five decay terms in the general equation [822] reduce the number of independent times, for all practical purposes, to two, which is insufficient to determine the three axes of the general ellipsoid. The possibility of combining anisotropy data with the additional information provided by the linear diffusion constant has been explored recently;[823] the authors conclude that caution should be exercised in drawing conclusions about the size and shape of macromolecules from anisotropy data alone. It was reported last year [816, 824, 825] that the fluorescence decays of NADH bound to glutamate and alcohol dehydrogenases (both oligomeric enzymes exhibiting allosteric properties) did not follow simple exponential time courses. These studies have been extended to the binding of NADH to octopine dehydrogenase, a simple monomeric enzyme with no allosteric properties (but with a claimed regulatory mechanism of a 'memory' type).[826] Binary and ternary complexes with a range of substrates and analogues all exhibited bi-exponential fluorescence decays, attributed to a heterogeneous environment in the excited state (whether this heterogeneity is also present in the ground state was unclear, but if it were the existence of more than one conformation might be involved in the 'memory' type of regulatory properties of this enzyme). The authors of this work [826] dispute the explanation that the bi-exponential decay of the NADH–alcohol dehydrogenase complex is due to a reversible excited state reaction which transforms a fluorescent residue to a non-fluorescent product;[825] they argue that this cannot lead to bi-exponential decay, since in order to be non-fluorescent the product must have a fast rate of decay, and consequently the rate of exchange between the two forms in the excited state would be large compared with the rate of deactivation of the fluorescent product. A theory has been devised to describe the decay of fluorescence polarization of groups embedded in a membrane which exhibit wobbling motions rather than free rotation; such motions are characterized by two constants: a wobbling diffusion constant and a degree of

[822] G. R. Penzer, in this series, 1974, vol. 5, p. 203.
[823] E. W. Small and I. Isenberg, *Biopolymers*, 1977, **16**, 1907.
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[826] J. C. Brochon, P. Wahl, M. O. Monneuse-Doublet, and A. Olomucki, *Biochemistry*, 1977, **16**, 4594.

orientational restraint.[827] The theory is not restricted to membranes; other applications include the internal motion of a residue within a macromolecule, or flexing motions in fibrous structures. The authors point out that wobbling rather than free rotation is quite common because certain orientations are favoured by the surrounding structure.

Energy Transfer. The accepted R_0 value for energy transfer from Tyr → Trp in an aqueous environment (14.0 or 14.8 Å, depending on whether the quantum yield of Tyr is taken to be 0.15 or 0.21)[828] was measured using the model system Trp.$(Gly)_n$.Tyr (n = 0, 2, 4).[829] However, glycine does not form α-helices and a number of conformations are available to such unhindered peptides. Attempts to overcome this uncertainty by measurements on Trp.$(Pro)_n$.Tyr (n = 1—5) were frustrated by the fact that the proline peptides existed as a mixture of *cis* and *trans* rotamers;[830] the authors conclude that in aqueous solution (although not apparently in EtOH)[831] proline is a poor spacer for energy transfer studies.

Claims that conformational changes accompany the binding of ligands to proteins are not always supported by decisive evidence or arguments. Koshland has described a method and illustrated its use in the galactose receptor of *Salmonella typhimurium* which, even within the limitations inherent in the analysis of energy transfer data, can provide convincing evidence of a conformational change.[832] The principle of the method is to select two suitable fluorescent reporter groups (either extrinsic or intrinsic), demonstrate by energy transfer that the minimum separation of the groups is much larger than the dimensions of the ligand, and show that the fluorescent properties of both groups respond in some way to the binding of ligand; since the ligand cannot physically interact with both groups then a conformational change must be involved. In the case of the galactose receptor the separation of the single Trp and a 5-iodacetamide fluorescein label was found to be in the range 32—52 Å using the acceptable limits to the value of the orientation factor κ^2 determined from the polarization spectrum. Binding of galactose altered the sensitivity of both fluorescent groups to quenching by KI; hence by the above argument conformational change must have occurred. The importance of the orientation factor κ^2 is illustrated in a study of the binding of ANS to D-amino acid oxidase.[833] When ANS binds to the holoenzyme, energy transfer between the dye and the coenzyme FAD is not observed, whereas it is observed if FAD is added to the dye–apoenzyme complex. However, in both cases the dye competes with the substrate D-alanine, and the authors conclude that it must bind in the same region close to the FAD binding-site irrespective of the order of binding; they suggest that the mode of binding is slightly different in the two cases and that in the former $\kappa^2 \sim 0$. Further evidence that the conducting transmembrane channel of gramicidin is a dimer and not a higher oligomer comes from the observation of energy transfer between dansyl-gramicidin C and *p*-phenylazo-

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[832] R. S. Zukin, P. R. Hartig, and D. E. Koshland, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1932.
[833] K. Yagi, F. Tanaka, N. Ohishi, and M. Morita, *Biochim. Biophys. Acta*, 1977, **492**, 112.

benzene-sulphonyl-(PABS) or 4-(diethylamino)-phenylazobenzene-4-sulphonyl-(DPBS) gramicidin (all labelled at Tyr 11).[834] The extent of quenching of the dansyl donor group (a measure of the amount of hybrid oligomer) varies linearly with the proportion of added acceptor gramicidin pointing to the formation of dimers; dependences on higher powers of that proportion would be expected for higher oligomers. In designing such experiments it is important to ensure that the surface density of fluorescent groups is sufficiently low that quenching can unambiguously be attributed to the formation of hybrid species (in this case the average separation >250 Å).[834] Equations have been developed for several 2- and 3-dimensional configurations of donors and acceptors on or near spherical membranes to enable calculation of the distance across membranes from energy transfer measurements; in general the curvature of cell surfaces can be neglected except for small vesicles.[835] A related approach was used to investigate the interaction of haemoglobin with red cell membranes.[836] Fluorescence energy transfer was observed between 12-(9-anthroyl) stearic acid embedded in the membrane and haemoglobin as quencher; the method depends on the fact that transfer only takes place to haemoglobin bound to the membrane (R_0 = 46 Å) and not to free molecules trapped within the ghost cells. In this connection, it is opportune to report here that a selection of naturally occurring conjugated polyene fatty acids (*e.g. cis*-parinaric acid) have properties which make them especially suitable probes for studies of membrane structure, and particularly of lipid–protein interactions.[837–841] Their fluorescence spectra should enable energy transfer to be observed [840] and, most importantly, they are biosynthetically incorporated into the membranes of *E. coli* [839] and cultured mammalian cells [841] into fairly well-defined locations with little, if any, perturbing effect on the physical properties of the membranes.

Radiationless energy transfer between Tb^{III} and Fe^{III} bound to the two metal sites on human transferrin leads to the conclusion that the two sites are separated by 25 ± 2 Å,[842] a result at variance with an earlier claim that the fluorescence of Tb^{III} was insensitive to the addition of Fe^{III}.[843] The discrepancy apparently resides in the procedure used in the earlier work to prepare the Fe^{III} species from apo-transferrin. The details have appeared of the use of bifunctional chelating reagents covalently linked to proteins in extending energy transfer measurements between two metal ions to systems containing natively one metal site.[844]

Fluorescence Probes.—*Covalent Labels.* A selection of some of the applications of covalent probes is collected in Table 2. Although *N*-dansylaziridine generally has a high specificity for sulphydryl groups,[845] reaction with other residues can

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[842] C. F. Meares and J. E. Ledbetter, *Biochemistry*, 1977, **16**, 5178.
[843] C. K. Luk, *Biochemistry*, 1971, **10**, 2838.
[844] C. S. H. Leung and C. F. Meares, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 149.
[845] W. H. Scouten, R. Lubcher, and W. Baughman, *Biochim. Biophys. Acta*, 1974, **336**, 421.

occur; thus bovine and human serum albumin (which each have only one free sulphydryl group) incorporate about three covalently bound dansyl labels per molecule as a result of the unusually high nucleophilicity of certain groups in the binding sites of the proteins.[846] The synthesis and characterization of some new probes have been reported.[847–849] Fluorescence depolarization has been used to investigate the stabilization of enzymes resulting from their incorporation on to solid supports or into gels.[850] The rotational relaxation time of dansyl-labelled chymotrypsin in polymethacrylate gels shows an abrupt inhibition of rotation of the enzyme at a threshold concentration of gel of 40%, which cannot be explained by the increase in microviscosity of the gel; it is concluded that the immobilization is due to multipoint interactions which occur in a co-operative manner. The polarization of dansyl-labelled α_2-macroglobulin points to the existence of structural domains within the dimer and a high degree of flexibility

Table 2 *Covalent probes and their applications**

Probe	*Application*	*Ref.*
N-[*p*-(2-Benzoxazolyl) phenyl]-maleimide	pyruvate dehydrogenase (E)	857
7-[Chloro-4-nitrobenzo-2-oxa-1,3-diazole] (NBD-Cl)	phosphofructokinase (E)	855
	6-phosphogluconate dehydrogenase	*a*
Dansyl	A-I apolipoproteins (P)	*b*
	$(Na^+ + K^+)$ATPase (P)	*c*
	bradykinin (E)	*d*
	chymotrypsin (P)	850
	gramicidin C (E)	834
	α_2-macroglobulin (P)	851
	myosin (P)	821
Dansylaziridine	bovine and human serum albumin	846
5′(*N*-Dansyl)cadaveryl-*p*-carboxy-methylpargyline	monoamine oxidase	*e*
Dibromofluorescein-isothiocyanate	ribosomes	*f*
N-(4-Dimethylamino-3,5-dichlorophenyl)-maleimide	pyruvate dehydrogenase (E)	857
4-Dimethylamino-4′-maleimido-stilbene	phosphofructokinase (E)	855
	pyruvate dehydrogenase (E)	857
N-(7-Dimethylamino-4-methyl-3-coumarinyl)-maleimide	thiol reagent (N)	848, 849
Fluorescamine	RNA polymerase (E)	861
Fluorescein	blood-clotting factor XIII (A)	854
Fluorescein mercuriacetate	thiol reagent	*g*
5-Iodoacetamido-fluorescein	galactose receptor (P)	832
N-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulphonate	glycogen phosphorylase *b* (P)	852

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[847] H. Khalaf and M. Rimpler, *Z. physiol. Chem.*, 1977, **358**, 505.
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Table 2 (*cont.*)

Probe	*Application*	*Ref.*
N-Iodoacetyl-*N'*-(1-sulphonyl-5-naphthyl) ethylene diamine (1,5-AEDANS)	muscle fibres (P)	853
	myosin (P)	821
1,5-AEDANS and 2,6 isomer	cytochrome *c* oxidase (E)	859
5-Isothiocyanato-1,8-naphthalene dicarbox-4-methylphenylimide	amino group reagent (N)	847
4(5)-(*N*-Maleinisoimido) rhodamine b	haemoglobin (P)	*h*
β-Naphthoquinone-4-sulphonate	myosin (L)	*i*
p-Phenylazobenzene-sulphonyl and 4-diethylamino derivative	gramicidin C (E)	834
N-Pyrene maleimide	chloroplast-coupling factor I (E)	860
	pyruvate dehydrogenase (E)	858
	ribosomes	*f*
Quinacrine	acetylcholine receptor (L)	*j*

* Some applications are characterized into the following classes: (A) protein association, (E) energy transfer, (L) ligand binding, (N) new probe, (P) polarization methods.

(*a*) A. Fontana, L. Mantovanelli, E. Bocca, and F. M. Veronese, *Int. J. Peptide and Protein Res.*, 1977, **9**, 329. (*b*) A. Jonas and J. J. Kajnovich, *J. Biol. Chem.*, 1977, **252**, 2194; A. Jonas, D. J. Kajnovich, and B. W. Patterson, *ibid.*, p. 2200; A. Jonas, S. M. Drengler, and A. M. Scanu, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 1424. (*c*) W. E. Harris and W. L. Stahl, *Biochim. Biophys. Acta*, 1977, **485**, 203. (*d*) E. S. Efromov, M. P. Filatova, T. O. Reutova, L. N. Stepanova, Z. Reissmann, and V. T. Ivanov, *Bioorg. Khimiya*, 1977, **3**, 1169. (*e*) R. R. Rando, *Mol. Pharmacol.*, 1977, **13**, 726. (*f*) D. Lavalette, B. Amand, and F. Pochon, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1407. (*g*) S. Takeuchi and A. Maeda, *J. Biochem.*, 1977, **81**, 971. (*h*) E. Y. Alfimova and B. O. Glotov, *Studia Biophysica*, 1977, **62**, 209. (*i*) A. Mahlrad, *Biochim. Biophys. Acta*, 1977, **490**, 79. (*j*) H. H. Grünhagen, M. Iwatsubo, and J. P. Changeux, *European J. Biochem.*, 1977, **80**, 225.

between these domains.[851] Fluorescence was only one of a range of techniques used to investigate the role of pyridoxal 5'-phosphate which is not involved in the chemical catalysis but is essential for the activity of glycogen phosphorylase *b*.[852]

Removal of pyridoxal 5'-phosphate resulted in structural changes which although limited in extent appeared to involve the reactive sulphhydryl group which had been labelled with *N*-(iodoacetylaminoethyl)-5-sulphonic acid (1,5-IAEDANS). Single, skinned, glycerinated muscle fibres from rabbit psoas have been labelled with 1,5-IAEDANS, reaction occurring almost exclusively on the reactive cysteine on SF1 of myosin.[853] Analysis of the polarization of fluorescence of these fibres, treated as a helical array of fluorophores, leads to the conclusion that the angle between the fibre axis and the direction of the emission dipole of 1,5-IAEDANS attached to SF1 is about 40°. The association–dissociation behaviour of factor XIII (a blood clotting factor) covalently labelled with fluorescein has been investigated by following the change in fluorescence polarization.[854] A point of general value from this study is the use of fluorescence methods to bridge the gap between high concentrations, where the molecular weights and dissociation behaviour can easily be characterized by physical

[851] J. P. Frenoy, R. Bourrillon, R. Lippoldt, and H. Edelhoch, *J. Biol. Chem.*, 1977, **252**, 1129.
[852] R. F. Steiner, L. Greer, and C. Gunther, *Biochim. Biophys. Acta*, 1977, **494**, 233.
[853] J. Borejdo and S. Putnam, *Biochim. Biophys. Acta*, 1978, **459**, 578.
[854] J. J. Holbrook, B. A. Lewis, J. M. Freyssinet, P. E. Bock, and J. D. Shore, *Trans. Biochem. Soc.*, 1977, **5**, 1247.

methods, and low concentration where information about the state of the protein is most physiologically interesting.

Several studies have appeared of the use of energy transfer between covalently labelled probes (or occasionally also using non-covalent but specifically bound probes) for mapping particular regions of multisubunit enzymes; systems investigated include phosphofructokinase,[855] aspartokinase,[856] pyruvate dehydrogenase complex.[857, 858] cytochrome *c* oxidase,[859] and chloroplast coupling factor I.[860] The use of fluorescence polarization to set limits to the value of κ^2, and hence the separation,[816] is increasing but not universal. Even when energy transfer is not observed because the separation of the groups is too large this information can be valuable. For example, the fact that the separation of the lipoic acid and the active sites on the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase enzymes is >40 Å is not consistent with a mechanism in which a single lipoic acid arm rotates between the catalytic sites.[858] Membrane-bound multienzyme systems, even when they can be obtained in a solubilized form, are not generally amenable to crystallographic study, and the fluorescence mapping approach is likely to be particularly important in providing information about the arrangement of the subunits in the membrane. The authors of the work on the solubilized chloroplast coupling factor I indicate their intention of extending their investigations to the structure of the membrane-bound form.[860] The binding of RNA polymerase to DNA has been studied by observing the energy transfer between fluorescamine covalently linked to the enzyme and ethidium bromide intercalated with the DNA.[861]

Non-covalent Probes. Table 3 summarizes some of the studies using non-covalent probes; for discussion they are divided according to whether or not they show some biological specificity.

Specific Probes. Theoretical calculations of the ground and excited states of 1,N^6-ethenoadenosine and the Y base of t-RNA^{Phe} indicate that their fluorescence properties should resemble those of the indolizines rather than purine.[862] Two formycin anhydronucleosides have been synthesized as models of formycin in *syn* and *anti* conformations; both have a stronger fluorescence emission than formycin and there is a bathochromic shift of their emission maxima.[863] The applications of *lin*-benzoadenosines, published in early 1977, were discussed in last year's report.[816] Other new probes (Table 3) have been designed for use with ribosomes[864] oestrogen-binding proteins,[865] choline acetyltransferase,[866] the glycoside receptor of (Na^+ + K^+)ATPase[867] and myosin.[868]

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[865] Y. J. Lee, A. C. Notides, Y. G. Tsay, and A. S. Kende, *Biochemistry*, 1977, **16**, 2896.
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[867] P. A. G. Fortes, *Biochemistry*, 1977, **16**, 531.
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Table 3 *Non-covalent fluorescent probes and their applications*

Probe	*Application*	*Ref.*
3-Aminopyridine-adenine dinucleotide	glyceraldehyde-3-phosphate dehydrogenase	875
ANS	D-amino-acid oxidase	833
	aminopeptidase I	*a*
	$(Na^+ + K^+)$ATPase	*b*
	choline actyltransferase	866
	glycogen phosphorylase *b*	852
	human glycophorin	*c*
	human serum albumin and fragments	*d*
	human serum pre-albumin, myosin	821, *e*
	phospholipase A_2	*f*
	polylysine and polyarginine	*g*
	pyruvate dehydrogenase	858
	ribulose-1,5-bisphosphate carboxylase	*h*
12(9-Anthroyl) stearic acid	membrane–protein interactions	836
Anthroyloubain	$(Na^+ + K^+)$ATPase	867
Auramine O	alcohol dehydrogenases	882
Aurovertin	mitochondrial ATPase	*i*
4,4′-Bis ANS	myosin	868
O-Carboxymethyl-4-methyl-umbelliferone	antibody–hapten binding	*j*
Chlorpromazine	plasma amine oxidase	*k*
Coumestrol	oestrogen receptor	865
Dansylaminoalkylthio galacto-pyranosides	*lac*-carrier protein	871
Dansylaminohexylthio galacto-pyranoside	*lac*-carrier protein	872
Dansylnorhexestrol	oestrogen receptor	865
1,N^6-Etheno ATP	aspartokinase	856
	chloroplast-coupling factor	*l*
1,N^6-Etheno ADP	F-Actin, myosin subfragment-1	*m*, 869
	chloroplast-coupling factor	*l*
1,N^6-Etheno CoA	choline acetyl transferase	866
Eu^{III}	thermolysin	815
FAD	D-amino-acid oxidase	833
	glucose oxidase	*n*
	pyruvate dehydrogenase	858
Flavinyl peptides	flavodoxins	*o*
Fluorescein-labelled erythromycins	ribosomes	*p*
Fluorescein-labelled oestradiol	receptor binding	*q*
Fluorescein thiocarbamyl t-RNA_{Tyr}	synthesis and characterization	*r*
Kynuramine	plasma amino oxidase	*s*
N-Methylacridium	acetylcholinesterase	*t*
N-Methylanilino-2-naphthalene-sulphonyl BSA	antibody–hapten binding	*u*
1-Methyl-7-hydroxy-quinolinium	acetylcholinesterase	*t*
4-Methylumbelliferyl-α-D-manno-pyranoside	concanavalin A	870
NAD^+/NADH	liver alcohol dehydrogenase	*v*
	liver aldehyde dehydrogenase	*w*
NADH	citrate synthase	*x*
	phosphoglycerate dehydrogenase	*y*
	UDP-galactose-4-epimerase	*z*

Table 3 (*cont.*)

Probe	*Application*	*Ref.*
NBD-norhexestrol	oestrogen receptor	865
Nitrobenzoxadiazole-alanine	antibody–hapten binding	*aa*
Polyene fatty acids	bovine serum albumin	840
Pyrene butyrate	antibody–hapten binding	*bb*
Pyrene butyric acid hydrazide linked to streptomycin	ribosomes	864
Quinacrine (and other agonists)	acetylcholine receptor	*cc*
Tb^{III}	thermolysin	815
	transferrin	842
	trypsin	814
Tetracyclin	ribosomes	*dd*
Tetramethylenerhodamine labelled α-bungarotoxin	characterization	*ee*
TNS	apomyoglobin	820
	glutamine synthetase	*ff*
	hydroxysteroid oxidoreductases	*gg*

(*a*) D. A. Deranleau and H. Zuber, *Int. J. Peptide and Protein Res.*, 1977, **9**, 258. (*b*) J. F. Halsey, D. B. Mountcastle, C. A. Takeguchi, R. L. Biltonen, and G. E. Lindenmayer, *Biochemistry*, 1977, **16**, 432. (*c*) J. A. Verpoorte, *Canad. J. Biochem.*, 1977, **55**, 942. (*d*) M. J. Geisow and G. H. Beaven, *Biochem. J.*, 1977, **163**, 477. (*e*) S. Y. Cheng, R. A. Pages, H. A. Saroff, H. Edelhoch, and J. Robbins, *Biochemistry*, 1977, **16**, 3707. (*f*) A. D. Purdon, D. O. Tinker, and L. Spero, *Canad. J. Biochem.*, 1977, **55**, 205. (*g*) S. Ochimura and M. Zama, *Biopolymers*, 1977, **16**, 1449. (*h*) R. Challet and L. L. Anderson, *Biochim. Biophys. Acta*, 1977, **482**, 228. (*i*) M. G. Douglas, Y. Koh, M. E. Dokter, and G. Schatz, *J. Biol. Chem.*, 1977, **252**, 8333. (*j*) B. M. Harina, A. A. Bothner-By, and T. J. Gill, *Biochemistry*, 1977, **16**, 4504. (*k*) J. B. Massey and J. E. Churchich, *Biochim. Biophys. Acta*, 1977, **480**, 70. (*l*) D. L. Vandermeulen and Govindjee, *European J. Biochem.*, 1977, **78**, 585. (*m*) S. C. Harvey, H. C. Cheung, and K. E. Thames, *Arch. Biochem. Biophys.*, 1977, **179**, 391. (*n*) B. Solomon and M. Shinitzky, *Biopolymers*, 1977, **16**, 1853. (*o*) H. J. W. G. Visser, T. M. Li, H. G. Drickamer, and G. Weber, *Biochemistry*, 1977, **16**, 4879, 4883. (*p*) R. Langlois, C. R. Cantor, R. Vince, and S. Pestka, *Biochemistry*, 1977, **16**, 2349; R. Langlois, C. C. Lee, C. R. Cantor, B. Vince, and R. Pestka, *J. Mol. Biol.*, 1977, **106**, 297. (*q*) W. B. Dandliker, A. N. Hicks, S. A. Levison, and R. J. Brown., *Biochem. Biophys. Res. Comm.*, 1977, **74**, 538. (*r*) A. Pingoud, R. Kownatski, and G. Maass, *Nuc. Acids Res.*, 1977, **4**, 327. (*s*) J. B. Massey and J. E. Churchich, *J. Biol. Chem.*, 1977, **252**, 8081. (*t*) T. L. Rosenberry and E. Neumann, *Biochemistry*, 1977, **16**, 3870. (*u*) M. Onodera and H. Shiokawa, *J. Biochem.*, 1977, **81**, 891. (*v*) M. C. DeTraglia, J. Schmidt, M. F. Dunn, and J. T. McFarland, *J. Biol. Chem.*, 1977, **252**, 3493. (*w*) A. K. H. McGibbon, P. D. Bukley, and L. F. Blackwell, *Biochem. J.*, 1977, **165**, 455; A. K. H. McGibbon, L. F. Blackwell, and P. D. Bukley, *ibid.*, **167**, 469. (*x*) M. H. Sadar, A. Queen, and H. W. Duckworth, *Canad. J. Biochem.*, 1977, **55**, 714. (*y*) R. Dubrow and L. I. Pizer, *Biochim. Biophys. Acta*, 1977, **484**, 244. (*z*) S. S. Wong and P. A. Frey, *Biochemistry*, 1977, **16**, 298. (*aa*) D. Lancet and I. Pecht, *Biochemistry*, 1977, **16**, 5150. (*bb*) C. Lovejoy, D. A. Holowka, and R. E. Cathou, *Biochemistry*, 1977, **16**, 3668. (*cc*) T. Heidmann, M. Iwatsubo, and J. P. Changeux, *Compt. rend.*, 1977, **284D**, 771. (*dd*) T. R. Tritton, *Biochemistry*, 1977, **16**, 4133. (*ee*) P. Ravdin and D. Axelrod, *Analyt. Biochem.*, 1977, **80**, 585. (*ff*) F. C. Wedler, B. A. Willis, and R. Srubas, *Experientia*, 1977, **33**, 1016. (*gg*) E. S. Szymanski and C. S. Furfine, *J. Biol. Chem.*, 1977, **252**, 205.

The fluorescence polarization of 1,N^6-etheno ADP bound to F-actin oriented in the field of flow shows that the emission dipole is nearly perpendicular to the long axis of F-actin.[869] Combining this observation with studies of the linear-dichroism and excitation polarization spectrum suggests that the etheno-adenine plane is perpendicular to the long axis. Several papers have appeared on the binding of 4-methylumbelliferyl mannopyranoside to dimeric and tetrameric

[869] M. Mike and K. Mihashi, *Biophys. Chem.*, 1977, **6**, 101.

concanavalin A;[870] equilibrium and fast kinetics results suggest that there is no detectable interaction between the sites in either of the protein forms. Fluorescent galactosidases have been used as probes of the *lac* carrier protein [871, 872] and the subject has recently been reviewed.[873] As the length of the methylene chain of a homologous series of (*N*-dansyl) aminoalkyl-1-thio-β-D-galactopyranosides (containing 2—6 methylene groups) was increased, the fluorescence of the dansyl residue first decreased by a factor of 10 (with a chain length of 4) and then increased again to its original intensity; in addition, the ability of *N*-methylpicolinium to quench the fluorescence was at a maximum with a chain length of four methylene groups, falling off rapidly as the chain was either lengthened or shortened.[871] The most reasonable explanation of the results is that with short chains the dansyl group experiences the hydrophobic environment around the carrier protein, and as the chain is extended the group is exposed to the aqueous environment, but as the chain is lengthened further the dansyl residue is reabsorbed into the hydrophobic region of the membrane. If this picture is correct then this would suggest that the sugar binding-site of the *lac* carrier protein is 5—6 Å from the membrane solvent interface. The binding of DG_6 (the aminohexyl derivative referred to above) to the *lac* protein in inverted vesicles of *E. coli* was dependent upon either efflux of internal lactose or the creation of a membrane potential.[872] Binding was similar to that previously observed with 'right side out' vesicles [874] (except that added ATP inhibited binding in the current case—confirming that the vesicles were inverted), leading the authors to conclude that the *lac* carrier protein behaves symmetrically with respect to binding of substrate analogues to the internal and external surfaces of the membrane.

The binding of 3-aminopyridine-adenine dinucleotide (AAD^+) (an NAD^+ analogue fluorescent at the pyridine moiety of the molecule) to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase supports the idea that the chief function of the pyridine ring (apart from its catalytic role) is to anchor the co-enzyme, leaving the adenine to produce the conformational changes required to generate negative co-operativity.[875] The interaction of ribosomes with yeast $t\text{-}RNA^{Phe}$, in which bases in the anticodon and dihydrouridine loops had been replaced by ethidium bromide, has been investigated using fluorescence and fluorescence polarization.[876] In the absence of codon–anticodon interactions there was considerable internal mobility of the t-RNA; addition of poly-U resulted in immobilization of both the anticodon and dihydrouridine loops, possibly involving for the latter, unfolding of the tertiary structure of the t-RNA and more direct interaction with the ribosome.

[870] F. G. Loontiens, R. M. Clegg, and T. M. Jovin, *Biochemistry*, 1977, **16**, 159; R. M. Clegg, F. G. Loontiens, and T. M. Jovin, *ibid.*, p. 167; F. G. Loontiens, R. M. Clegg, A. van Landschoot, and T. M. Jovin, *European J. Biochem.*, 1977, **78**, 465; F. G. Loontiens, R. M. Clegg, and T. M. Jovin, *Arch. int. Physiol. Biochem.*, 1977, **85**, 184; A. van Landschoot, R. M. Clegg, F. G. Loontiens, and T. M. Jovin, *ibid.*, p. 203.

[871] S. Schuldiner, R. Weill, D. E. Robertson, and H. R. Kaback, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1851.

[872] J. R. Lancaster and P. C. Hinkle, *J. Biol. Chem.*, 1977, **252**, 7662.

[873] S. Schuldiner and H. R. Kaback, *Biochim. Biophys. Acta*, 1977, **472**, 399.

[874] S. Schuldiner, G. K. Kerwar, H. R. Kaback, and R. Weil, *J. Biol. Chem.*, 1975, **250**, 1361.

[875] Y. I. Henis and A. Levitski, *J. Mol. Biol.*, 1977, **117**, 699.

[876] J. M. Robertson, M. Kahar, W. Wintermeyer, and H. Zachau, *European J. Biochem.*, 1977, **72**, 117.

Non-specific Probes. The environmental sensitivity of the fluorescence of ANS and related compounds continues to be the subject of investigation. A study of the depolarization of ANS fluorescence in glycerol–water mixtures supports the view that the fluorescent form is nearly planar, and stabilized by weak hydrogen bonds between the N—H and sulphonate groups which can be disrupted by increasing temperature and water content of the solvent.[877] The suggestion [878] that the greater environmental sensitivity of *N*-substituted arylaminonaphthalene-sulphonamides compared with the corresponding sulphonates was due to some specific solvent polarity effects has not been substantiated by recent work. This shows the sulphonamides to exhibit the same intramolecular charge transfer behaviour as the sulphonates and to have fluorescence properties consistent with the general scheme for ANS excited state processes.[879] Intense irradiation of a glycerol solution of *N*-alkyl-2-phenylaminonaphthalene-6-sulphonates leads to the production of a highly fluorescent photoproduct from a protonation reaction of the vibrationally excited molecule;[880] this product (λ_{em} = 405 nm) can mask or confuse the desired spectrum. Probes such as ANS are often used to determine the capacity of proteins bound to membranes to bind fluorophoric groups; the chief parameters characterizing this interaction, n, the capacity/unit mass of protein and K, the dissociation constant, are commonly determined from Scatchard plots, having first related the fluorescence intensity to concentrations of bound dye using double reciprocal plots. It has recently been shown [881] that the plots usually employed are based upon a mistaken interpretation of the formal relationship, which ultimately leads to erroneous values. Correct procedures for determining n and K are outlined. The dye Auramine O shows many of the useful properties associated with fluorescence probes such as sensitivity to solvent polarity and conformational changes in proteins, but being cationic may be used to provide information about sizes which are inaccessible to anionic ligands.[882] Picosecond spectroscopy has been used to study the lifetimes and environmental sensitivity of fluorescein and three halogenated derivatives: eosin, erythrosin, and rose bengal.[883]

Intrinsic Fluorescence.—A comprehensive study of the effects of anions [884] and cations [885] on the fluorescence spectra of proteins in neutral aqueous solution shows that most anions are good quenchers of both Trp and Tyr fluorescence (in general following a collisional mechanism with an order of effectiveness which resembles the Hofmeister series), whereas, of the cations examined, only Cs^+ brought about quenching by direct interaction with the fluorescent residue. Other quenchers recently developed include 2,2,2-trichloro-ethanol,[886] a hydro-

[877] F. E. Treloar, *Chem. Scripta*, 1977, **10**, 215.
[878] F. C. Greene, *Biochemistry*, 1977, **14**, 747.
[879] H. Doduik and E. M. Kosower, *J. Phys. Chem.*, 1977, **81**, 50.
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[884] W. Altekar, *Biopolymers*, 1977, **16**, 341.
[885] W. Altekar, *Biopolymers*, 1977, **16**, 369.
[886] M. R. Eftink, J. L. Zajicek, and C. A. Ghiron, *Biochim. Biophys. Acta*, 1977, **491**, 473.

phobic quencher with properties resembling those of acrylamide; quaternary salts of 4-picoline, which are several orders of magnitude more effective than I^- or Cs^+, and which have been used in studies of membrane proteins;[887] and diethylformamide, which is an effective quencher of indole fluorescence, mimicking the effect of a peptide bond.[888] The specific quenching of Tyr fluorescence by phosphate (previously thought to be due mainly to collisional quenching of the excited state[889]) is accompanied by the occurrence of a new emission peak at 345 nm, as a result of ground state interactions.[890]

Protein Conformation. Table 4 summarizes some of the structural and related studies of intrinsic fluorescence. The effect of water on protein structure has been investigated by examining the Trp fluorescence of protein powders in the temperature range 0— −196 °C.[891] In the absence of water, protein molecules have a fairly rigid structure, possibly due to a strong increase in intramolecular electrostatic interactions owing to the reduced dielectric constant. On increasing the water content of the powder, the protein structure becomes more flexible and mobile, reflected by a shift in the emission maximum to longer wavelength; a water content of 0.3 $g\,g^{-1}$ protein, the amount of water necessary to form the first monolayer, is sufficient to complete the structural change. The acrylamide quenching reaction is very discriminating in sensing the exposure of Trp groups and giving information on the dynamic fluctuations in protein structure.[892] The quenching rate constants for some proteins, such as aldolase and human serum albumin, are smaller than expected for simple collisional quenching and also are independent of solvent viscosity, indicating that the quenching reaction is limited by penetration of the quencher through the matrix of the protein by a mechanism thought to involve the formation of free spaces by conformational fluctuations. The effect of temperature on this rate of penetration can be used to characterize the nature of the protein matrix surrounding the fluorescent group in terms of enthalpic and entropic contributions to the activation barrier. The heterogeneity of the environments of single Trp residues revealed by fluorescence decay measurements was commented on last year;[816] a collection of abstracts of a symposium on site heterogeneity in protein luminescence summarizes the present position.[893] The single Trp of azurin shows fine structure in its fluorescence spectrum at 77 K, indicating the absence of hydrogen-bonding or other polar interactions which tend to make the spectrum structureless.[894] A dramatic reduction in the intensity of the Trp fluorescence of malate dehydrogenase from extremely halophilic bacteria accompanies the transfer of the enzyme into dilute buffer solution from its natural environment (>4M-NaCl); the rates of inactivation and fluorescence change follow the same first-order time

[887] M. Shinitzky and B. Rivnay, *Biochemistry*, 1977, **16**, 982.
[888] P. M. Froehlich, D. Gantt, and V. Paramasigamani, *Photochem. Photobiol.*, 1977, **26**, 639.
[889] R. F. Chen and P. F. Cohen, *Arch. Biochem. Biophys.*, 1966, **114**, 514.
[890] O. Shimizu and K. Imakubo, *Photochem. Photobiol.*, 1977, **26**, 541.
[891] E. A. Permyakov and E. A. Burstein, *Studia Biophysica*, 1977, **64**, 83.
[892] M. R. Eftink and C. A. Ghiron, *Biochemistry*, 1977, **16**, 5546.
[893] J. W. Longworth, *Biophys. J.*, 1977, **19**, 72; C. Formoso, *ibid.*, p. 73; A. Grinvald and I. Z. Steinberg, *ibid.*, p. 74; S. S. Lehrer, *ibid.*, p. 77; A. H. Maki, *ibid.*, p. 78; J. Schlessinger and I. Z. Steinberg, *ibid.*, p. 80.
[894] E. A. Burstein, E. A. Permyakov, V. A. Yashin, S. A. Burkhanov, and A. Finozzi Agro, *Biochim. Biophys. Acta*, 1977, **491**, 155.

Table 4 *Structure studied by intrinsic protein fluorescence*

Protein	*Application*	*Ref.*
ATPases	Denaturation by guanidium	*a*
Azurin	Trp environment	894
(*Pseudomonas aeruginosa*)	Trp, Tyr environment	*b*
(*Pseudomonas fluorescens*)	Tyr environment	*b*, 902
Bacteriophage f2 capsid	Trp environment, assembly	*c*
Bovine and human serum albumins	quenching mechanisms	886
Brome mosaic virus	assembly	*d*
Dihydrofolate reductase	Trp environment	*e*
Galactose oxidase	Trp environment	*f*
Hemocyanin	Trp, Tyr fluorescence	*g*
Histones H1 and H5	Tyr fluorescence	900
Lactate dehydrogenase	refolding and reactivation	*h*
Lysozymes (phage T4)	Trp, Tyr environments	898
α_2-Macroglobulin	general structure	851
Malate dehydrogenase (halophilic bacterium)	general structure	895
Monellin	Tyr–Trp energy exchange	*i*
Myosin (native and modified)	general structure	*j*
Phosphorylase kinase	Trp quenching (I^-)	*k*
Pyruvate kinase	refolding and reactivation	*l*
Ribonuclease A	denaturation by temperature and urea	899
Ribosomal proteins S8 and S15	Tyr fluorescence	901
Subtilisins (*Carlsberg* and *Novo*)	Tyr–Trp energy exchange	897
Subtilisin inhibitor	denaturation by temperature and urea	*m*
Superoxide dismutase	Phe fluorescence	903
Thermolysin	role of metal ions	*n*
Tobacco mosaic virus (and mutants)	Trp environment; Tyr–Trp energy exchange	*o*

(*a*) M. Niento and J. A. Ayala, *Biochem. J.*, 1977, **161**, 321. (*b*) K. Ugurbil, A. H. Maki, and R. Bersohn, *Biochemistry*, 1977, **16**, 901. (*c*) B. B. Kitchell, S. P. Merrill, and R. W. Henkens, *Biochim. Biophys. Acta*, 1977, **475**, 536. (*d*) M. Herzog, D. Gerard, L. Hirth, and G. Laustriat, *Biochim. Biophys. Acta*, 1977, **493**, 167. (*e*) V. A. Reddy and N. A. Rao, *Archiv. Biochem. Biophys.*, 1977, **183**, 90—97. (*f*) R. E. Weiner, M. J. Ettinger, and D. J. Kosman, *Biochemistry*, 1977, **16**, 1602. (*g*) A. Klarman, N. Shaklai, and E. Daniel, *Biochim. Biophys. Acta*, 1977, **490**, 322. (*h*) R. Rudolph, I. Heider, E. Westhof, and R. Jaenicke, *Biochemistry*, 1977, **16**, 3384. (*i*) J. G. Band and R. H. Cagan, *Biochim. Biophys. Acta*, 1977, **493**, 178. (*j*) Z. I. Vishnevskaya, N. J. Vedekina, and M. V. Georgadze, *Biokhimiya*, 1977, **41**, 1293. (*k*) D. Dimitrov, *Int. J. Biochem.*, 1977, **8**, 369. (*l*) J. M. Cardenas, D. R. Hubbard, and S. Anderson, *Biochemistry*, 1977, **16**, 191. (*m*) T. Komiyama, M. Miwa, S. Sato, and S. Murao, *Biochim. Biophys. Acta*, 1977, **493**, 188. (*n*) A. Fontana, C. Vita, E. Boccu, and F. M. Veronese, *Biochem. J.*, 1977, **165**, 539. (*o*) A. Magne, D. Gerard, L. Hirth, and G. Lanstriat, *Biochim. Biophys. Acta*, 1977, **495**, 189.

course.[895] Thermodynamic analysis suggests that at moderate concentrations the salt mainly screens the fixed charges of the subunits, whereas at concentrations higher than 0.8 M, it stabilizes hydrophobic interactions between the subunits.

Energy transfer from Tyr-1 to Trp-4 has been used to measure the separation of the two residues in [Trp^4, Met^5]enkephalin (which possesses the same affinity for opiate receptors as [Met^5]enkephalin).[896] With the assumption that $\kappa^2 = \frac{2}{3}$

[895] M. Mevarech, H. Eisenberg, and E. Neumann, *Biochemistry*, 1977, **16**, 3781; M. Mevarech and E. Neumann, *ibid.*, p. 3786.

[896] P. W. Schiller, *Biochem. Biophys. Res. Comm.*, 1977, **79**, 493.

the separation was found to be 9.3 Å, a value closely similar to the phenol–phenyl separation in morphine derivatives which enkephalin mimics. Although Tyr → Trp energy exchange is common in proteins, it is not usually amenable to detailed analysis unless the number of residues involved is very small (*cf.* last year's report on luliberin [816]) or the existence of suitable closely related species makes a comparative approach possible. Thus, although subtilisin *Carlsberg* has only one Trp (at position 113), subtilisin *Novo* has three (Trp 105, 113, 241);[897] there is significant energy transfer from Tyr → Trp in the latter, but very little with the former, suggesting that Trp 105 and 241 are the usual acceptors of Tyr emission in subtilisin *Novo*. The use of mutants can also contribute to the understanding of the interactions in the Tyr and Trp. Wild type phage T_4 lysozyme has three Trp, all in the upper lobe of a bilobed protein, and six Tyr, three in the upper and three in the lower lobes; two mutant enzymes have been isolated in which either one or all three Trp are replaced by Tyr.[898] The emission maximum shifts from 338 nm (W type) to 329 nm (mutant 1) and finally to 310 nm for the mutant in which no Trp is present.

An increasing amount of work is being done on the Tyr fluorescence of class A proteins (which contain no Trp). Reports have appeared this year on ribonuclease A,[899] histones H1 and H5,[900] *E. coli* ribosomal proteins S_8 and S_{15} [901] and azurin from *Pseudomonas fluorescens*.[902] The most significant general conclusion is that it appears necessary to reassess the view (based primarily on the behaviour of RNAase A) that Tyr residues on the surface fluoresce (relatively) strongly, whereas buried residues show weak fluorescence. Quenching experiments with I^- suggest that the two Tyr residues of azurin are buried although they fluoresce strongly.[902] In addition, the intensity of the single Tyr in histone H1 increases four-fold as the protein is converted from a random-coil to a folded state.[900] Both sets of authors argue that although not observed before, this behaviour might be expected of residues buried in a hydrophobic environment away from any peptide or carboxyl quenching groups.

The spectra of class A proteins are dominated by the fluorescence of Tyr, without any traces of Phe emission. Class C proteins (devoid of Trp and Tyr) are fairly rare. Earlier reports on the spectra of horse hepatocuprein, and parvalbumins from fish muscle have been added to by a recent study of superoxide dismutase which contains nine Phe;[903] the excitation maximum at 265 nm is similar to that of aqueous Phe, but the emission (282 nm) is shifted to a slightly longer wavelength. The very low quantum yield (*ca.* 1%, compared with aqueous Phe 3.8%) was attributed to close contact with the Cu or Zu ions.

Ligand Binding. The use of intrinsic protein fluorescence to study ligand binding is widespread, often comprising a small part of a much wider ranging investi-

[897] M. F. Brown, S. Omar, R. A. Raubach, and T. Schleich, *Biochemistry*, 1977, **16**, 987.
[898] M. L. Elwell and J. A. Schellmann, *Biochim. Biophys. Acta*, 1977, **494**, 367.
[899] N. Barboy and J. Feitelson, *Photochem. Photobiol.*, 1977, **26**, 561.
[900] V. Giancotti, M. Fonda, and C. Crane-Robinson, *Biophys. Chem.*, 1977, **6**, 379.
[901] B. Lux, D. Gerard, and G. Laustriat, *F.E.B.S. Letters*, 1977, **80**, 66.
[902] K. Ugurbil and R. Bersohn, *Biochemistry*, 1977, **16**, 895.
[903] E. A. Permyakov, E. A. Burstein, Y. Sawada, and I. Yamakazi, *Biochim. Biophys. Acta*, 1977, **491**, 149.

gation, hence any attempt at comprehensive coverage is pointless; a selection of the studies reported is collected in Table 5.

The binding of the tripeptide Lys-Trp-Lys to native, denatured, and u.v. irradiated DNA was interpreted in terms of a model involving two types of binding complex: in the first, the interactions were purely electrostatic between

Table 5 *Binding studied by intrinsic protein fluorescence*

Protein	*Ligand*	*Ref.*
Alcohol, lactate, and glyceraldehyde-3-phosphate dehydrogenases	NAD^+ analogues	905
D-Amino-acid oxidase	FAD	*a*
Glucoamylase	gluconolactone	*b*
Glyceraldehyde-3-phosphate dehydrogenase	NAD^+	*c*
Homogeneous antibodies	oligosaccharides (as antigens)	*d*
Human antithrombin III	heparin	*e*
lac Repressor	isopropyl-β-D-thiogalactoside	*f*
Liver alcohol dehydrogenase	NAD^+	*g*
Lysozyme	oligosaccharides	*h*
Myosin subfragment 1	chromophoric nucleotides	*i*
Octopine dehydrogenase	NADH	826
Phosphatidyl exchange protein	phospholipids	*j*
Phosphoglycerate dehydrogenase	serine	*k*
Ribosomal elongation factors T_u and T_s	GTP, GDP	906

(*a*) Y. Nishina, K. Horiike, K. Shiga, Y. Miyake, and T. Yamano, *J. Biochem.*, 1977, **81**, 1455. (*b*) M. Ohnishi, T. Yamashita, and K. Hiromi, *J. Biochem.*, 1977, **81**, 99. (*c*) C. W. Niekamp, J. M. Sturtevant, and S. F. Velick, *Biochemistry*, 1977, **16**, 436. (*d*) H. Maeda, A. Schmidt-Kessen, J. Engel, and J. C. Jaton, *Biochemistry*, 1977, **16**, 4086. (*e*) R. Einarsson and L. O. Anderson, *Biochim. Biophys. Acta*, 1977, **490**, 154. (*f*) R. B. O'Gorman and K. S. Matthews, *J. Biol. Chem.*, 1977, **252**, 3572. (*g*) J. K. Wolfe, C. I. Weidig, H. R. Halvorson, J. D. Shore, D. M. Parker, and J. J. Holbrook, *J. Biol. Chem.*, 1977, **252**, 433. (*h*) M. Schindler, Y. Assaf, N. Sharon, and D. M. Chipman, *Biochemistry*, 1977, **16**, 423. (*i*) J. F. Eccleston and D. R. Trentham, *Biochem. J.*, 1977, **163**, 15. (*j*) K. W. A Wirtz and P. Moonen, *European J. Biochem.*, 1977, **77**, 437. (*k*) R. Dubrow and L. I. Pizer, *J. Biol. Chem.*, 1977, **252**, 1527.

lysine and the phosphate group of DNA, and the fluorescence quantum yield of the Trp was the same as in the free peptide; whereas in the second, stacking of Trp with the bases also occurred, resulting in complete quenching of the fluorescence; intercalation of the Trp was favoured by single-stranded base regions.[904] The quenching of protein fluorescence of three dehydrogenases by a range of six NAD^+ analogues was one of the techniques used to compare the interactions of co-enzymes with the co-enzyme domain in the crystal (where catalysis is not complete) with that in solution (where catalysis occurs).[905] The results suggest that although the 2′- and 3′-OH groups on the adenine ribose are necessary for efficient catalysis and productive complex formation, these groups do not contribute significantly to the free energy change of substrate binding. Tyrosine fluorescence dominates the spectrum of the complex formed between GDP and elongation factor Tu (which contains two Trp and ten Tyr), but the contribution from Trp becomes much more evident on unfolding the Tu–GDP

[904] J. J. Toulmé and C. Hélène, *J. Biol. Chem.*, 1977, **252**, 244.
[905] R. J. Suhadolnik, M. B. Lennon, T. Uematsu, J. A. Monahan, and R. Baur, *J. Biol. Chem.*, 1977, **252**, 4125.

complex with 1% dodecylsulphate, or on replacement of GDP by GTP.[906] The nature of the Trp quenching in the Tu–GDP complex is not clear, but the major conformational differences depending on whether GTP or GDP is bound are presumably important in facilitating the release of the Tu factor from the ribosome upon hydrolysis of GTP. The single Trp of the amphipathic peptide melittin is very sensitive to binding to phospholipids which elicit a blue-shift from 352 nm to 333 nm.[907] Binding in this model lipid–protein interaction is thought to occur *via* an initial electrostatic interaction between the phosphate or carboxyl residues on the polar head of the lipid with basic residues in melittin, followed by the insertion of hydrophobic residues into the membrane bilayer involving at least the Trp, but probably all of the hydrophobic part of the peptide.

[906] K. Arai, T. Arai, M. Kawaki, and Y. Kazino, *J. Biochem.*, 1977, **81**, 1335.
[907] J. Duforcq and J. F. Faucon, *Biochim. Biophys. Acta*, 1977, **467**, 1.

3
Peptide Synthesis

BY E. ATHERTON AND R. C. SHEPPARD
Appendices compiled by
A. HALLETT AND A. V. STACHULSKI

1 Introduction

The general arrangement of this chapter is similar to that in previous volumes. The Proceedings of the Fifth American Peptide Symposium [1] were published (with commendable promptness) during the period under review. These Proceedings include the text of the first Alan E. Pierce Award Lecture (Peptide synthesis: an undiminished challenge) given by M. Bodanszky,[2] and other surveys by Sakakibara [3] (Solution synthesis of complex peptides by the maximum protection procedure), Feurer [4] (Special features of large scale peptide synthesis), and Merrifield *et al.*[5] (Some recent developments in solid phase synthesis). This last subject is also reviewed by Sheppard [6] (Solid phase peptide synthesis, a reassessment) in the Proceedings of the Endocrinology '77 Symposium.[7] Other papers from these symposia are referred to elsewhere in this chapter.

Some other general reviews noted were by Blaha (Linear peptides: synthetic methods),[8] Geiger (Classical synthesis of polypeptides),[9] and Hirschmann (Recent developments in the synthesis of biologically active peptides).[10]

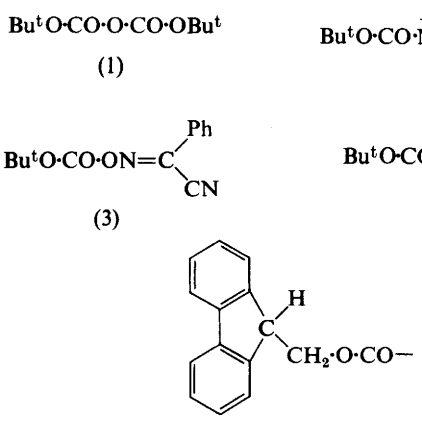
The customary lists of synthetic peptides and useful amino-acid derivatives are presented in the form of Appendices to this chapter.

2 Methods

Protective Groups.—*Established Methods of Amino-group Protection.* Reagents alternative to the dangerous [11] azide for the introduction of the t-butoxycarbonyl group were discussed in last year's Report (p. 315). Of these, the pyrocarbonate (1) has now also been used in a simple preparation of the water-soluble reagent (2).[12] The latter reacts smoothly with amino-acids in aqueous alkaline solution giving, for example, Boc-proline in 96% yield. Mention has also been

[1] 'Peptides', Proceedings of the 5th American Symposium, San Diego, 1977, ed. M. Goodman and J. Meienhofer, Academic Press, New York, 1977.
[2] M. Bodanszky, ref. 1, p. 1.
[3] S. Sakakibara, ref. 1, p. 436.
[4] M. Feurer, ref. 1, p. 448.
[5] R. B. Merrifield, G. Barany, L. Cosand, M. Engelhard, and S. Mojsov, ref. 1, p. 488.
[6] R. C. Sheppard, ref. 7, p. 43.
[7] 'Molecular Endocrinology', Proceedings of Endocrinology '77, ed. I. MacIntyre and M. Szelke, Elsevier, Amsterdam, 1977.
[8] K. Blaha, *Int. Rev. Sci: Org. Chem., Ser.* 2, 1976, **6**, 73.
[9] R. Geiger, *Excerpta Med. Int. Congr. Ser.* 1976, **374**, 40.
[10] R. F. Hirschmann, *Med. Chem., Proc. Internat. Symp. 5th 1976*, 1977, 63.
[11] P. Feyen, *Angew. Chem. Internat. Edn.*, 1977, **16**, 115.
[12] E. Guibé-Jampel and M. Wakselman, *Synthesis*, 1977, 772.

made of use of the pyrocarbonate (1) in the preparation of Boc-derivatives of polyfunctional amino-acids.[13] Full details have now appeared of the use of oxime carbonates bearing electron withdrawing substituents as t-butoxycarbonylating reagents.[14] The phenylacetonitrile (3) and malonate (4) derivatives appear to be the derivatives of choice with (3) favoured because of the ease of removal of the co-product, 2-(hydroxyimino)-2-phenylacetonitrile. Yields of Boc-amino-acids were uniformly high. Similarly constituted reagents for the introduction of benzyloxycarbonyl, *p*-methoxybenzyloxycarbonyl, βββ-trichloroethoxycarbonyl, and 1-cyclopropylethoxycarbonyl protecting groups have been prepared.[14] The last-named group is removed under conditions similar to those required for cleavage of Boc-derivatives (*cf.* Vol. 9 of these Reports, p. 320).



New Methods of Amino-group Protection. The design of new methods for the protection of amino-functions continues to attract the ingenuity of organic chemists. Acid-labile groups have for many years dominated most synthetic strategies, but in recent volumes of these Reports we noted increasing interest in protecting groups cleaved by base and other specific treatments. These may be used in a complementary manner to the established acid-labile groups (biphenylisopropoxycarbonyl, t-butoxycarbonyl, and benzyloxycarbonyl), which already span the useful range of acidic cleavage conditions (AcOH to liq. HF). The need for protecting groups cleavable under non-acidolytic conditions has increased as synthetic targets have become more ambitious, since the vigorous acidic conditions commonly used in final deprotection procedures are clearly damaging to many of the larger synthetic peptides and small proteins. A significant recent development is the independent resuscitation in two laboratories[15–17] of the fluorenylmethoxycarbonyl group (5), which has lain essentially dormant since its

[13] V. F. Pzodnev, *Bioorg. Khim.*, 1977, 3(12) 1605.
[14] M. Itoh, D. Hagiwara, and T. Kamiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 718.
[15] E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, and B. J. Williams, *J.C.S. Chem. Comm.*, 1978, 537.
[16] E. Atherton, H. Fox, D. Harkiss, and R. C. Sheppard, *J.C.S. Chem. Comm.*, 1978, 539.
[17] C.-D. Chang and J. Meienhofer, *Internat. J. Peptide Protein Res.*, 1978, **11**, 246.

introduction several years ago by Carpino and Han.[18] This group shows quite remarkable lability to secondary (as opposed to tertiary) amines and essentially complete acid stability. Its use has enabled solid-phase syntheses of dihydrosomatostatin[17] and β-endorphin[16] to be carried out under exceptionally mild conditions (p. 335), and further consideration of its value in solution synthesis may be indicated.

Carpino and his colleagues have also introduced β-(trimethylsilyl)ethoxycarbonyl (Teoc) derivatives (6) for amino-protection.[19] These are readily prepared by way of the chloro- or azido-formates and undergo a specific fluoride ion-induced fragmentation (Scheme 1) giving only gaseous co-products. The preferred

$$Me_3Si{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}CO{\cdot}NHR \longrightarrow Me_3SiF + CH_2{=}CH_2 + CO_2 + NH_2R$$

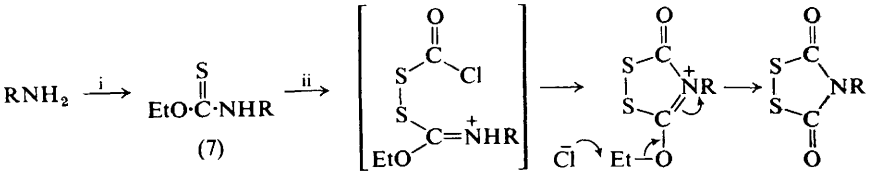
(6)

Reagents: $Et_4N^+F^-/CH_3CN$, 50 °C, 4—5 h

Scheme 1

reagent is tetraethylammonium fluoride in a range of polar organic solvents (acetonitrile, dimethylformamide, *etc.*). Potassium fluoride is relatively ineffective; its reactivity might be increased in the presence of crown ethers. The new protecting group is stable to the conditions of catalytic hydrogenolysis and to treatment with piperidine, ethanolamine, and liquid ammonia. It is, however, rapidly cleaved by acidic reagents, notably trifluoroacetic acid. A similar system has been applied to carboxy group protection (p. 315).

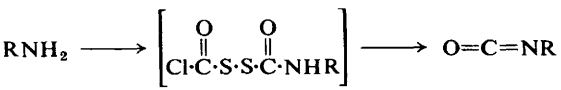
Barany and Merrifield have proposed[20, 21] the use of 1,2,4,-dithiazolidine-3,5-dione (Dts) derivatives (8) for amino group protection. The protected amines are prepared in a two-step process by reaction of the intermediate ethoxythiocarbonyl derivative (7) with chlorocarbonylsulphenyl chloride (Scheme 2). Other potential routes, *e.g.* direct reaction between bis(chlorocarbonyl)-disulphane and primary amine, failed, giving instead the isocyanate (Scheme 3) and supporting the



(7) (8)

Reagents: i, EtOC SSMe; ii, ClSCOCl

Scheme 2



Reagents: ClCOSSCOCl

Scheme 3

[18] L. A. Carpino and G. Y. Han, *J. Amer. Chem. Soc.*, 1970, **92**, 5748.
[19] L. A. Carpino, J.-H. Tsao, H. Ringsdorf, E. Fell, and G. Hettrich, *J.C.S. Chem. Comm.*, 1978, 358.
[20] G. Barany and R. B. Merrifield, *J. Amer. Chem. Soc.*, 1977, **99**, 7363.
[21] G. Barany and R. B. Merrifield, *Fed. Proc.*, 1977, **36**, 864.

mechanism outlined in Scheme 2. Unfortunately Dts derivatives could not be prepared directly from free amino-acids although methyl, t-butyl, or trimethylsilyl amino-acid esters reacted smoothly. The ester group was finally cleaved by refluxing HCl–AcOH or by HBr–AcOH at room temperature to give the *N*-protected amino-acids. The Dts-moiety is completely stable to strongly acidic and mildly basic reagents, and to photolysis at $\lambda > 330$ nm. It is rapidly cleaved by thiolysis, especially in the presence of tertiary amine. Preliminary application to peptide synthesis has been reported.[20] The group is regarded [20] as potentially suitable in orthogonal [22] systems (*i.e.* those using amino, carboxy, and side-chain protecting groups cleavable by completely independent reactions), particularly in combination with a photo-labile *C*-terminal protecting group or resin linkage and t-butyl-based side-chain derivatives. Preparation of the latter may, however, present difficulties.

A short paper has at last appeared on the use of vinyloxycarbonyl (Voc) derivatives (9) for amino-group protection.[23] This supplements information released in 1973 in patent applications and a commercial brochure (see Vol. 6 of these Reports, p. 270). The protected amino-acids are readily prepared in mildly alkaline solution using vinyl chloroformate. Deprotection may be achieved by a number of reagents capable of attacking the particularly reactive olefinic bond. Thus reaction with anhydrous HCl–dioxan generates the unstable adduct (10) which decomposes in warm ethanol as indicated (Scheme 4). Other acidic reagents

$$\underset{(9)}{CH_2{=}CH{\cdot}O{\cdot}CO{\cdot}NHR} \xrightarrow{i} \underset{(10)}{Me{\cdot}CHCl{\cdot}O{\cdot}CO{\cdot}NHR} \xrightarrow{ii}$$

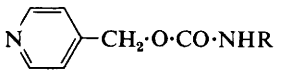
$$Me{\cdot}CH(OEt){\cdot}O{\cdot}CO{\cdot}NHR \longrightarrow Me{\cdot}CH(OEt)_2 + R\overset{+}{N}H_3\overset{-}{Cl} + CO_2$$

Reagents: i, HCl–dioxan; ii, EtOH, 50 °C

Scheme 4

(HBr–AcOH, HCl–AcOH, HBr–CH_2Cl_2) cause cleavage in one-step processes. Similarly, one equivalent of bromine reacts instantaneously with Voc-derivatives giving unstable dibromo adducts which decompose to the amine hydrobromide, bromacetal, and carbon dioxide on addition of alcohol. This cleavage can apparently be achieved without concomitant removal of Boc groups. Deprotection can also occur in reaction with other electrophiles, notably mercuric ion.

A detailed account has also appeared [24] of the use of isonicotinyloxycarbonyl (iNoc) derivatives (11) for amino protection, supplementing a symposium account in 1972 (see Vol. 6 of these Reports, p. 300). This group is completely stable to the action of acids even resisting cleavage by liquid hydrogen fluoride, and is therefore particularly advocated for side-chain protection of lysine residues. It is



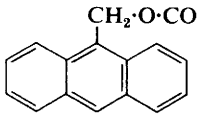
(11)

[22] R. B. Merrifield, G. Barany, W. L. Cosand, M. Engelhard, and S. Mojsov, ref. 1, p. 488.
[23] R. A. Olofson, Y. S. Yamamoto, and D. J. Mancowicz, *Tetrahedron Letters*, 1977, 1563.
[24] D. F. Veber, W. J. Paleveda, jun., Y. C. Lee, and R. Hirschmann, *J. Org. Chem.*, 1977, **42**, 3286.

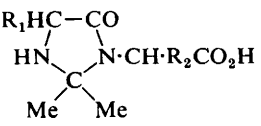
introduced using isonicotinyl *p*-nitrophenyl carbonate (in preference to the analogous hydroxysuccinimide derivative previously advocated). Removal of iNoc groups is easily achieved by catalytic or Zn–AcOH reduction.

A new method for reversibly protecting amines makes use of the remarkable susceptibility of anthrylmethyl derivatives to specific nucleophilic displacement reactions.[25] Thus anthrylmethoxycarbonyl derivatives (12), prepared *via* the *p*-nitrophenyl carbonate, are cleaved within minutes by mercaptide ion at room temperature, and within a few hours at −20 °C. In contrast, they are resistant to cleavage by nitrogen nucleophiles, *e.g.* by ethylamine during 24 h. As expected, acidic reagents (*e.g.* trifluoroacetic acid) also cause rapid deprotection. The anthrylmethoxy group has previously been advocated for carboxy protection by the same authors.[26]

Further discussion has been given of the use of acid-labile diphenylphosphinothioyl derivatives for amino-protection.[27] (See also last year's Report, p. 322.) Dimethylphosphinothioyl derivatives show greater acid lability, particularly to the hydrochloride salt of triphenylphosphine. This last reagent is claimed



(12)



(13)

to have special application to solid-phase synthesis. Since cleavage of phosphinothioyl derivatives does not give rise to carbonium ions, the protecting group is thought to be particularly suitable for the synthesis of tryptophan-containing peptides.

Dipeptides (other than those containing *C*-terminal proline) may be protected by condensation with acetone to yield *NN'*-isopropylidene derivatives (13).[28] Peptide synthesis using these isopropylidene derivatives may be carried out with dicyclohexylcarbodi-imide without racemization, and the protecting group removed by hydrolysis under mild, neutral conditions. This ease of deprotection and their relative water solubility has suggested [28] possible value in partial synthesis. In similar vein, addition of *N*-protected cysteic acid residues to the *N*-termini of peptides has been proposed for increasing water solubility and facilitating purification.[29] The cysteic acid residue is removed (after cleavage of its own amino-protecting group) by one cycle of Edman degradation.

Protection of Carboxy-groups. The use of caesium salts for the attachment of protected amino-acids to chloromethylated polystyrene [30] has been extended to esterification in solution.[31] The reaction can be carried out with simple *N*-protected amino-acids or more usefully with protected peptides. Esters prepared

[25] N. Kornblum and A. Scott, *J. Org. Chem.*, 1977, **42**, 399.
[26] N. Kornblum and A. Scott, *J. Amer. Chem. Soc.*, 1974, **96**, 590.
[27] M. Ueki, S. Ikeda, and F. Tonegawa, ref. 1, p. 546.
[28] P. M. Hardy and D. J. Samworth, *J.C.S. Perkin I*, 1977, 1954.
[29] A. Hubbuch, W. Danho, and H. Zahn, ref. 1, p. 540.
[30] B. F. Gisin, *Helv. Chim. Acta*, 1973, **56**, 1476.
[31] S-S. Wang, B. F. Gisin, D. P. Winter, R. Makofske, I. D. Kuleshec, C. Tzougraki, and J. Meienhofer, *J. Org. Chem.*, 1977, **42**, 1291.

include methyl, benzyl, *o*-nitrobenzyl, *p*-methoxybenzyl, α-methylphenacyl, trityl, and t-butyl. Reaction with the appropriate alkyl halide takes place under mild conditions and, except for the very hindered t-butyl and trityl derivatives, yields are generally good. The sulphur atom of methionine residues is not attacked under the conditions used.

N-Protected amino-acids are also easily converted into trimethylsilylethyl esters (14).[32, 33] The preferred method is reaction between benzyloxycarbonyl-amino-acids and trimethylsilylethanol using dicyclohexylcarbodi-imide in the

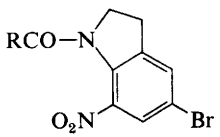
$$RCO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}SiMe_3$$

(14)

presence of pyridine. Poor results are obtained with asparagine derivatives under these conditions, but otherwise yields are generally excellent. The trimethylsilylethyl esters are stable under a variety of conditions, including hydrogenolytic removal of the benzyloxycarbonyl group using isopropanol as solvent. Cleavage of the ester is achieved specifically with fluoride ion using a tetra-alkylammonium fluoride in dimethylformamide or dimethylsulphoxide. No evidence was found for racemization during this cleavage reaction. The group is also removed by acids but sufficiently slowly to allow preferential cleavage of t-butoxycarbonyl derivatives. A careful study of the reactivity of other common protecting groups under the conditions for cleavage of trimethylsilylethyl esters by fluoride ion has been carried out. t-Butoxycarbonyl, biphenylisopropoxycarbonyl, *N*- and *S*-trityl, *S*-acetamidomethyl, and t-butyl ethers are stable, but methyl, t-butyl, and especially benzyl esters undergo slow cleavage and benzyloxycarbonyl groups may be partly converted to hydantoins. Aspartic β-t-butyl esters and carboxy terminal asparagine derivatives present special problems associated with

$$RCO{\cdot}O{\cdot}CH_2{\cdot}S{\cdot}CH_3$$

(15)



(16)

ring closure reactions, and disulphides undergo rapid disproportionation. These results are also of interest in relation to the similarly constituted trimethylsilylethoxycarbonyl amino-protecting group (see p. 312).

Methylthiomethyl esters (15) may be prepared in good yield under mild conditions by reaction of potassium salts with chloromethyl methyl sulphide in the presence of catalytic amounts of sodium iodide and 18-crown-6.[34] The esters are cleaved by mercuric ion. Phenyl esters are obtained in high yield using the benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate reagent.[35] The method fails with asparagine derivatives. Acyl derivatives of 5-bromo-7-nitro indolines (16) have been prepared in a multi-step process.[36] They

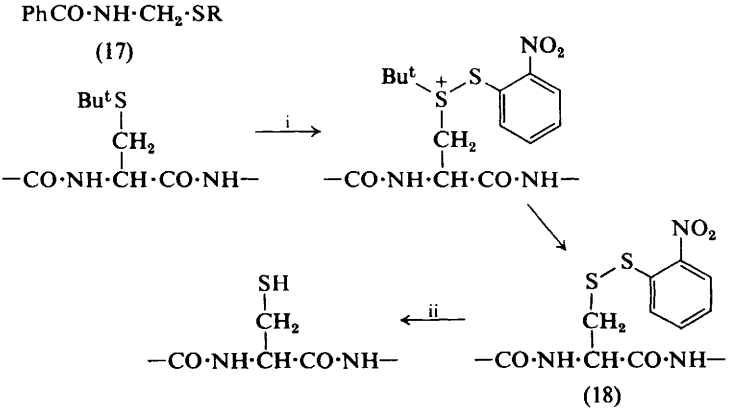
[32] P. Sieber, *Helv. Chim. Acta*, 1977, **60**, 2711.
[33] P. Sieber, R. H. Andreatta, K. Eisler, B. Kamber, B. Riniker, and H. Rink, ref. 1, p. 543.
[34] L. G. Wade, J. M. Gerder, and R. P. Wirth, *Tetrahedron Letters*, 1978, 731.
[35] B. Castro, G. Evin, C. Selve, and R. Seyer, *Synthesis*, 1977, **6**, 413.
[36] G. Goissi, B. W. Erickson, and R. B. Merrifield, ref. 1, p. 559.

are cleaved by photosolvolysis, to free acids or esters. Use of carboxy-terminal arginine methyl ester or amide residues as protecting groups has been suggested.[37] Removal is by a two-step enzymic procedure using trypsin (to cleave the ester or amide) and then carboxypeptidase B.

Further papers have appeared on side-chain protecting groups for glutamic and aspartic acids, particularly for applications in solid phase synthesis (q.v.). γ-Phenacyl and *p*-nitrobenzyl esters of glutamic acid are resistant to acidolysis, even by liquid hydrogen fluoride.[38] They may be removed reductively by Zn–AcOH and catalytic hydrogenolysis respectively.[38] β-*p*-Bromobenzyl esters of aspartic acid derivatives are usefully more stable to cleavage by trifluoroacetic acid than simple benzyl derivatives.[39]

Derivatives of α-picolyl-γ-t-butyl and α-*p*-nitrobenzyl-γ-t-butyl as well as α-unprotected derivatives of glutamic acid have been used successfully in new routes to γ-linked glutamyl peptides.[40, 41]

Side-chain Protection and Reactions of Amino-acids. Benzamidomethyl derivatives (17) have been suggested[42] as convenient alternatives to the corresponding acetamidomethyl compounds for protection of the thiol group of cystine and its relatives. They are readily prepared by reaction of the thiol with benzamidomethanol in anhydrous trifluoroacetic acid, and proved particularly appropriate in the case of *N*-methyl cysteine. The *S*-t-butyl thioether derivative of cysteine is finding increasing application and has been advocated[43] as a simpler and advantageous alternative to *S*-acetamidomethylcysteine. The thioether group is cleaved under the same mild conditions (mercuric acetate, pH 4;[44] *o*-nitrophenyl-



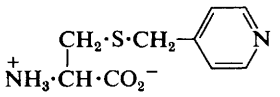
Reagents: i, *o*-$NO_2C_6H_4SCl$–AcOH

ii, $HOCH_2CH_2SH$, $NaBH_4$, or $HSCH_2CO_2H$

Scheme 5

[37] J. Glass and M. Pelzig, *Proc. Nat. Acad. Sci., U.S.A.*, 1977, **74**, 2739.
[38] K. Suzuki, N. Endo, and Y. Sasaki, *Chem. Pharm. Bull.*, 1977, **25**(10), 2613.
[39] D. Yamashiro, *J. Org. Chem.*, 1977, **42**(3) 523.
[40] C. N. C. Drey and G. Priestley, ref. 1, p. 468.
[41] C. N. C. Drey and G. Priestley, *J.C.S. Chem. Comm.*, 1977, 144.
[42] P. K. Chakravarty and R. K. Olsen, *J. Org. Chem.*, 1978, **43**(6), 1270.
[43] J. J. Pastuszak and A. Chimiak, *Roczniki Chem.*, 1977, **51**(7—8), 1567.
[44] A. M. Felix, M. H. Jimenez, and J. Meienhofer, ref. 1, p. 532.

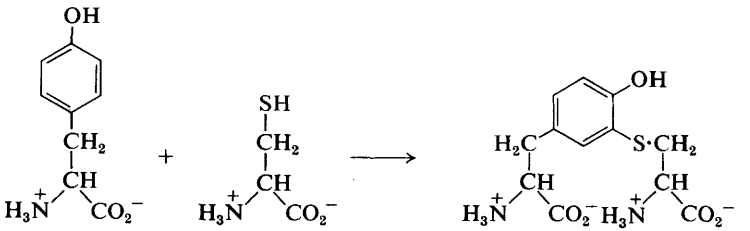
sulphenyl chloride in acetic acid [43]) as are acetamidomethyl derivatives. In the sulphenylchloride cleavage reaction (Scheme 5) the initial product is the mixed disulphide (18) between the cysteine residue and *o*-nitrothiophenol. The disulphide (18) may be isolated and conceivably used in interchange reactions to establish disulphide bridges, or it may be reduced directly to the free thiol compound. Advantages claimed [43] for the use of *S*-t-butylcysteine in synthesis (over *S*-acetamidomethylcysteine) are simplicity of preparation (from cysteine, t-butanol, and dil. hydrochloric acid), freedom from contamination by thiazolidine (thiaproline) derivatives, and greater resistance to racemization.



(19)

Details have been published [45] of the preparation and use in synthesis of *S*-4-picolyl-L-cysteine (19). The picolyl group is cleaved by electrolytic reduction. Cystine bis-t-butyl ester has been prepared and used in synthesis of simple peptides.[46] The *S*-benzyl, *p*-methylbenzyl, and *p*-methoxybenzyl protecting groups have been used comparatively in solid phase synthesis (q.v.).[47] The substituted benzyl derivatives were preferred.

The sulphydryl group of cysteine has been shown to substitute into the phenolic ring of tyrosine residues (Scheme 6) under vigorous acidic conditions.[48] It is



Reagents: 40% hydrobromic acid, 24 h

Scheme 6

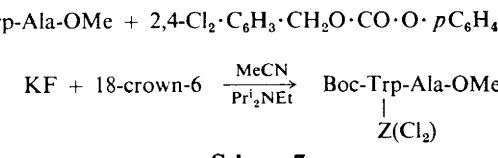
conceivable that this and similar reactions may be involved in irreversible aggregation phenomena sometimes observed when cysteinyl peptides are subjected to strongly acidic (hydrogen fluoride) deprotection conditions.

The sensitivity of the indole ring in tryptophan to adventitious oxidation and electrophilic substitution reactions has long been recognized. This undesirable reactivity is reduced by the electron withdrawing effect of N^{in}-acylation, and the N^{in}-formyl derivative has achieved some popularity in synthesis. A full account has now been published [49] of the introduction of other acyl groups into the indole nitrogen in the presence of unsolvated fluoride ion. Experiments using other bases for formation of the indolide cation, *e.g.* sodium hydride, were unsuccessful.

[45] A. Gosden, R. Macrae, and G. T. Young, *J. Chem. Res.* (S), 1977, 22.
[46] M. Joaquina, S. A. Amarcel, M. A. Macedo, and M. I. A. Oliveira, *J.C.S. Perkin I*, 1977, 205.
[47] D. H. Live, W. C. Agosta, and D. Cowburn, *J. Org. Chem.*, 1977, **42**(22), 3556.
[48] S. Ito and G. Potu, *J.C.S. Chem. Comm.*, 1977, 251.
[49] Y. S. Klausner and M. Choreo, *J.C.S. Perkin I*, 1977, 627.

Both the amino and carboxy groups of the tryptophan residue must be blocked and it is therefore convenient to introduce the indole protecting group at the dipeptide stage. A typical example is shown in Scheme 7. The dichlorobenzyloxycarbonyl derivative was preferred because of its greater acid stability. Cleavage is by catalytic hydrogenation, liquid hydrogen fluoride, or hydrazine.

A careful investigation in two laboratories has confirmed the danger of t-butylation of the indole ring of tryptophan during the introduction or cleavage



Scheme 7

of t-butyl groups elsewhere in the molecule.[50] Model experiments showed that N^{in}-tryptophan is the main product of alkylation, but that a range of mono- and poly-*C*-alkylated derivatives may also be formed. The reaction is potentially serious during the cleavage of t-butyl ethers and esters by trifluoroacetic acid, and especially so by liquid HF when the yields of alkylation products may be up to 50%. No comment is made on the effect of carbonium ion scavengers in this reaction.

The *o*-nitrobenzyl group has been introduced for the photo-labile protection of the phenolic hydroxyl function of tyrosine residues.[51] Efficient photolysis requires the presence of an aldehyde scavenging agent (*e.g.* semicarbazide hydrochloride) in the reaction medium. This also minimizes but does not eliminate formation of coloured by-products formed during the cleavage step. Details have appeared of the preparation and use in synthesis of *O*-4-picolyltyrosine.[45] The 4-picolyl protecting group is removed by electrolytic reduction. A kinetic study has been reported[52] of the *O*-acylation of side-chain unprotected tyrosine residues under peptide synthesis conditions.[52] The rate of side-chain acylation by *p*-nitrophenyl esters is dependent on base (triethylamine) concentration, whereas the rate of *N*-acylation is not. *O*-Acylation of tyrosine has been encountered during an active ester coupling in the insulin series.[53] In several simple model couplings, *O*-acylation of tyrosine, threonine, and serine derivatives was observed using Boc-glycine *p*-nitrophenyl ester. Benzyloxycarbonyl serine amide underwent *O*-acylation whereas the corresponding ethyl ester formed the dehydroalanine derivative by β-elimination.[53] Extensive serine *O*-acylation by activated ester derivatives has been encountered in histidine-containing peptides.[54] In subsequent model studies,[55] it was observed that *O*-acylation by both hydroxysuccinimide and *p*-nitrophenyl esters was extensively catalysed by added imidazole and to a lesser

[50] E. Wünsch, E. Jaeger, L. Kisfaludy, and M. Low, *Angew. Chem. Internat. Edn.*, 1977, **16**, 317.
[51] B. Amit, E. Hazum, M. Fridkin, and A. Patchornik, *Internat. J. Peptide and Protein Res.*, 1977, **9**, 91.
[52] S. K. Girin and Yu. P. Shvachkin, *Zhur. obshchei Khim.*, 1977, **47**(9) 2139.
[53] S. K. Girin and Yu. P. Shvachkin, *Zhur. obshchei Khim.*, 1977, **47**(5), 1182.
[54] Y. S. Klausner and H. Bodanszky, *J. Org. Chem.*, 1977, **42**, 147.
[55] M. Bodanszky, M. L. Fink, Y. S. Klausner, S. Natarajan, K. Tatemoto, A. E. Yiotakis, E. Athanasios, and A. Bodanszky, *J. Org. Chem.*, 1977, **42**, 149.

extent by hydroxybenzotriazole, but remarkably a mixture of the two catalysts largely suppressed *O*-acylation by the latter esters. Nevertheless, caution is clearly indicated in the widespread use of activated esters in minimum protection strategies, and the use of excess active esters in such situations is inadvisable.[55]

General Deprotection of Peptides. A further paper has appeared on the use of transfer hydrogenation for the cleavage of benzyl-based protecting groups.[56] Benzyl ester, ether, urethane, and *im*-benzyl groups are all cleaved by palladium–charcoal in the presence of cyclohexene, although the quantity of (re-usable) catalyst needed is quite large. The method is claimed to be superior over catalytic hydrogenation for the removal of the nitro group from nitroarginine, and is also useful for the removal of benzyloxycarbonyl groups from peptides having sulphur-containing amino-acids.

Catalytic hydrogenolysis of benzyloxycarbonyl groups in liquid ammonia solution has been used extensively in a recent synthesis of somatostatin.[44] The average yield for twelve successive hydrogenations was 87%; divalent sulphur (in *C*-terminal *S*-t-butylcysteine) was present at every step.

Ethane dithiol has been preferred to anisole and mercaptoethanol in the deprotection of *p*-methoxybenzyloxycarbonyl peptides by trifluoroacetic acid.[57] No coloration is produced with tryptophan-containing peptides by the trifluoroacetic acid–ethane dithiol combination.

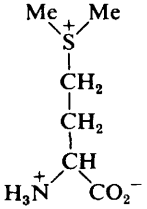
A careful study has been carried out on the use of dilute solutions of various alkyl and aryl sulphonic acids in acetic acid or methylene chloride for the cleavage of t-butoxycarbonyl and *p*-methoxybenzyloxycarbonyl groups.[58] Complete selectivity could be obtained over side-chain benzyl ester (aspartic acid) and benzyloxycarbonyl (lysine) groups. Cleavage of *p*-methoxybenzyloxycarbonyl groups was complete within 1 h at room temperature in the concentration range 0.5—4N, and of t-butoxycarbonyl groups within 3 h in the range 0.5—1.0N.

Methanesulphonic acid, in contrast to trifluoromethanesulphonic acid, does not appear to promote N^{ϵ}-benzylation during the cleavage of N^{ϵ}-benzyloxycarbonyl lysine.[59]

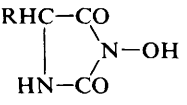
A full paper has appeared on the reaction between anisole and methionine peptides under acidic conditions used in deprotection reactions.[60] Anisole is cleaved quantitatively in 24 h by methanesulphonic acid or trifluoromethanesulphonic acid in the presence of methionine to form the *S*-methyl sulphonium cation (20). In the absence of methionine, no decomposition of the anisole takes place. The salt (20) and lesser amounts of the corresponding *S*-t-butyl or *S*-benzyl sulphonium derivatives were formed when *N*-t-butoxycarbonyl or benzyloxycarbonylmethionine was similarly treated with methanesulphonic acid. The importance of this side-reaction with anisole in the presence of other acidic reagents is apparently not known. It may be avoided in synthesis by use of methionine sulphoxide which is unaffected under these conditions.

[56] G. M. Anantharamaiah and K. M. Swanandaiah, *J.C.S. Perkin I*, 1977, 490.
[57] F. Tamura, H. Ogawa, N. Fujii, H. Yajima, K. Miyata, M. Nakamura, and A. Tanaka, *Chem. Pharm. Bull. Japan*, 1977, **25**, 767.
[58] H. Yajima, H. Ogawa, N. Fujii, and S. Funakoshi, *Chem. Pharm. Bull.*, 1977, **25**, 740.
[59] N. Fujii, S. Funakoshi, T. Sasaki, and H. Yajima, *Chem. Pharm. Bull.*, 1977, **25**, 3096.
[60] H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *Chem. Pharm. Bull.*, 1977, **25**, 2929.

Methionine sulphoxide may appear in synthetic peptides by either chance or design, and a detailed study[61] of its reduction back to methionine is therefore very welcome. Of the commonly used reagents (mercaptoacetic acid, dithiothreitol, and mercaptoethanol), mercaptoacetic acid was most effective at pH 3.5 and was approximately equiactive with dithiothreitol at pH 8.5. However, use



(20)



(21)

of mercapto-acids for reduction of sulphoxide in aqueous acetic acid solution caused substantial acetylation of amino-groups, presumably due to the formation of intermediate thioglycollides. This problem was circumvented by use of the new neutral reagent, *N*-methylmercaptoacetamide, which proved very satisfactory for the reduction of a range of peptides and proteins.

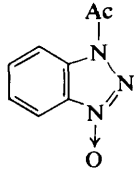
Sulphoxides are also reduced to sulphides in organic media under very mild conditions by hexamethyldisilthiane and hexamethylcyclotrisilthiane.[62]

A study of the electrochemical reductive cleavage of *N*-tosyl and benzyloxycarbonyl groups has appeared.[63]

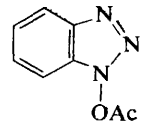
Formation of the Peptide Bond.—The 3-hydroxyhydantoins (21; R = Me and Me_2CHCH_2) have joined the list of hydroxylamine derivatives useful in peptide coupling reactions.[64] They are readily prepared from alanine or leucine *N*-carboxyanhydride and *O*-benzylhydroxylamine with cleavage of the benzyl group by hydrogenolysis or acidolysis. High yields of simple model peptides were obtained using preformed esters of both hydroxyhydantoins, or when they were added to dicyclohexylcarbodi-imide condensation reactions. An interesting feature is the presence of a chiral centre in (21) leading to the possibility of asymmetric induction in peptide bond formation. Reaction of the preformed ester of benzyloxycarbonyl-L-alanine with optically active (21; R = $Me_2CH.CH_2$) with excess DL-alanine ethyl ester gave seemingly optically pure L,L-dipeptide. This very high stereoselectivity is determined by both asymmetric centres in the activated carboxy component, since the preformed ester from benzyloxycarbonylglycine gave only 13% of the L-dipeptide when coupled with DL-alanine ethyl ester. A higher degree of selectivity was obtained in the latter case when the *N*-acetyl derivative of the hydantoin was employed.

Crystalline *N*- and *O*-acetyl [(22) and (23)] and phenylacetyl derivatives of hydroxybenzotriazole have been isolated and shown to form equilibrium mixtures in solution.[65] The corresponding benzyloxycarbonylglycine derivative also exists

[61] R. A. Houghten and C. H. Li, ref. 1, p. 458.
[62] H. S. D. Soysa and W. P. Weber, *Tetrahedron Letters*, 1978, 235.
[63] V. G. Mairanovskii and N. F. Loginova, *Bioorg. Khim.*, 1976, **2**(11), 1497.
[64] T. Teramoto, T. Kurasaki, and M. Okawara, *Tetrahedron Letters*, 1977, 1523.
[65] K. Horiki, *Tetrahedron Letters*, 1977, 1897.

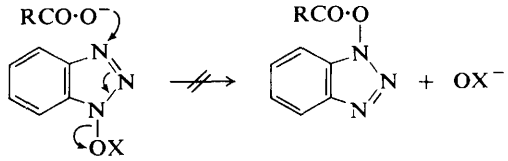


(22)



(23)

as a mixture of *N*-acyl and *O*-acyl forms in solution.[65] Mechanisms for the hydrolysis of acyl hydroxybenzotriazole derivatives have been discussed.[66] The use of hydroxybenzotriazole in ester forming reactions between acylamino-acids and alcohols, *e.g.* in attachment to soluble or insoluble polymers, is contra-indicated since *O*-alkyl derivatives of hydroxybenzotriazole may be formed.[67] Thus attempts to attach acylamino-acids to polyethyleneglycol using hydroxybenzotriazole esters gave a highly u.v. absorbing polymer.[67] Displacement reactions of *O*-substituted derivatives of hydroxybenzotriazole do not take place by attack at the opposite nitrogen (arrows, Scheme 8). Thus reaction between

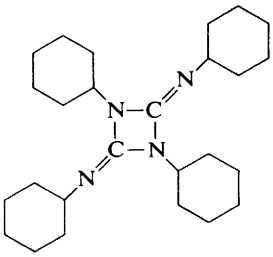


Scheme 8

^{18}O-labelled benzoate and benzotriazolyloxy(trisdimethylamino)phosphonium hexafluorophosphate [X = $-OP^{+}(NMe_2)_3PF_6^{-}$] to form the hydroxybenzotriazolyl benzoate ester resulted in equal distribution of label between this ester and hexamethylphosphoramide.[68] The reaction therefore takes place in two steps with initial attack at the phosphorous atom. A similar mechanism applies in the activation of carboxylic acids by Itoh's reagent (X = $ArSO_2$).[68]

The formation of *N*-acylureas in dicyclohexylcarbodi-imide-mediated coupling reactions has been studied quantitatively.[69] In the reaction between a benzyloxycarbonylamino-acid and glycine ethyl ester, *N*-acylurea formation varied in methylene chloride solution from 3.2% (leucine) to 14.6% (valine), and in tetrahydrofuran from 18.7% (phenylalanine) to 37.7% (valine). Other benzyloxycarbonylamino-acids gave intermediate values. The use of unsymmetrical carbodi-imides rather than the universal dicyclohexyl derivative is claimed to reduce *N*-acylurea formation.[70] In the coupling of 2,4-dinitrophenylsulphenylglycine with valine methyl ester, *N*-benzyl-*N'*-ethylcarbodi-imide gave 4% *N*-acylurea compared with 16% using dicyclohexylcarbodi-imide. More favourable results were also obtained with racemization studies using unsymmetrical carbodi-imides (p. 326). The dimer (24) is formed from dicyclohexylcarbodi-imide in the presence

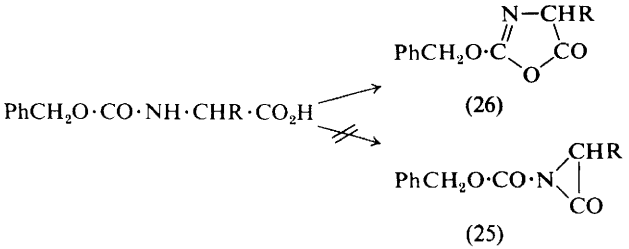
[66] K. Horiki, *Tetrahedron Letters*, 1977, 1901.
[67] B. Hemmasi and E. Bayer, *Tetrahedron Letters*, 1977, 1599.
[68] B. Castro, J.-R. Dormoy, G. Evin, and C. Selve, *J. Chem. Res.*, 1977, 182.
[69] J. Izdebski, M. Lebek, and S. Drabenek, *Roczniki Chem.*, 1977, **51**, 81.
[70] H. Ito, N. Takamatsu, and I. Ichikizaki, *Chem. Letters*, 1977, 539.



(24)

of hydroxybenzotriazole under peptide synthesis conditions but in the absence of amino and carboxy components.[71]

The formation of benzyloxycarbonylaziridinones (25) by cyclodehydration of benzyloxycarbonylamino-acids (Scheme 9) has been disputed.[72] The product from



Reagent: PCl_5–ether

Scheme 9

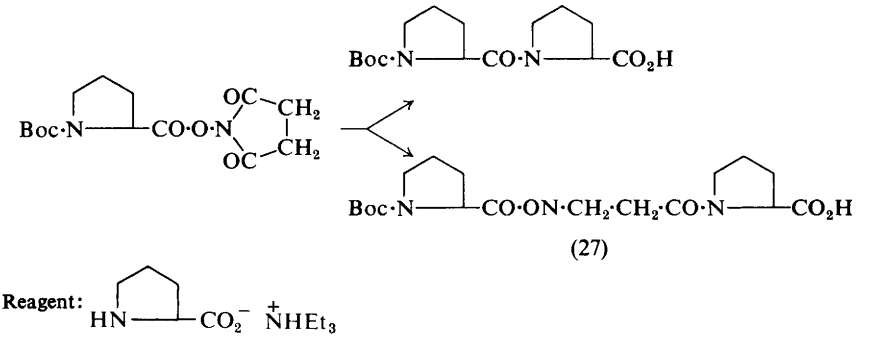
benzyloxycarbonylphenylalanine has been shown by ^{15}N–^{13}C coupling studies to be the alkoxyoxazolone (26). Thus the ^{15}N-labelled compound gave the same ^{13}C-n.m.r. spectrum as the unlabelled product, except that the ring C-2 and C-4 singlet resonances, but not that of the carbonyl group C-5, became doublets due to coupling with the nitrogen atom. The aziridone structure (25) would show ^{15}N–^{13}C coupling with three carbon atoms. The oxazolone (26) is the first to be derived from a benzyloxycarbonylamino-acid, and its formation cautions future assumptions that racemization of urethane-protected amino-acids by the oxazolone mechanism is entirely prohibited. In fact, it seems likely that asymmetric alkoxyoxazolones of this type will, in general, prove to be optically more stable than analogous oxazolones derived from other acyl (non-urethane) amino-acids.[72]

The use of large excesses of acylating species in solution (as opposed to solid-phase) peptide forming reactions sometimes leads to difficulties in eliminating unreacted acylating agent. This problem was overcome in the repetitive excess mixed anhydride (REMA) synthesis procedure by alkaline hydrolysis of excess mixed anhydride.[73] It has now been shown that the azide coupling procedure can be used in a similar manner with destruction of excess acylamino-acid azide by hydrolysis at pH 8.[74] Protection of serine and threonine side-chains is unnecessary,

[71] H. D. Jakubke and C. Klessen, *J. prakt. Chem.*, 1977, **319**(1), 159.
[72] J. H. Jones and M. J. Witty, *J.C.S. Chem. Comm.*, 1977, 281.
[73] M. A. Tilak, *Tetrahedron Letters*, 1970, 849.
[74] M. A. Tilak and J. A. Hoffman, *J. Org. Chem.*, 1977, **42**, 2098.

but other advantages of the procedure are not apparent with the very simple examples given. The mixed anhydride coupling procedure has been re-examined with special regard to problems likely to arise in the REMA and other minimal purification procedures.[75] Appreciable (up to 8%) attack at the 'wrong' carbonyl group of mixed anhydrides of t-butoxycarbonylamino-acids and isovaleric acid was observed under simulated REMA conditions, especially with sterically hindered carboxy components. Contamination of the final (protected) product with chain terminated isovaleryl derivatives must therefore be anticipated.

A major side-reaction has been encountered in use of hydroxysuccinimide esters for coupling with the triethylammonium salts of free amino-acids.[76] Reaction between t-butoxycarbonylproline hydroxysuccinimide ester and the triethylammonium salt of proline in dimethylformamide gave up to 40% of (27), clearly arising by attack at the succinimide carbonyl group rather than at that of the activated ester (Scheme 10). The analogous product was obtained when

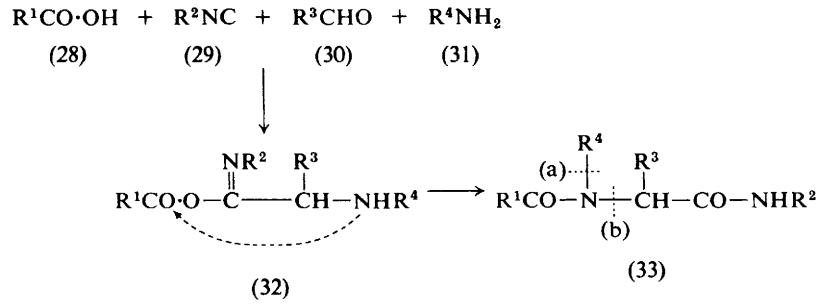


Scheme 10

thiazolidine-4-carboxylic acid was used in place of proline. This side-reaction may be particularly associated with formation of the difficult Pro–Pro sequence. It was not observed in a similar preparation of t-butoxycarbonylprolylglycine.

The present status of peptide synthesis by four component condensation has been reviewed by Ugi and his colleagues,[77] and a new side-product identified.[78] The fundamental process is assembly of carboxylic acid (28), isonitrile (29), aldehyde (30), and amine (31) components into an intermediate complex (32) which then undergoes rapid intramolecular rearrangement to (33) with formation of new amide bonds (Scheme 11). Two variants have been recognized. In 'four component synthesis' a new amino-acid residue is generated from the original amine (31) and aldehyde (30) components and appears in the centre of a tripeptide sequence (33), the first amino-acid residue corresponding to the carboxylic acid and the third to the isonitrile. For completion this synthesis requires cleavage of the unwanted substituent on the central nitrogen atom (cleavage a). 'Four

[75] M. Bodanszky and J. C. Tolle, *Internat. J. Peptide Protein Res.*, 1977, **10**, 380.
[76] J. Šavrda, *J. Org. Chem.*, 1977, **42**, 3199.
[77] I. Ugi, G. Eberle, H. Eckert, I. Lagerlund, D. Marquarding, G. Skorna, R. Urban, L. Wackerle and H. v. Zychlinski, ref. 1, p. 484.
[78] A. Gieren, B. Dederer, G. George, D. Marquarding and I. Ugi, *Tetrahedron Letters*, 1977, 1503.

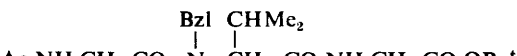


Scheme 11

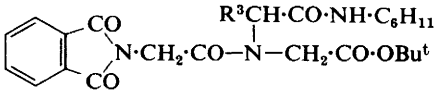
component synthesis' poses serious stereochemical problems since the central amino-acid residue derives from an achiral aldehyde and the last residue from an isonitrile of uncertain optical stability. These problems have been extensively studied and methods for asymmetric induction of the central residue developed.[77] The second variant, 'four component fragment condensation', treats the same rearrangement product (33) differently with elimination of all the atoms derived from the aldehyde and isonitrile units. Overall this procedure results in simple union of carboxy- and amino-components and the aldehyde–isonitrile combination functions as an intramolecular condensing agent. Completion of the synthesis by this route requires cleavage of the central N—C bond (cleavage b).

It is encouraging to see that aspects of both types of four component condensations have now been examined by a group other than the original discoverers.[79, 80] In four component synthesis of *N*-acetylglycyl-DL-*N*-benzylvalylglycine t-butyl ester (34) (a model target which avoids the stereochemical and *N*-substituent problems), yields were highly solvent dependent, ranging from 75% in methanol to 20—30% in a wide range of non-alcoholic organic solvents. Amine components other than benzylamine used in these studies gave generally poorer yields.

The 'four component fragment condensation' procedure was tested initially by preparation of the simple model dipeptide phthaloylglycylglycine t-butyl ester by way of the intermediate (35), and later by preparation of the tetrapeptide (36) by way of (37). Cyclohexylisonitrile and a range of aldehyde components were



(34)



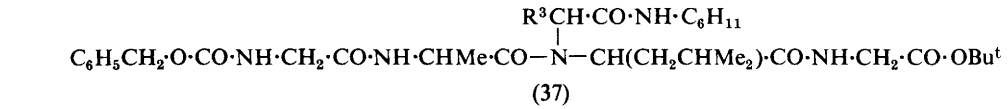
(35)

$Z \cdot Gly \cdot Ala \cdot Leu \cdot Gly \cdot OBu^t$

(36)

[79] M. Waki and J. Meienhofer, *J. Amer. Chem. Soc.*, 1977, **99**, 6075.
[80] M. Waki and J. Meienhofer, *J. Org. Chem.*, 1977, **42**, 2019.

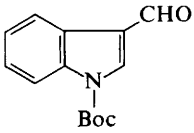
used, of which 2-nitrobenzaldehyde gave (35; R^3 = 2-nitrophenyl) and (37; R^3 = 2-nitrophenyl) in 67 and 71% yield respectively, and *N*-t-butoxycarbonyl-3-formylindole (38) gave (35; R^3 = *N*-Boc-3-indolyl) in 53% yield. Photolysis (cleavage b) of the nitrobenzaldehyde adducts gave the protected dipeptide (61%) and tetrapeptide (78%) derivatives while the indolic dipeptide derivative



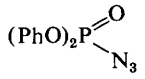
(37)

was similarly cleaved by trifluoroacetic acid in better than 80% yield. The authors conclude that their results warrant 'a continuation and expansion of efforts to further develop a more routine use of four component condensation in practical peptide synthesis'. There seems no doubt that the fragment condensation procedure is superficially much the most attractive of the two variants in a practical sense. The general concept of intramolecular peptide bond formation following prior association of amino- and carboxy-components does indeed warrant continuing study (see also Vol. 8 of these Reports, p. 263).

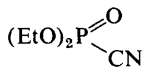
The use of phosphorous derivatives in peptide bond forming reactions continues to attract attention. The tertiary phospine–hexachloroethane combination [81] provides a new variant on the triphenylphosphine–carbon tetrachloride procedure. Coupling yields using triphenyl–phosphine or resin-bound phosphines were high but so was racemization in the Young and Anderson tests. Diphenylphosphoroazidate (DPPA) (39) and diethylphosphorocyanatidate (40) have been used as



(38)



(39)



(40)

coupling agents in synthetic studies on secretin,[82] VIP,[83] and GIP.[84] In the VIP study, a pentapeptide was prepared by a stepwise, racemization-free process as well as by fragment condensation using the phosphoroazidate. Identity of the two products suggests 'that fragment condensations by the DPPA method may be free of racemization'.[83] In the work on secretin, comparison of fragment couplings by the DPPA and azide procedure revealed 'that the former may be as good as the latter' (46% yield using DPPA; 51% by conventional azide coupling).

Several papers describe the use of phosphorus-containing [85] and other [87] peptide bond forming reagents for the preparation of thiol and selenol esters which are themselves of interest in peptide synthesis. The reagents studied include diphenylphosphoroazidate and diphenylphosphorocyanatidate,[85] di-

[81] R. Appel and L. Willms, *Chem. Ber.*, 1977, **110**, 3209.
[82] K. Ozawa, T. Shioiri and S.-i. Yamada, *Chem. Pharm. Bull.*, 1977, **25**, 122.
[83] Y. Hamada, T. Shioiri, and S.-i. Yamada, *Chem. Pharm. Bull.*, 1977, **25**, 221.
[84] Y. Hamada, S. Rishi, T. Shioiri and S.-i. Yamada, *Chem. Pharm. Bull.*, 1977, **25**, 224.
[85] Y. Yokoyama, T. Shioiri and S.-i. Yamada, *Chem. Pharm. Bull.*, 1977, **25**, 2423.
[87] H.-J. Gais, *Angew. Chem. Internat. Edn.*, 1977, **16**, 244.

phenylphosphoryl chloride,[86] dicyclohexylcarbodi-imide-hydroxybenzotriazole,[86] carbonyldi-imidazole and carbonylditriazole.[87] Good yields of thiol and selenol esters were readily obtained.

Water-soluble *o*-nitro-*p*-sulphophenyl esters have found application in synthesis in aqueous solution, as well as in dimethylformamide.[88] They may have additional application in partial synthesis of peptides and proteins where aqueous media are sometimes mandatory.

This year has seen a distinct revival of interest in peptide bond formation through the reverse action of proteolytic enzymes.[89–94] Indeed the use of enzymes as condensing agents offers in principle at least a number of advantages which could conceivably contribute to the outstanding problem of the union of large peptide fragments. Enzymic reactions are noted for their stereochemical and structural selectivity. In peptide bond formation this implies complete absence of racemization (even stereoselective synthesis from racemic components) and ready differentiation between α- and ω-carboxyl groups and possibly also between α- and ε-amino functions. Thus side-chain protecting groups may conceivably be dispensed with. On the other hand, the equilibrium between peptide bond formation and hydrolysis usually lies far on the side of hydrolysis for the common proteolytic enzymes, and some means has to be found for displacing this equilibrium towards bond synthesis. A number of recent examples have used precipitation from solution for displacement of this equilibrium. Thus stringent solubility limitations are presently imposed since the starting components must necessarily

Boc-Asn-Arg(NO_2)-Val-Tyr(Bzl) + Val-His(Bzl)-Pro-Phe-OEt

(41) (42)

↓

Boc-Asn-Arg-(NO_2)-Val-Tyr(Bzl)-Val-His(Bzl)-Pro-Phe-OEt

Reagent: papain

Scheme 12

be reasonably soluble in an aqueous or partially aqueous medium in which the enzyme is not denatured, and the product largely insoluble. Enzymes recently studied include papain[89, 90] thermolysin,[89] Nagarse,[89, 90] pepsin,[89, 91] and chymotrypsin.[92–94] A substantial number of small terminally protected peptides have been prepared, some in very high yield. However, one paper notes that the outcome of individual coupling reactions is at present unpredictable.[92] The outstanding achievement so far is enzymic synthesis of a protected angiotensin by combination of two protected tetrapeptide derivatives [(41) and (42) Scheme 12].[90] The product precipitated after incubation for 2 h at 38 °C with papain and after recrystallization gave the angiotensin derivative in 78% yield.

[88] Y. S. Klausner, T. H. Meiri, and E. Schneider, ref. 1, p. 536.
[89] Y. Isowa, M. Ohmori, T. Ichikawa, H. Kurita, M. Sato, and K. Mori, *Bull. Chem. Soc. Japan*, 1977, **50**, 2762.
[90] Y. Isowa, M. Ohmori, M. Sato, and K. Mori, *Bull. Chem. Soc. Japan*, 1977, **50**, 2766.
[91] A. Pellegrini and P. L. Luisi, ref. 1, p. 556.
[92] R. Saltman, D. Vlach and P. L. Luisi, *Biopolymers*, 1977, **16**, 631.
[93] P. L. Luisi, R. Saltman, D. Vlach, and R. Guarnaccia, *J. Mol. Catalysis*, 1977, **2**, 133.
[94] K. Morihara and T. Oka, *Biochem. J.*, 1977 **163**(3), 531.

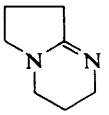
Racemization.—H.p.l.c. has been used to separate the diastereoisomers of benzoylphenylalanylalanine benzyl ester, providing a new sensitive racemization test.[95] A variety of coupling procedures both in solution and on a solid phase have been examined by this method.[95] Similarly, the ion-exchange separation of diastereoisomers has been extended to include the Merrifield–Dorman test tetrapeptide, Leu-Ala-Gly-Val.[96] The D-Leu and D-Ala diastereoisomers were separated from each other and from the all-L compound on a standard amino-acid analyser. No racemization was detected in the stepwise solid phase synthesis of this peptide.[96] Racemization in the oxidation–reduction condensation and other methods has been studied through synthesis of a tetrapeptide from two dipeptides, and determination of D-*allo*-isoleucine in the product. The best results were obtained using the triphenylphosphine–2-mercaptopyridine *N*-oxide combination.[97] The effect of a range of isonitroso compound additives on racemization in synthesis by the carbodi-imide procedure has been studied.[98,99] Several simple isonitroso derivatives eliminated racemization completely.

The absolute value of *all* racemization tests on simple model compounds is to be questioned in view of results obtained in large practical applications (see, for example, p. 320). Racemization and peptide bond formation are concurrent competing processes. The extent of the former will be substantially affected by the rate of the latter. In the synthesis of large peptides, concentrations and hence bimolecular coupling rates are necessarily much reduced and racemization, even by reagents with a clean bill of health in many tests, may be severe.

Repetitive Methods of Peptide Synthesis.—*Solid-phase Synthesis.* Several accounts of solid-phase synthesis have appeared. One is 'a reassessment' – a survey of improvements which have taken place since an earlier review.[100] It discusses particularly the role of the solid support and the development of polar, polyamide-based resins. Another, whilst describing the basic features of solid-phase synthesis, also outlines areas where improvements are needed, again particularly in the nature of the support system.[101] Recent methodological developments have also been discussed [102] by Merrifield and his collaborators with special emphasis on new protecting groups and fragment condensation strategies.

Potassium salts of N^{α}-protected amino-acids have been used to form resin linkages in high yield.[103] Oxytocin and vasopressin were synthesized after attaching the carboxy terminal residue to the resin by this method. Salts of the complex amines (43) and (44) have also been utilized to form the benzyl ester resin linkage.[104] Good yields were obtained by use of 0.5 equivalent of (43) or (44) with the N^{α}-protected amino-acid in dimethylformamide at 50 °C for 48 h

[95] M. Goodman, P. Keogh, and H. Anderson, *Bioorganic Chem.*, 1977, **6**, 239.
[96] S. B. H. Kent, A. R. Mitchell, G. Barany, and R. B. Merrifield, *Anal. Chem.*, 1978 **50**(1), 155.
[97] R. Matsueda, H. Takahagi, and T. Mukaiyama, *Chem. Letters*, 1977, 719.
[98] J. Przybylski, H. Jeschkeit, and G. Kupryszewski, *Roczniki Chem.*, 1977, 939.
[99] J. Przybylski, H. Jeschkeit, and G. Kupryszewski, *Roczniki Chem.*, 1977, 949.
[100] R. C. Sheppard, ref. 7, p. 43.
[101] J. M. Stewart, in 'Polymer Grafts in Biochemistry', ed. H. F. Hixson and E. P. Goldberg, Dekker, New York, 1976.
[102] R. B. Merrifield, G. Barany, W. L. Cosand, M. Engelhard, and S. Mojsov, ref. 1, p. 488.
[103] Academia Sinica, Shanghai, *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao*, 1975, **7**, 23. Read in abstract only (*Chem. Abs.*, 1977, **86**, 90 224d).
[104] K. Suzuki and N. Endo, *Chem. Pharm. Bull.*, 1977, **25**, 1143.



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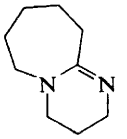
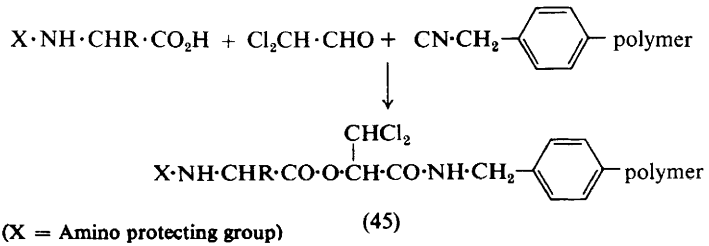


(44)

without detectable racemization. A novel way of attaching the C-terminal amino-acid to isocyanomethylpolystyrene according to Scheme 13 has been proposed.[105] The product (45) could presumably be used in chain elongation in the normal manner and the peptide finally cleaved from the resin by cobalt-I-phthalocyanine anion. *p*-Dimethylaminopyridine is achieving some popularity as a catalyst in the formation of benzyl esters from hydroxymethyl polymers and protected amino-acids. Imidazole has been proposed as an alternative catalyst.[106] Good results were obtained in the reaction between Boc-amino-acid active esters and hydroxymethyl resins in the presence of imidazole. No alloisoleucine was formed in the reaction of Boc-isoleucine indicating absence of racemization in this procedure.



(X = Amino protecting group) (45)

Scheme 13

A phenolic resin, obtained by deacetylation with hydrazine of an acetoxystyrene, styrene, and divinylbenzene copolymer, has been used in a versatile way to prepare enkephalin and some analogues.[107] The phenyl ester bond between the first residue (methionine) and the resin was formed from the symmetrical anhydride of the protected amino-acid in the presence of pyridine as catalyst. Unreacted hydroxyl groups were blocked by acetylation. The phenyl ester resin linkage is intrinsically reactive to aminolysis and after cleavage of Boc-protecting groups the resin was therefore neutralized in the presence of the acylating species. Removal of the final peptide from the resin was achieved by transesterification with dimethylaminoethanol. Alternatively, monomeric cyclic peptides could be obtained by intramolecular attack of the terminal amino group on the phenyl ester linkage. The rate of this last reaction was doubled in the presence of *p*-dimethylaminopyridine, but was inhibited by hydroxybenzotriazole.

A comparison of benzhydrylamine and chloromethylated polystyrene resins has been made in synthesis of oxytocin and some analogues.[108] The benzhydrylamine resin was obtained reproducibly by reductive amination of benzoylated

[105] I. Ugi, G. Eberle, H. Eckert, I. Lagerlund, D. Marquarding, G. Skorna, R. Urban, L. Wackerle, and H. v. Zychlinski, ref. 1, p. 484.
[106] M. Bodanszky and D. T. Fagan, *Internat. J. Peptide Protein Res.*, 1977, **10**, 375.
[107] D. Hudson, I. MacIntyre, R. Sharpe, M. Szelke, G. Fink, and G. W. Kenner, ref. 7, p. 269.
[108] V. J. Hruby, D. A. Upson, and N. S. Agarwal, *J. Org. Chem.*, 1977, **42**, 3552.

polystyrene. Similar yields of oxytocin analogues were obtained from the two resins, but a substantial improvement (29% to 49% yield) was noted when the benzhydrylamine polystyrene was used in conjunction with the 3,4-dimethylbenzyl group for cysteine protection. An *N*-methyl-benzhydrylamine resin has been used in the synthesis of neurotensin *N*-methylamide.[109] The *N*-terminal tetradecapeptide of lysozyme has been synthesized using ultrasonic waves for resin agitation.[110]

Side-chain phenacyl (see also Vol. 9, p. 324) and *p*-nitrobenzyl esters of glutamic acid have been advocated for solid-phase synthesis.[111] Both ester groups are retained during treatment with liquid hydrogen fluoride thus avoiding side-reactions arising from the presence of free carboxyl groups during this treatment. The *p*-nitrobenzyl ester can be removed finally by hydrogenation, and the phenacyl derivative is cleaved by the action of zinc and acetic acid. *p*-Halogenated benzyl-based side-chain protecting groups for serine, threonine, and aspartic acid have been prepared.[112] The halogen substituent increases the stability to acid compared with the unsubstituted benzyl ether or ester, but they are still removed efficiently by liquid hydrogen fluoride.

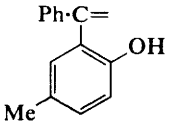
Protection of the sulphydryl group of cysteine has been a major problem in solid-phase synthesis and has probably not yet been fully resolved. Several *S*-protecting groups have been used in a comparative synthesis of apamin.[113] It now seems highly likely that liberation of sulphydryl groups during treatment with hydrogen fluoride commonly results in aggregation reactions (see p. 337) which seriously lower the yield of final products. There is therefore considerable interest in cysteine protecting groups which are retained during the HF reaction. t-Butylmercapto derivatives proved to be unstable during the HF reaction. Ethylmercaptocysteine proved to be more suitable, the *S*-protecting group being cleaved by reduction with tributylphosphine. Likewise acetamidomethylcysteine was retained until its final cleavage by mercuric acetate. Both derivatives gave substantially higher yields of biologically active apamin than did a comparable synthesis using *p*-methoxybenzylcysteine although it was noted that use of the last-named protecting group provided by far the easiest isolation of final peptide.

Use of *S*-benzyl, *p*-methoxybenzyl, and *p*-methylbenzyl protecting groups for cysteine have been compared in recent syntheses of oxytocin.[114] The two substituted benzyl derivatives gave equally good results with overall yields of 55% of HPLC homogeneous, fully active product. Inferior results were obtained using the simple *S*-benzyl protecting group, presumably due to the need to carry out the final hydrogen fluoride cleavage reaction at higher temperature. 2-Hydroxy-5′-methyl-α-phenylbenzyledine derivatives (46) have been suggested for the side-chain protection of lysine and ornithine.[115] In earlier investigations this group was found to be too stable for N^{α} protection in solid-phase synthesis,

[109] J. E. Rivier, L. H. Lazarus, M. H. Perrin, and M. R. Brown, *J. Medicin. Chem.*, 1977, **20**, 1409.
[110] S. Kumajae and Y. Shimonishi, *Bull. Chem. Soc. Japan*, 1977, **50**, 3073.
[111] K. Suzuki, N. Endo, and Y. Sosaki, *Chem. Pharm. Bull.*, 1977, **25**, 2613.
[112] D. Yamashiro, *J. Org. Chem.*, 1977, **42**, 523.
[113] J. Van Rietschoten, E. Pedroso Muller, and C. Granier, ref. 1, p. 522.
[114] D. H. Live, W. C. Agosta, and D. Cowburn, *J. Org. Chem.*, 1977, **42**, 3556.
[115] A. Abdipranoto, A. P. Hope, and B. Halpern, *Austral. J. Chem.*, 1977, **30**, 2711.

presumably because the aqueous 80% acetic acid is largely excluded from the polystyrene resin. However, the group is potentially useful for side-chain protection.

Several new alkyl ethers of tyrosine have been prepared and their value in solid-phase synthesis studied.[102] Their acid stability as reflected in percentage



(46)

loss per synthetic cycle, or hydrogen fluoride-induced rearrangement, has been measured (Table 1).

Table 1 *Acid-stability of Protected Tyrosine Derivatives* (iBor = *isobornyl*)

Compound	*Loss/Cycle* (TFA)	3-*Alkyltyrosine formation* (HF-*anisole*)
Tyr (iBor)	100%	0%
Tyr (Bzl)	0.76	15
Tyr (cHex)	0.006	0.5
Tyr (iPr)	0.00017	3.5
Tyr (2,6-Cl_2Bzl)	0.00014	5

The *O*-cyclohexyl derivative appeared to possess the best combination of properties.[102] Its acid stability was satisfactory for a multi-step process and although the extent of rearrangement was not zero, it was considered to be acceptably low for many purposes. This cyclohexyl ether was used in a solid-phase synthesis of angiotensin II and the unfractionated product shown to contain only 0.3% of the 3-cyclohexyl tyrosine isomer.

The diphenylphosphinothioyl protecting group (47) has been proposed for the solid-phase synthesis of tryptophan, containing peptides. This group can be removed by the same reagents as are conventionally used for the cleavage of Boc groups, but without the generation of reactive carbonium ions. Addition of triphenylphosphine to the acidic deprotection reagent was beneficial.[116] Two tripeptides (48) and (49) were synthesized using the oxidation–reduction condensation procedure in combination with this new protecting group.

(47)

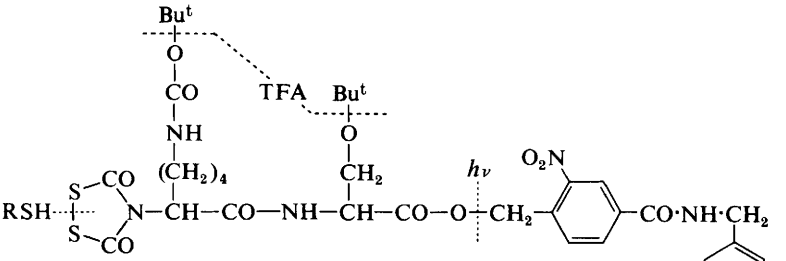
Ph_2PS-Ala-Trp-Gly-OMe (48)

Ph_2PS-Trp-Trp-Trp-OMe (49)

Practitioners of the solid-phase method have long realized the inflexibility dictated by the conventional methodology, especially with regard to the graded acid lability of N^{α}-protecting groups on the one hand and ω-protecting groups and resin linkage on the other. There is a clear need for a series of protecting groups and resin linkage capable of independent cleavage, preferably by mechanistically distinct processes. Such a combination has been termed 'orthogonal'.[102] Merri-

[116] M. Ueki and S. Ikeda, *Chem. Letters*, 1977, 869.

field and his colleagues have proposed [102] the use of the dithiasuccinoyl group [117] (see p. 312) for N^{α}-protection in combination with t-butyl-based side-chain derivatives and a photo-labile resin linkage for orthogonal protection, as in (*50*). If successful, such a combination could provide an approach to true solid-phase fragment condensation strategies (synthesis of protected peptide fragments on solid supports, detachment with all protecting groups intact, purification in

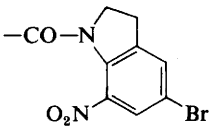


(50)

solution, and reassembly, again on a solid support (*cf.* p. 333). Further developments are awaited with great interest.

A new approach to photo-labile peptide–resin linkage is one involving photosolvolysis. An example of reactivity of this sort is provided by acyl derivatives of 5-bromo-7-nitroindoline (51).[118] Photosolvolysis of (52) and (53) in an aqueous–organic mixture containing acetic acid cleaved the indoline protecting group quantitatively and released some 80% of the free Boc-dipeptide acid. Photosolvolysis in benzyl alcohol-containing media gave varying proportions of benzyl ester and free acid.

Direct transesterification has also been used for the solid-phase preparation of protected peptide esters. Cleavage of resin-bound insulin B(24–30) heptapeptide by 1 M-triethylamine in benzylalcohol gave the corresponding benzyl ester in 44% yield which compared favourably with the same peptide obtained by solution methods.[119] Transesterification induced by potassium cyanide has been advocated as a method for removing fully protected peptides from Merrifield resins.[120] The



(51)

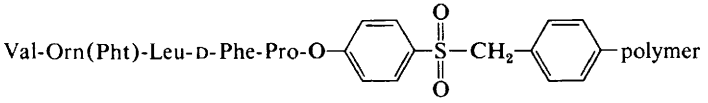
Boc-Gly-Val-Bni Boc-Leu-Ala-Bni

(52) (53)

[Bni = 1-(5-bromo-7-nitroindolyl)]

[117] G. Barany and R. B. Merrifield, *Fed. Proc.*, 1977, **36**, 864.
[118] G. Goissis, B. W. Erickson, and R. B. Merrifield, ref. 1, p. 559.
[119] G. Losse, B. Meisegeier, M. Maukr, and H. Klengal, *Tetrahedron*, 1977, **33**, 1993.
[120] G. Moore and D. McMaster, ref. 1, p. 518.

rate of release of methyl or benzyl esters of Boc-glycine and Boc-valine by 1% potassium cyanide in benzyl or methyl alcohols was enhanced in the presence of crown ethers. No racemization was detected in the transesterification procedure. The potassium cyanide–crown ether complex is also effective for the direct displacement of peptides from oxyacyl-type resins.[121] The cyclic dipeptide, diphthalyl-gramicidin-S, has been prepared by direct cyclodimerization of the resin-bound pentapeptide derivative (54).[122] Evidently interchain aminolysis of the reactive ester linkage occurs first followed by intrachain cyclization. In



(54)

relatively non-polar solvents the cyclic dimer is the main product whereas in polar solvents such as dimethylformamide substantial amounts of higher oligomers could be detected. This reaction has been applied to the synthesis of cyclic oligomers of a tripeptide sequence up to the cyclic 24-peptide.[123]

Two papers describing hydrogenolysis as a mild method of cleaving the benzyl ester resin linkage have appeared.[124, 125] Palladium(II) acetate dissolved in dimethylformamide is allowed to diffuse into the polystyrene resin before hydrogenolysis is carried out at 40 °C and 60 psi. Ninety per cent of leucine enkephalin was cleaved from a polystyrene resin by this procedure. Other catalysts such as bistriphenylphosphine palladium chloride and bistriphenylphosphine rhodium chloride, although soluble in dimethylformamide, did not catalyse hydrogenolysis.

An interesting development is the application of fluorenylmethoxycarbonyl derivatives in solid-phase synthesis.[15–17] Two groups of authors have recognized that the damaging effects of prolonged acid treatment, in cleavage of t-butoxycarbonyl derivatives and particularly in the use of very strong acids for detachment of synthetic peptides from resins may be minimized by use of this N^{α} protecting group. It has been employed in combination with tertiary butyl-based side-chain protecting groups and the equally labile *p*-alkoxybenzyl ester resin linkage; thus the only acid treatment necessarily required is the single final cleavage which takes place under mild conditions. Stepwise cleavage of the N^{α}-fluorenylmethoxycarbonyl group is achieved by treatment with 20%[15, 16] or 50%[17] piperidine or by the weaker secondary amine piperazine.[15] This new combination of protecting groups and resin linkage has been used both in a polystyrene-based synthesis of dihydrosomatostatin[17] and notably in a new synthesis of β-endorphin on a polydimethylacrylamide-based resin. Comparison with an earlier synthesis of β-endorphin on the same solid support but using conventional tertiary butyl and benzyl-based protecting groups and resin linkage shows a four-fold improvement in yield by the new procedure. A major con-

[121] J. P. Tann, W. F. Cunningham-Rundles, B. W. Erickson, and R. B. Merrifield, *Tetrahedron Letters*, 1977, 4001.
[122] M. Rothe, A. Sander, W. Fischer, W. Mastle, and B. Nelson, ref. 1, p. 506.
[123] L. T. Scott, J. Rebek, L. Ovsyanko, and C. L. Scrims, *J. Amer. Chem. Soc.*, 1977, **99**, 625.
[124] J. M. Schlatter, R. H. Mazur, and O. Goodmonson, *Tetrahedron Letters*, 1977, 2851.
[125] D. A. Jones, *Tetrahedron Letters*, 1977, 2853.

taminant, formation of which had been ascribed to degradation by the action of hydrogen fluoride, was almost completely absent in the more recent synthesis. It should be noted that the combination of N^{α}-fluorenylmethoxycarbonyl and tertiary butyl-based side-chain protecting groups with a benzyl ester resin linkage cleavable by hydrogenolysis (see above) constitutes a potential orthogonal system for fragment condensation. Preliminary experiments using the polydimethylacrylamide resin showed that hydrogenolysis was effective.

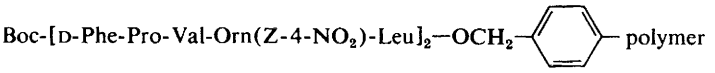
Fragment Condensation on Solid Supports. Probably the next important stage in the development of solid-phase methods will be the realization of practical fragment condensation strategies. Clear distinction should be drawn between stepwise assembly on solid supports of protected peptide fragments, themselves obtained by chemical solution synthesis, and similar assembly of solid-phase synthesized fragments. In the former approach, the solid-phase element is used primarily as an adjunct to solution synthesis providing essentially an aid to isolation and purification. Relatively few of the peptide bonds are formed in the solid phase and the advantages of speed and simplicity in the solid-phase method are lost. In the second, true solid-phase fragment condensation strategy, individual peptides of a length capable of complete purification are synthesized by stepwise solid-phase methods, cleaved from the resin with protecting groups intact, purified in solution, and reassembled again on a solid support. This approach takes full advantage of the favourable features of the solid-phase method and extends its applicability to areas where the completely stepwise assembly gives products incapable of adequate purification. It requires an 'orthogonal' (see p. 327) combination of protecting groups. Although some interesting proposals have been made in this direction, practical realization of a general method does not seem to have been achieved thus far. However, a number of examples have shown that the fragment condensation steps themselves may be achieved on solid supports in adequate yield using only moderate excesses of peptide components with dicyclohexylcarbodi-imide–hydroxybenzotriazole as the coupling agent.

The coupling of the dipeptide benzyloxycarbonylphenylalanylarginine onto a resin-bound 12-residue peptide has been investigated using a variety of condensing agents.[126] By far the most efficient was dicyclohexylcarbodi-imide in the presence of hydroxybenzotriazole in a dimethylformamide solution. A coupling yield of 96% was obtained with no racemization detectable by L-amino-acid oxidase digestion of an acid hydrolysate. This result is of particular promise for the solid-phase reassembly of appropriately protected tryptic fragments of natural proteins (p. 339). Condensation of a heptapeptide azide to a resin-bound 12-residue peptide gave a yield of 50—55%.[127]

Gramicidin S has been the subject of detailed comparative solid-phase syntheses using both stepwise and small fragment condensation strategies.[128] Assembly of the resin-bound decapeptide (55) was carried out by sequential addition of single amino-acid residues, dipeptide, or tripeptide units onto a leucyl-polymer, the dipeptide unit synthesis being completed using a single *N*-terminal residue.

[126] C. Di Bello, A. Marigo, O. Buso, and A. Lucchiari, *Tetrahedron Letters*, 1977, 1135.
[127] G. A. Zheltukina, M. V. Sidorova, E. I. Filippovich, and R. P. Evstigneeva, *Zhur. obshchei Khim.*, 1977, **47**, 1208.
[128] K. Sato, H. Abe, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 1999.

Four-fold excesses of the carboxy components were used with dicyclohexylcarbodi-imide as the coupling agent, hydroxybenzotriazole being added for the peptide additions. Better overall coupling yields were seemingly obtained in the fragment syntheses, but the gramicidin S samples finally obtained by all three routes were indistinguishable.



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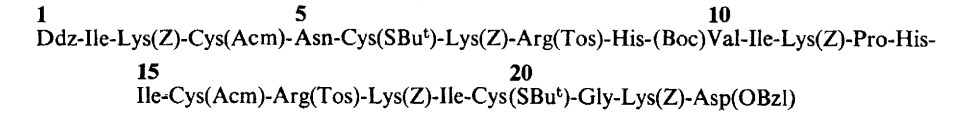
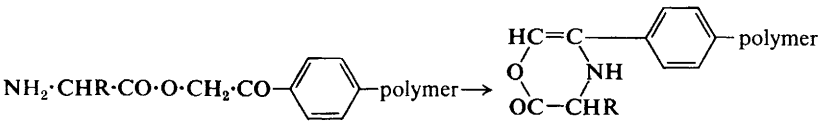


(56)

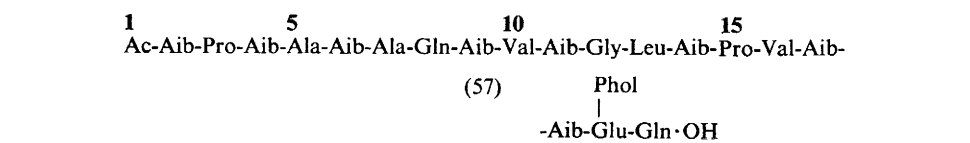
A solid-phase fragment assembly of the 22-residue bee venom constituent, mast cell degranulating (MCD) peptide (56), has been described.[129] Ddz-Amino-acids and a lightly cross-linked polystyrene gel were used. Individual protected peptide fragments were synthesized using a oxyacyl-type resin linkage with detachment by hydrolysis with Triton B. Complications were encountered in the early stages due to cyclization (Scheme 14) and diketopiperazine forming reactions of the



Scheme 14

somewhat activated phenacyloxycarbonyl resin linkage. Solid-phase assembly of the solid-phase synthesized fragments followed a (7 + 4) + (4 + 7) scheme with coupling yields of 53% and 24% for the two intermediate resin-bound fragments and 32% for the final coupling. The dicyclohexylcarbodi-imide–hydroxybenzotriazole method was used in all cases. This work was seemingly incomplete at the time of publication and results from the isolation, de-protection, and characterization of the synthetic product will be awaited with great interest.

The difficult 19-residue sequence of alamethicin has been tackled by fragment condensation methods.[131, 132] The precise structure of this membrane-active peptide is uncertain, both cyclic and open chain structures having been proposed. Eight residues of the highly hindered α-amino-isobutyric acid are present in the sequence (57) chosen as the initial synthetic target. On the other hand, the general



(57)

(Phol = phenylalaninol)

[129] C. Birr, M. Wengert-Muller, and A. Buku, ref. 1, p. 510.
[131] B. F. Gisin, S. Kobayashi, and J. E. Hall, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 115.
[132] B. F. Gisin, S. Kobayashi, D. G. Davis, and J. E. Hall, ref. 1, p. 215.

absence of amino-acid side-chains requiring protection provided an exceptional opportunity for use of a conventional *N*α-Boc-protecting group and benzyl ester resin linkage combination in a fragment condensation strategy. Fragments of length 4, 4, 9, and 2 residues were synthesized in stepwise fashion and solid phase assembled in a {[(4 + 4) + 9] + 2} sequence. Yields ranged from 33 to 71%. The product cleaved from the resin was not identical with natural alamethicin. A second synthesis was carried [132] out using as the target sequence a revised structure in which the phenylalaninol residue was moved to the carboxy terminus. The product this time was indistinguishable in chromatographic behaviour from the main component of natural alamethicin.

Crystalline glucagon has been obtained by solid-phase assembly of four chemically synthesized fragments.[133] Coupling efficiencies of better than 95% are claimed giving an overall yield of 17%.

Low yield (0.5% and 0.1%) syntheses of the 60 amino-acid residue Taiwan cobra venom cardiotoxin by stepwise solid-phase and 'solid-phase-assembly-of-short-peptides-synthesized-in-solution' strategies have been reported.[134]

Supports Used for Solid-phase Synthesis. An inorganic support based on silica gel has been prepared.[135] The gel coated with titanium oxide was treated with the silylating agent (58) and then chloromethylated in the usual way. The dipeptide Gly-Ala was prepared.

The Kel-F support grafted with polystyrene (see Vol. 8 of these Reports, p. 274) has been used in combination with polystyrene for simultaneous synthesis of peptides related to human 3a anaphylatoxins.[136] Separation of the polymers was by flotation of the lighter polystyrene in dichloromethane. By labelling the Kel-F-linked peptide with tritium and that attached to polystyrene with ^{14}C, separations

$$Cl_3Si{\cdot}CH_2{\cdot}CH_2{\cdot}Ph$$

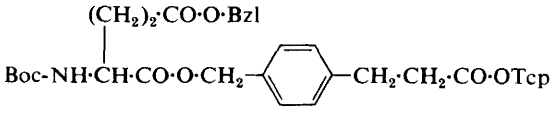
(58)

of better than 99% could be demonstrated. This is a useful approach which ensures that two related peptides are synthesized under very similar reaction conditions.

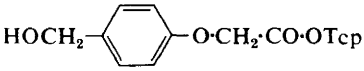
The value and versatility of polar polyamide supports have been further demonstrated by two syntheses of the 31-residue opiate human β-endorphin. In the first [137, 138] a spacer arm was first constructed on the amino functionalized polydimethylacrylamide resin by addition of further residues of β-alanine and norleucine. The latter provided a useful irreversibly attached internal reference amino-acid by which the efficiency of amino-acid assembly could be judged. A benzyl ester-type resin linkage was then established by use of the activated ester reagent (59) incorporating the first amino-acid residue of the β-endorphin sequence. Subsequent residues were added using preformed Boc-amino-acid

[133] Protein Synthesis Group, Academia Sinica, Shanghai, *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao*, 1975, **7**, 119. Read in abstract only (*Chem. Abs.*, 1977, **86**, 90 223c).
[134] Kung-Tsung Wang and Chi-Huey Wong, ref. 1, p. 528.
[135] V. N. Postnov, K. A. Makarov, V. B. Aleskovskii, and S. I. Kol'tsov, *Doklady Akad. Nauk S.S.S.R.*, 1977, **235**, 599. Read in abstract.
[136] T. E. Hugly and B. W. Erickson, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1826.
[137] E. Atherton, M. Caviezel, H. Over, and R. C. Sheppard, *J.C.S. Chem. Comm.*, 1977, 819.
[138] E. Atherton and R. C. Sheppard, ref. 1, p. 503.

anhydrides in the usual way, incorporation being monitored by ninhydrin tests, amino-acid analysis, and complete Edman degradation. It is noteworthy that coupling reactions appear to be exceptionally rapid using this support system, presumably because its polar character enables it to be used in combination with polar organic media, especially dimethylformamide. No repeated acylation or



(59)



(60)

deprotection reactions were necessary in the entire assembly, in contrast to common practice using polystyrene supports. After cleavage from the resin with liquid hydrogen fluoride and purification, an overall yield of 10% of biologically active β-endorphin was obtained.

In the second synthesis,[16] the same polyamide resin was employed but with a new combination of protecting groups and resin linkage designed to reduce the severity of repetitive deprotection and final cleavage reactions. A more acid-labile *p*-alkoxybenzyl ester resin linkage was employed, established through the linkage agent (60). The ester bond was formed by acylation with the Bpoc-derivative of γ-t-butyl glutamate in the presence of 4-dimethylaminopyridine. Subsequent residues save the last were added as the anhydrides of base-labile fluorenylmethoxycarbonylamino-acids, with 20% piperidine in dimethylformamide used for intermediate de-protection reactions. The synthesis was conveniently terminated with a Boc-amino-acid residue and then all the protecting groups and the resin linkage cleaved with a single trifluoroacetic-acid treatment. In this synthesis the very substantial degradation by hydrogen fluoride previously observed was avoided, and the overall yield increased four-fold to over 40%. Notable features of this synthesis are its mildness of reaction conditions and simplicity. The same solvent (dimethylformamide) is employed for all the repetitive coupling and deprotection reactions and no separate neutralization steps are required. The number of reagent addition and washing operations per cycle were reduced by half compared with the first Boc-amino-acid-based synthesis.

Techniques for Monitoring Solid-phase Synthesis. A double labelling technique has been used as an aid in assessing product purity in solid-phase synthesized apamin.[139] Incorporation of a ^{14}C-labelled residue at one end of the peptide chain and a tritiated residue at the other and purification to constant isotope ratio provides an indication of the removal of chain-terminated sequences. Single radiolabels have been used on several occasions as aids to isolation and other purposes,[107, 131, 136, 137] but a note of caution has been sounded in the case of

[139] W. L. Cosanel and R. B. Merrifield, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2771.

β-endorphin tritiated to the extent of 3.45 mCi/mmole in the penultimate residue (glycine).[137] The resin-bound peptide was apparently particularly susceptible to oxidation at the nearby methionine, and over a period of months was converted substantially to the sulphone form.

An orange dyestuff has been used for high sensitivity detection of amino-groups on a Merrifield resin and studies on acylation rates carried out with its aid.[140] Further details of a titration monitoring system which allows fully automatic feedback control of a solid-phase synthesizer have been published.[141] The picric acid [114, 131] and chloride titration method [128] continue to find use for quantitating free amino groups on resins. For assessing completion of acylation reactions, the qualitative ninhydrin reaction is now nearly universally applied. Low colour yields are sometimes observed with this reagent, *e.g.* with an *N*-terminal β-benzyl aspartate sequence.[142] In these cases use of fluorescamine has again been found advantageous.[142] Automated Edman degradation continues to find useful application for detecting amino-acid omission at levels of less than 1% in resin-bound [137] and free peptides [142, 143] (see also p. 338). The rationale of this technique applied to solidphase synthesized peptides has been carefully described.[142]

Side-reactions in Solid-phase Synthesis. Evidence continues to accumulate that the use of liquid hydrogen fluoride in the final stages of solid-phase syntheses can be excessively damaging, especially as synthetic targets become longer and more sensitive. The well-established conversion of glutamyl residues to anisyl ketones by hydrogen fluoride and anisole (see Vol. 8 of these Reports, p. 277) is not the only deleterious side-reaction. This particular transformation is associated with the appearance of u.v. absorbing, glutamic acid-free by-products, but anomalously u.v. absorbing species containing the full complement of glutamic acid have also been observed, sometimes in massive amounts (*e.g.* ref. 137, *cf.* ref. 16). Formation of side-chain phenyl esters is a possible explanation in view of the established de-alkylation of anisole under acidic conditions, particularly in the presence of methionine.[60] Difficulties with cysteine-containing sequences have also been frequently encountered and are mentioned again in the current literature.[108, 113] Generally these are characterized by the formation of intractable high molecular weight products. Although intermolecular disulphide formation has been considered as a possible cause of this aggregation, reduction by the usual disulphide cleaving reagents has often been found ineffective in your Reporters' laboratory. The recent observation [48] that under vigorous acidic conditions the sulphydryl group of cysteine may substitute into the phenolic ring of tyrosine is of interest in this connection, and it is attractive to suggest that similar reactions involving cysteine and tyrosine or tryptophan residues may occur in anhydrous hydrogen fluoride. Such substitutions might proceed through sulphenium ions formed by proton-induced cleavage of disulphides.

[140] A. Yu. Bilibin, N. Yu. Kozhevnikova, and G. P. Vlasov, *Zhur. obshchei Khim.*, 1977, **47**, 217. Read in abstract only.
[141] T. Christensen, P. Villemoes, and K. Brunfeldt, ref. 1, p. 569.
[142] G. W. Tregear, J. Van Rietschoten, R. Sauer, H. D. Niall, H. T. Keutmann, and J. T. Potts, jun., *Biochemistry*, 1977, **16**, 2817.
[143] M. Rosenblatt, H. T. Keutmann, G. W. Tregear, and J. T. Potts, jun., *J. Med. Chem.*, 1977, **20**, 1452.

Adventitious detachment of peptide from the resin support is a continuing problem increasing in severity as synthetic targets become longer. In a recent assembly of an immunoglobulin light chain variable region,[144] 82% of the peptide was cleaved from the resin. It was noted that repetitive de-protection by HCl–dioxan was superior to CF_3CO_2H–CH_2Cl_2 in this repect.

A careful analysis using automatic Edman degradation has thrown some light on by-products arising during an assembly of residues 44—68 of human parathyroid hormone.[143] This sequence contains two residues of glutamic acid (at positions 61 and 65), and partial cyclization to pyroglutamyl derivatives occurred at both. This was not observed for other regions of parathyroid hormone such as the 1—34 sequence, despite the presence of three residues of glutamic acid in the latter. A number of other short peptide derivatives were found in the product from the 44—68 HPTH synthesis, corresponding to chain termination in the residues 56—60 region. The cause of this chain termination was not established.

Other Repetitive Methods. A number of papers have reported use of repetitive methods in solution without isolation of intermediates, using either conventional or polymeric protecting groups. The repetitive excess mixed anhydride (REMA) method has been discussed by Tilak [145] and applied in the synthesis of various enkephalins.[146] The probability of contaminants arising through attack at the alternate carbonyl group has been investigated,[75] and the use of acid azides as alternatives to mixed anhydrides suggested.[74] Synthesis of angiotensin II in a two-part liquid system using a water-soluble carbodi-imide in conjunction with hydroxybenzotriazole has been described.[147] An overall yield of 74% was obtained. A photo-labile linkage has been used for attachment to soluble polyethyleneglycol.[148] Cleavage by irradiation at 350 nm was far more efficient than in the case of polystyrene. A repetitive method based on reaction between soluble polyethyleneglycol-bound peptide and insoluble polystyrene-bound acylamino-acid activated esters has been described in glowing terms, but no significant examples of its application are recorded in the paper.[149] In an alternative approach, insoluble α-polystyrylbenzyloxycarbonylamino-acids were coupled repetitively with the soluble amino-component. Cleavage of the polymeric *N*-protecting group liberates the peptide derivative into solution at each stage.[150] Leuteinizing hormone releasing factor has been obtained in 26% overall yield by this procedure using dicyclohexylcarbodi-imide–hydroxysuccinimide as the coupling reagent.[151] Soluble polymer-supported triphenylphosphine has been used in combination with carbon tetrachloride as a condensing agent in peptide synthesis.[152] Free amino-acids and peptides have been used as amino-components for chain extension with polymeric *N*-hydroxysuccinimide esters of Boc-amino-

[144] J. Burton, M. N. Margolies, and E. Haber, ref. 1, p. 525.
[145] M. A. Tilak, D. S. Wedel, and E. A. Sible, *Macromol. Synth.*, 1977, **6**, 7.
[146] A. R. Day, R. J. Freer, and D. I. Marlborough, ref. 1, p. 114.
[147] S. Nozaki, A. Kimura, and I. Muramatsu, *Chem. Letters*, 1977, 1057.
[148] F.-S. Tjoeng, W. Staines, S. St. Pierre, and R. S. Hodges, *Biochim. Biophys. Acta*, 1977, **490**, 489.
[149] G. Hensel, G. Borermann, W. Gohring, and G. Jung, *Angew. Chem.*, 1977, **16**, 642.
[150] F. Hartmut, H. Meyer, and H. Hagenmaier, *Chem.-Ztg.*, 1977, **101**, 188.
[151] F. Hartmut, H. Hagenmaier, E. Bayer, and D. M. Desiderio, ref. 1, p. 514.
[152] R. Appel and L. Willms, *J. Chem. Res.*, 1977, 84.

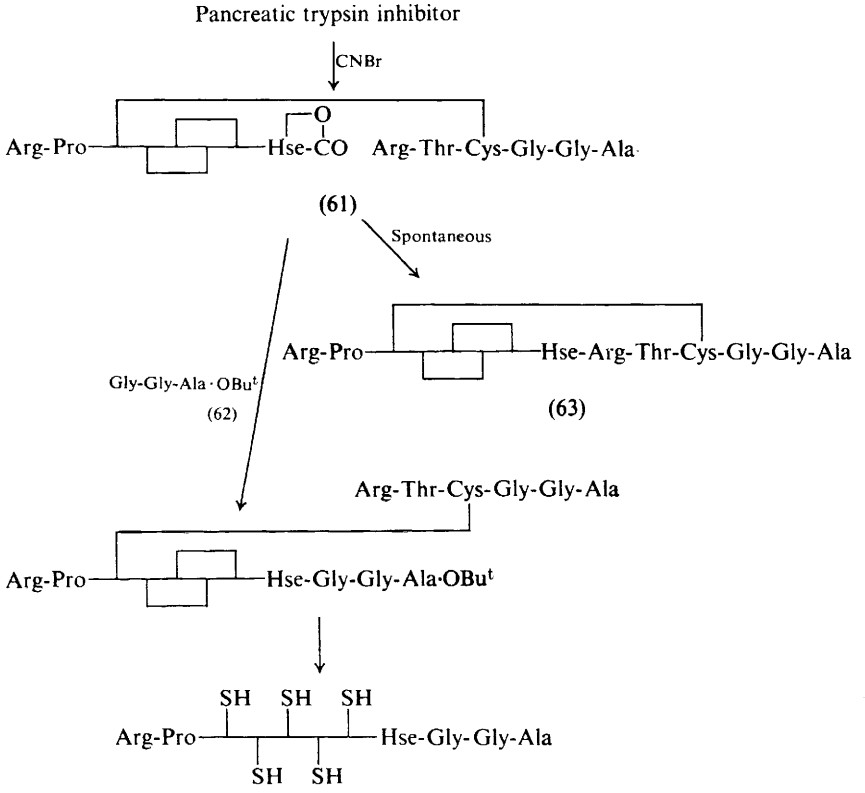
acids.[153] Dimethyl sulphoxide was used as the solvent in which the amino-component (*e.g.* triglycine) dissolved during three days in the presence of the insoluble activated ester.

Synthetic Operations with Peptides of Biological Origins.—The literature on peptide and protein partial synthesis is dominated this year by publication of the proceedings of the First International Symposium on Protein Semisynthesis.[154] This collection of 31 papers reflects increasing recognition of the potential of partially synthetic methods. Substantial progress is recorded in the insulin, myoglobin, cytochrome, and trypsin inhibitor fields. The general impression is that greatest success has been achieved in those cases where advantage has been taken of particular properties of individual proteins. Thus the specific interaction between trypsin and its inhibitors has enabled elegant amino-acid replacement reactions at the active sites of both the soybean [155, 156] and pancreatic [157] proteins. Reconstitution of cyanogen bromide-cleaved cytochrome *c* and the preparation of hybrid analogues has been achieved [158] by virtue of the spontaneous 'restitching' reaction between terminal homoserine lactone and amino-groups in non-covalently associated complexes. Partially synthetic studies on insulin have involved either selective acylation reactions of the individual terminal and side-chain amino-groups,[159–161] or modifications in the carboxy terminus of the B chain deriving from specific tryptic cleavage at the single arginine residue.[159, 162–164] Replacement of the amino-terminal 14-residue peptide in myoglobin has been achieved following cleavage at one of the two tryptophan residues in the protein.[165] Much less success has been obtained in attempts to utilize natural proteins in a more general partially synthetic way, *e.g.* by widespread proteolysis before or after side-chain protection, fragment separation, and reassembly studies (*cf.* these Reports, Vol. 9, p. 350). The search for general methods of protein partial synthesis may be particularly elusive. Detailed discussion of the individual contributions to this symposium which are collected in a single volume is, unfortunately, not possible within the space available in this Report.

Full details have been published [166] of studies on the use of protein fragments terminating in homoserine lactone in partial synthesis (see also Vol. 8 of these Reports, p. 284). Bimolecular reaction between cyanogen bromide-cleaved

[153] S. M. Andreev, V. A. Tsiryapkin, N. A. Sansoilova, N. V. Mironova, Yu. A. Davidovich, and S. V. Rogozhin, *Synthesis*, 1977, 303.
[154] 'Semisynthetic Peptides and Proteins', ed. R. E. Offord and C. Di Bello, Proceedings of the International Symposium on Protein Semisynthesis, Bressanone-Brixen, 1977, Academic Press, London, 1978.
[155] M. Laskowski, jun., ref. 154, p. 255.
[156] D. Kowalski, ref. 154, p. 263.
[157] H. Jering and H. Tschesche, ref. 154, p. 283.
[158] H. A. Harbury, ref. 154, p. 73.
[159] R. Geiger, V. Teetz, W. Konig, and R. Obermeier, ref. 154, p. 141.
[160] H.-J. Friesen, J. Weimann, J. Nowak, and D. Brandenburg, ref. 154, p. 161.
[161] D. J. Saunders, ref. 154, p. 213.
[162] H.-G. Gattner, E. W. Schmitt, and V. K. Naithani, ref. 154, p. 181.
[163] G. Weitzel, F.-U. Bauer, and A. Rehe, ref. 154, p. 193.
[164] R. Obermeier, ref. 154, p. 201.
[165] C. C. Wang, R. D. Dimarchi, and F. R. N. Gurd, ref. 154, p. 59.
[166] D. F. Dyckes, H. Kini, and R. C. Sheppard, *Internat. J. Peptide Protein Res.*, 1977, **9**, 340.

pancreatic trypsin inhibitor (61) and the model peptide (62) was effective provided that a high concentration (1 M) and excess of the peptide component was used in a denaturing medium (dimethyl sulphoxide or aqueous guanidinium chloride). Under these conditions, competition from intramolecular reaction to form the trypsin inhibitor analogue (63) was minimized (Scheme 15).

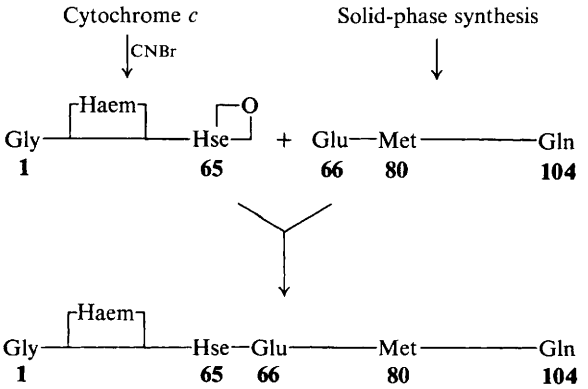


Scheme 15

In the case of cyanogen bromide-cleaved cytochrome c, the corresponding intramolecular reaction has been utilized in a partial synthesis of this protein from natural and synthetic fragments [167] (see also ref. 158 and Vol. 7 of these Reports, p. 281). The haem-containing 1—65 sequence terminating in homoserine lactone was combined with a totally synthetic preparation of residues 66—104 obtained by solid-phase synthesis. Evidence is presented for the formation of reconstituted (homoseryl) protein (Scheme 16) with spectroscopic and electron transfer properties indistinguishable from the original (methionyl) cytochrome. The overall yield is presently very low but this reflects also inadequacies in the solid-phase synthesis. The technique employed was a conventional one containing some features known to cause difficulties (*e.g.* use of benzyloxy-

[167] L. E. Barstrow, R. S. Young, E. Yakali, J. J. Sharp, J. C. O'Brien, P. W. Berman, and H. A. Harbury, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 4248. See also ref. 1, p. 136.

carbonyl side-chain protection for the eight lysine residues) and the actual content of the substantial target sequence might have been very low. No serious purification was carried out on the crude solid-phase product and no characterization reported. Reliance was placed on selectivity in the recombination reaction which is recognized as requiring specific non-covalent association between the two fragments prior to re-formation of polypeptide chain.

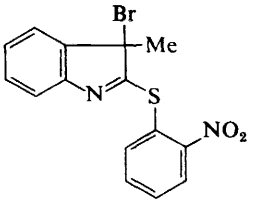


Scheme 16

A range of acylated methionine derivatives has been investigated as amino protecting groups cleavable by cyanogen bromide.[168, 169] The benzyloxycarbonyl derivative has been used for the temporary protection of B1, B29 amino-groups in insulin during the preparation of A1-substituted analogues.[170] Other routes for the preparation of A1-modified insulins have been summarized.[171] Partial syntheses of insulin derivatives by way of the hexamethyl ester have been further investigated and the formation of substantial imide from the *C*-terminal asparagine residue confirmed.[164, 172] Notwithstanding this serious side-reaction, which results ultimately in the formation of mixtures of A21 asparagine and isoasparagine derivatives, procedures involving saponification of insulin methyl esters are still being used for the preparation of analogues with sequence modifications in the B-chain.[173]

The *N*-terminal tetradecapeptide sequence of myoglobin has been removed and replaced using synthetic fragments.[165, 174] The fully acetimidylated protein was cleaved at Trp-14 by a large excess of 2-(2-nitrophenylsulphenyl)-3-bromo-3-methylindolenine (64) in the presence of phenol. The yield was 55—65%. Trp-14 was added as a single residue hydroxysuccinimide ester followed by the protected peptide azides corresponding to residues 6—13 and 1—5. The overall yield in the reconstruction steps was about 3%, but there seems to be no mention of removal of protecting groups from the reconstituted product.

[168] D. J. Saunders and R. E. Offord, *Biochem. J.*, 1977, **165**, 479.
[169] C. W.-T. Yeung, F. H. Carpenter, and W.-D. Busse, *Biochemistry*, 1977, **16**, 1635.
[170] D. J. Saunders and R. E. Offord, *Z. Physiol. Chem.*, 1977, **358**, 1469.
[171] R. Geiger, ref. 7, p. 27.
[172] H. G. Gattner and E. W. Schmitt, *Z. Physiol. Chem.*, 1977, **358**, 105.
[173] G. Weitzel, F.-U. Bauer, and A. Rehe, *Z. Physiol. Chem.*, 1977, **352**, 1573.
[174] F. R. N. Gurd, W. H. Garner, R. D. Dimarchi, and C.-C. Wang, ref. 1, p. 480.



(64)

Some transformations involving replacement of Trp-9 by phenylalanine in α-MSH are noted in this section,[175] although the starting material involved was in fact a synthetic sample (65; X = Trp), bearing terminal and side-chain protecting groups. This was cleaved with trypsin into the expected two fragments (66) and (67; X = Trp) which were separated and the latter degraded by one cycle of Edman degradation. The methylsulphonylethyloxycarbonyl protecting group on the lysine side-chain is appropriately stable to the acidic degradation conditions. The degraded peptide (68) was extended by reaction with Boc-Phe-ONSu to give the analouge (67; X = Phe) identical with a totally synthetic sample. Reconstitution of the 13-residue peptide was achieved using dicyclohexylcarbodi-imide and hydroxybenzotriazole with a yield of 54%.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg—X—Gly-Lys(Msc)-Pro-Val-NH_2

(65)

Trypsin (X = Trp) ↓ ↑ DCCI/HOBt (X = Phe)

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg + X—Gly-Lys(Msc)-Pro-Val-NH_2

(66) (67)

PhNCS (X = Trp) ↓ ↑ Boc-Phe-ONSu

Gly-Lys(MSc)-Pro-Val-NH_2

(68)

Scheme 17

3 Syntheses Achieved

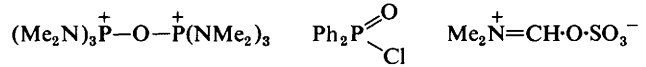
Lysozyme.—The Bakerian Lecture 'Towards Synthesis of Proteins', by G. W. Kenner, which was mentioned in last year's Report, has now been published.[176] It contains a masterly account of the problems intrinsic to protein synthesis, the minimization of these problems by judicious choice of target sequence, and the strategies to be adopted which, in the author's view, are most likely to overcome them. The target in the work described is a 129-residue analogue of lysozyme which contains 28 changes from the hen egg white sequence. In devising this revised sequence, the overall aim was to reduce the difficulties of the synthesis to

[175] J. W. Van Nispen, P. J. H. Smeets, E. H. A. Poll, and G. I. Tesser, *Internat. J. Peptide and Protein Res.*, 1977, **9**, 203.

[176] G. W. Kenner, *Proc. Roy. Soc.*, 1977, **B197**, 237.

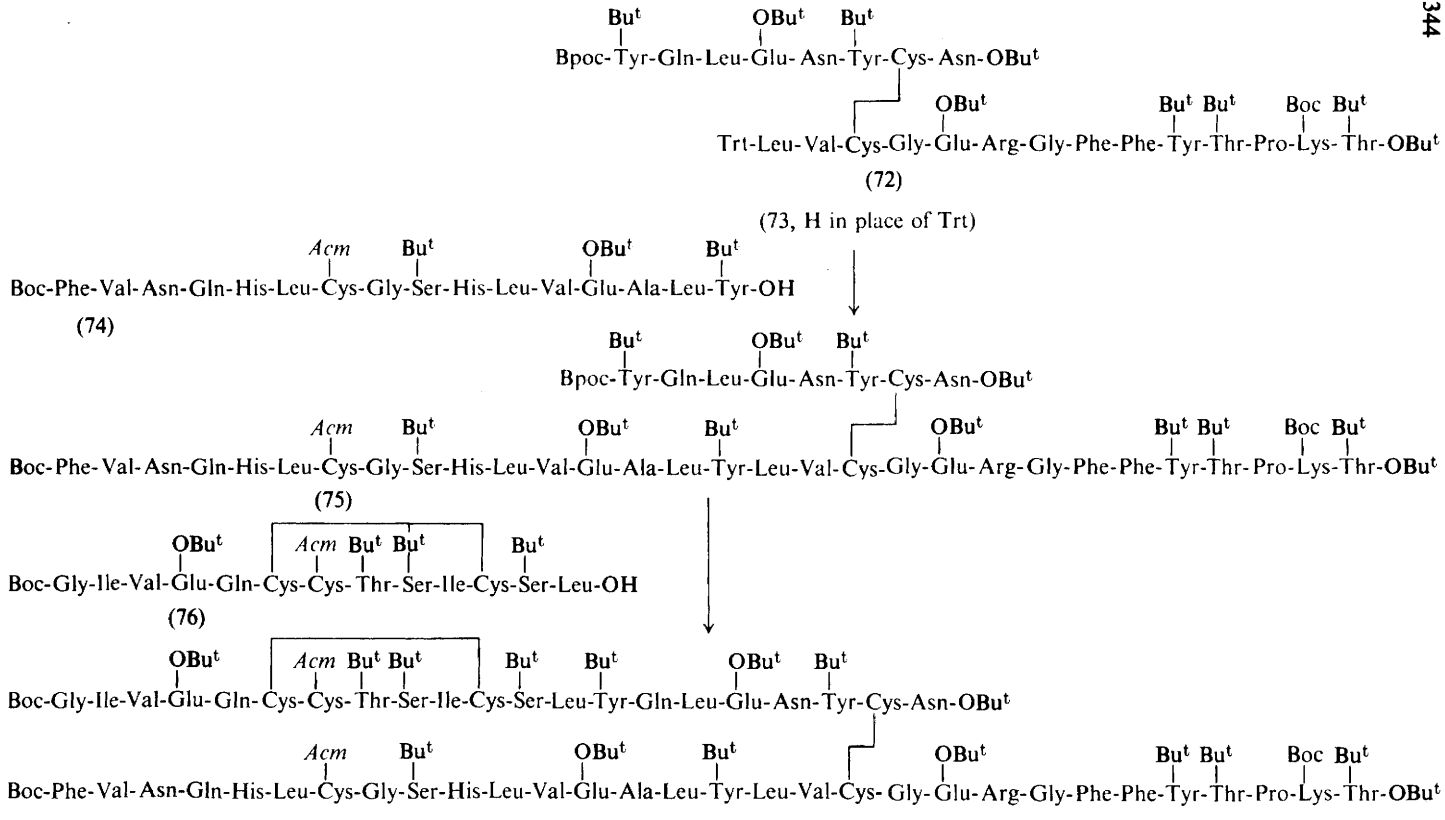
manageable proportions while still producing a molecule with essentially the same compact folded structure as the active enzyme. It was appreciated that while it was possible to satisfy the space filling requirements of a globular structure in a modified sequence, lack of information about the precise processes involved in the attainments of protein structures (*i.e.* protein folding mechanisms) left open the possibility of disruption of the mechanistic pathway. It was hoped that the study of this and similarly conceived analogues would in fact shed light upon the protein folding process. Individual amino-acid replacements were made after consideration of change and conservation within natural variants, factors favouring stability within highly ordered secondary structures (helices, *etc.*), and only after construction and study of a complete atomic model of the natural protein. This model enabled changes made in one part of the sequence to be sterically compensated by complementary changes elsewhere. No modification was permitted of residues thought to be involved in the enzymic process. Thus both methionine residues, the single histidine, and all but one of the surface arginine residues were replaced. Six additional glycines were introduced and also a number of unnatural residues (norleucine, norvaline) as aids to the formidable analytical problems anticipated.

The strategy of essentially complete side-chain protection was adopted; a seemingly wise choice in view of the increasing realization (*e.g.* these Reports, Vol. 8, p. 260) of involvement of free carboxyl groups, *etc.* in minimal protection strategies. The solubility problems consequently anticipated did not prove overwhelming, special devices such as chromatography on polyacrylmorpholide in *N*-methylpyrrolidone being used for purification in difficult cases. Particular care was taken in the selection of permanent side-chain protecting groups so as to confer adequate protection and complete stability throughout the very many synthetic operations involved. Thus side-chain t-butoxycarbonyl protection of lysine and ornithine was discarded in favour of the more stable adamantyloxycarbonyl derivatives. On the other hand, temporary protecting groups for terminal amino- and carboxy-functions were chosen with equal care to be removable under the mildest possible conditions. Benzyloxycarbonyl and biphenylisopropoxycarbonyl were used for α-amino groups and notably the labile phenyl esters for α-carboxy groups. The very ready cleavage of the latter at pH 10.5 in the presence of peroxide anion is an important design feature in view of the presence of multiple -Asp-Gly- and -Asn-Gly- units in the target sequence.



(69) (70) (71)

The 129-residue sequence was divided into 12 sections, each having glycine at its carboxy terminus. These have been assembled into two major fragments comprising residues 1—75 and 76—129. Dicyclohexylcarbodi-imide in combination with hydroxysuccinimide was used as the principal condensing agent, but other reagents are mentioned, notably the bisphosphonium salt (69), diphenylphosphinic chloride (70), and even the early dimethylformamide–sulphur trioxide complex (71), all of which are original introductions of this research group.

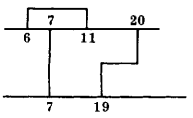

Bpoc-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OBut
Trt-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OBut
(72)
(73, H in place of Trt)
Boc-Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-OH
(74)
Boc-Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OBut
(75)
Boc-Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-OH
(76)
Boc-Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OBut
Acm
But
OBut

Scheme 18

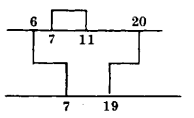
Which particular method will prove most suitable for the difficult union of the two macromolecular peptides thus far synthesized remains to be established, but the preliminary results are encouraging. The successful completion of this carefully conceived programme is awaited with the greatest of interest.

Insulin.—Full details of the end steps of the total synthesis of human insulin have now been published,[177] and clearly illustrate the finesse required in a synthesis of this complexity. Although the peptide [(72) Scheme 18] contains in all 12 acid-labile protecting groups including biphenylisopropoxycarbonyl and trityl derivatives, only the last was removed by pH-controlled acidolysis in trifluoroethanol. Coupling of the partially deprotected product (73) with the B(1—16) sequence (74) using DCCI/HOBt (which amongst modern methods has probably the cleanest bill of health in model racemization tests) resulted in some 60% racemization of the terminal tyrosine residue. Removal of the 30% D-tyrosine containing contaminant could only be achieved at the end of the synthesis. A significant side-reaction (*N*-amidino-imidazole formation) at the histidine residue B5 was also observed. Selective cleavage of the Bpoc-group in (75) was achieved by warming in trifluoroethanol, and the product coupled with the disulphide-containing A(1—13) sequence (76) again using DCCI/HOBt. This reaction proceeded much more smoothly with no evidence for racemization of the leucine at A13 and only slight side-reaction at the histidine residue. Treatment of the product with iodine under carefully controlled conditions before or after cleavage of all the acid-labile protecting groups caused closure of the 85-membered disulphide ring in over 70% yield. Pure human insulin (50%) was isolated after extensive purification by countercurrent distribution.

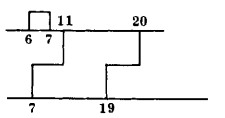
All previous syntheses of insulin have involved simultaneous formation of the three disulphide bonds through a general oxidation reaction. The stepwise formation of individual disulphides in the above synthesis permits specific variation in the cysteine pairings, and a recent paper [178] reports preparation of analogues (78) and (79) of human insulin (77) in which essentially the size of the disulphide loop in the A-chain is varied. The starting material was the previously obtained peptide



(77)



(78)



(79)

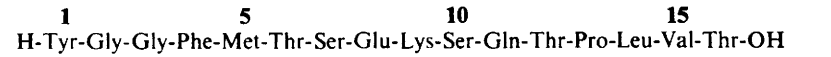
[(75) Scheme 18] containing the A20, B19 bridge, which was combined with disulphide variants of the A(1—13) sequence. Both synthetic isomers proved to be distinctly less stable than human insulin under alkaline conditions, especially in the presence of added thiol, and gave varying amounts of insulin and polymeric material. Under acidic and neutral (physiological) conditions all three species were of comparable stability, and had qualitatively indistinguishable biological

[177] P. Sieber, B. Kamber, A. Hartmann, A. Johl, B. Riniker, and W. Rittel, *Helv. Chim. Acta*, 1977, **60**, 27.

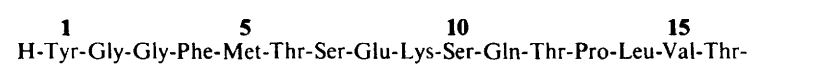
[178] P. Sieber, K. Eisler, B. Kamber, B. Riniker, W. Rittel, F. Marki, and D. de Gasparo, *Z. Physiol. Chem.*, 1978, **359**, 113.

activities in several test systems. Potencies of the analogues were less (15—37%) than those of natural insulin. It is concluded that the 'essential regions of the tertiary structure of these isomers may be similar to corresponding regions of the insulin molecule', but it is also conceivable that disulphide interchange initiated by thiol groups at or close to the receptor site may be responsible for the rather unexpected biological results.

Endorphin.—Interest in opiate peptides has continued, and the early syntheses of enkephalins and α (80), β (81), and γ (82) endorphins noted in last year's Report have been supplemented by several others. Two solid-phase syntheses of



(80)



(81)

(82)

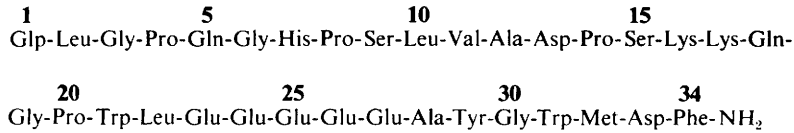
human β-endorphin using a polar polydimethylacrylamide support and in one case a mild procedure avoiding strongly acidic reagents have been described above (p. 335).[16, 137] A synthesis of the human hormone by a more conventional procedure has also been reported.[179] In this case a brominated polystyrene support was used, minimizing adventitious loss of peptide from the resin. Acidolytic cleavage of the benzyl ester resin linkage was only 0.03% per cycle compared with a more normal 1.4% for unbrominated polystyrene. A weak base (*N*-methylmorpholine) was employed for neutralization steps. An overall yield of 32% was obtained after cleavage of the complete peptide from the resin with liquid hydrogen fluoride and anisole and purification by gel filtration, ion-exchange, and partition chromatography. This is a truly remarkable result for a polystyrene-based synthesis involving hydrogen fluoride cleavage. Comparison between syntheses using and avoiding hydrogen fluoride have shown that resin-bound β-endorphin is often substantially destroyed by this reagent. The paper gives no clue as to the special technique employed which minimizes this destruction, but equally high yields (23—35%) have been reported from the same laboratory in similar syntheses of a number of analogues.[180]

Other solid-phase syntheses of human β-endorphin and some analogues have been described briefly.[181] Di-isopropylcarbodi-imide (which gives a more soluble

[179] C. H. Li, D. Yamishiro, L.-Fu. Tseng, and H. H. Loh, *J. Med. Chem.*, 1977, **20**, 325.
[180] D. Yamashiro, L.-F. Tseng, B. A. Doneen, H. H. Loh, and C. H. Li, *Internat. J. Peptide and Protein Res.*, 1977, **10**, 159.
[181] D. H. Coy, P. Gill, A. J. Kastin, A. Dupont, L. Cusan, F. Labrie, D. Britton, and R. Fertel, ref. 1, p. 107.

urea) was used as the coupling agent in an otherwise similar polystyrene approach. Overall yields were 10—15%. Syntheses of the 16- and 17-residue α- and γ-endorphins have also been reported.[182]

Human Somatotropin.—A solid-phase synthesis of an *S*-protected 54-residue *N*-terminal fragment has been described which has a number of features of general interest and importance.[183] The problem of reversible protection of cysteine residues was avoided in this synthesis since the biological objectives could be met through preparation of a stable *S*-carboxyamidomethyl derivative. Other side-chain protecting groups used were *o*-bromobenzyloxycarbonyl (lysine, tyrosine), tosyl (arginine), t-butoxycarbonyl (histidine), benzyl (serine, threonine, aspartic, and glutamic acids). Asparagine was introduced as its side-chain unprotected *p*-nitrophenyl ester. Substantial chain termination was encountered in a previous unsuccessful synthesis, only 39% of the amino-groups remaining free. Fractionation of this product gave low molecular weight material amounting to 33% of the total and consisting of a mixture of truncated sequences. Since these had a significant content of fluorine, their formation was ascribed to chain termination by trifluoroacetylation. Two principal changes in technique seem to have been adopted to minimize this serious side-reaction. Conventional coupling by *in situ* activation of t-butoxycarbonylamino-acids with dicyclohexylcarbodi-imide was replaced by use of symmetrical anhydrides preformed outside the resin-containing reaction vessel. This minimizes the possibility of contact of residual trifluoroacetic acid in the resin with activating carbodi-imide, and is a procedure now widely recognized as advantageous. Additionally, multiple washing procedures with base (di-isopropylethylamine) were used to facilitate removal of the last traces of trifluoroacetic acid. A further aliquot of base was also added during the course of the coupling reactions. These modifications seem to have been successful, and in the second synthesis a final resin was obtained which retained 68% of free amino-groups. From this resin the amide peptide was liberated by the action of hydrogen fluoride and subjected to extensive purification using Sephadex G-10 and G-25, isoelectric focusing, and partition chromatography on Sephadex G-50. An impressive range of criteria were used in attempting to establish the degree of homogeneity of the final product.



(83)

Human Big Gastrin.—This (presumed) prohormone (83) contains 17 *N*-terminal amino-acid residues additional to those present in the longer known 17-residue gastrins. As in other hormone–prohormone related peptides, two basic residues (lysine) are present at the junction of the two sequences. Two syntheses of the

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amino-acid sequence assigned to human big gastrin have been reported.[184, 185] The first [184] is an interesting collaborative effort between the Liverpool (Kenner and co-workers) and Munich (Wünsch and co-workers) groups, and reflects to some extent the individual prejudices and predilections of the two teams. Fortunately, these coincide in many respects. Wünsch and his colleagues have also completed a second, independent synthesis.[185]

In the first synthesis,[184] the *N*-terminal 1—19 sequence was prepared in Liverpool. The design principles involved are clearly analogous to those of the lysozyme work described above. The sequence was assembled from three fragments comprising residues 1—6, 7—12, and 13—19, each synthesized in stepwise manner from a *C*-terminal amino-acid phenyl ester. Benzyloxycarbonyl groups were used exclusively for N^{α}-protection with cleavage by hydrogenolysis, and acid-labile, notably adamantyloxycarbonyl, derivatives for side-chain protection. Active ester and pivalic mixed anhydride couplings were used during the stepwise assembly apart from the histidine residue (azide). The free fragments were combined by the DCCI/HOBt or HONSu procedures.

The 20—34 sequence was constructed in Munich using a somewhat more elaborate procedure. Hydroxysuccinimide esters of DCCI/HONSu were used exclusively for peptide bond formation, both in stepwise assembly and union of the intermediate 20—22, 23—27, 28—31, and 32—34 fragments. t-Butyl-based side-chain protection was used throughout. For N^{α}-protection, the differential acidic cleavage of *o*-nitrophenylsulphenyl derivatives was favoured although in the absence of methionine use was also made of N^{α}-benzyloxycarbonyl derivatives. Terminal carboxy groups were generally left unprotected. It is not obvious in which country the union of the two major fragments (by DCCI/HONSu) took place, but purification, using principally column electrophoresis, was clearly carried out in Liverpool.

The second (Munich) synthesis employs generally similar strategies.[185] Extensive use is made of hydroxysuccinimide derivatives in coupling reactions, and terminal carboxy-groups are commonly left unprotected. Acid-labile t-butyl-based side-chain protecting groups are used exclusively save for the single histidine residue. This was introduced as the *im*-unprotected N^{α}-benzyloxycarbonyl azide, but subsequent fragment condensation reactions evidently required side-chain protection of this residue. This was effected by reaction with adamantyloxycarbonyl fluoroformate at the octapeptide level. Although only published thus far in preliminary form, this paper gives a good impression of the scale of effort still required in syntheses of substantial sequences by solution methods.

4 Appendix 1: A List of Syntheses Reported during 1977

Natural Peptides, Proteins, Analogues, and Partial Sequences.—The syntheses are listed under the name of the peptide or protein to which they relate, as in previous years.

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[185] E. Wünsch, G. Wendlberger, A. Hallett, E. Jaeger, S. Knof, L. Moroder, R. Scharf, I. Schmidt, P. Thamm, and L. Wilschowitz, *Z. Naturforsch.*, 1977, **32c**, 495.

Peptide	*Ref.*
Adipokinetic hormone	
Locust adipokinetic hormone	1
Adrenocorticotropic hormone (ACTH)	
Human and porcine ACTH	2
[(3,5-$^3H_2$)Tyr^{23}]-Human ACTH	3
Human ACTH and [(3,5-I_2)Tyr^2]-, [(3,5-I_2)Tyr^{23}]-, and [(3,5-I_2)Tyr^{2,23}]-ACTH	4
Human and porcine ACTH-(22—39)-peptides	5
ACTH-(11—19)- and -(11—21)-peptides	6
N-Ethoxycarbonyl-ACTH-(6—17)-peptide	7
Anaphylatoxin	
Human C3a-(70—77)-, -(70—77)-Gly, -(65—77)-, and -(65—77)-Gly peptides	8
Angiotensin	
Angiotensin I and renin substrate	9
[Asp^1, Val^5, Ser^9]-Angiotensin I	10
Angiotensin II	11
[Asn^1, Val^5]-Angiotensin II	12
[Sar^1, Ser(Me)^8]-and four position 1 analogues of [Thr^8]-angiotensin II	13
[Trp^1, Val^5]-Angiotensin II	14
Five analogues of angiotensin II	15
Octanoyl-[Leu^8]-angiotensin II	16
Four analogues of [des-Asp^1]-angiotensin II	17
[Des-Asp^1, Ala^8]-Angiotensin II	18
Apamin	
[Orn^{13,14}]- and [Har^4]-Apamin	19
Apolipoprotein	
Apolipoprotein-A-II-(65—77)-, -(56—77)-, -(47—77)-, and -(40—77)-peptides	20
Asalin	
Some analogues	21

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Peptide	*Ref.*
Bradykinin	
Four position 8 analogues	22
Replacement of Arg with 4-guanidinophenyl-L-Ala	23
Analogues with photoactive labels	24
Bradykinin-potentiating peptides	25, 26
Bungarotoxin	
Some protected fragments	27
Cholecystokinin-pancreozymin (CCK-PZ)	
[Ser(SO_3H)^{27}]-CCK-PZ-(27—33)-peptide	28
Cobrotoxin	
[Tyr^{29}]- and [Ala^{24}]-Cobrotoxin-(16—62)-peptides and a peptide with cobrotoxin activity	29
Cytochrome *c*	
Horse heart cytochrome *c*-(66—76)-, -(66—80)-, -(77—87)-, and -(88—104)-peptides	30—32
Cytochrome *c*-(1—18)-haem-peptide	33
N-Terminal haem-peptide fragments	34
Parsnip cytochrome *c*-(56—60)-peptide	35
Eledoisin	
[11-Homocysteine]-eledoisin-(6—11)-peptide	36
Endorphin	
Human-β-endorphin	37, 38
[D-Tyr^1]-, [D-Ala^2]-, [D-Phe^4]-, and [D-Met^5]-Camel-β-endorphins	39
Some fragments of camel-β-endorphin	40

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Peptide	*Ref.*
Heptapeptide endorphin fragment	41, 42
Enkephalin	
[Leu^5]-Enkephalin	42—45
Seven [Leu^5]-analogues	44
[Met^5]-Enkephalin	18, 42, 45—48
Five [Met^5]-analogues	48
Some enkephalin amides	49, 50
Fibrinopeptide A	
Human fibrinopeptide A-(12—16)-peptide	51
Gastric inhibitory polypeptide (GIP)	
GIP-(1—10)-peptide	52
Gastrins	
Human big gastrin I and its [Leu^{32}]-analogue	53
[Leu^{15}]-(5—17)-Human gastrin, 'minigastrin', and a by-product	54
Three analogues of tetragastrin	55
Glucagon	56
Duck glucagon	57
Gonadotropin	
Human chorionic gonadotropin-(117—147)- and -(111—145)-fragments	58
Gonadotropin releasing factor (LRF/FRF)	59
Granuliberin-R	60, 61
Growth hormone (GH)	
[Cys(Cam)^{53}]-Human-GH-(1—54)-peptide	62

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Peptide	*Ref.*
Human GH-(172—191)-peptide	63
Haemoglobin	
β-Chain-S-(1—13) and related peptides	64
β-Chain-(70—77)-peptide	65
Histones	
Three fragments of histone fraction HI	66
Calf thymus histone fraction F2al-(35—40)-peptide	67
Histone fraction F2al modified by sarcolysine	68
Insulin	
[Leu^{B10}]-Human insulin	69
Two unnatural disulphide-bond isomers of human insulin	70
[Dab^{A2}, Glu^{A19}]-Sheep insulin	71
B-chain fragments	72—79
Lipotropin	
Three short fragments including analogues	47, 80
Luteinizing hormone releasing hormone (LH-RH)	81—83
A[D-Ala] analogue	81, 82
[Cpc^1, D-Phe^2, Pro^3, D-Phe^6]-, [Glu^1, D-Phe^2, Pro^3, D-Phe^6]-, and [D-Phe^2, Pro^3, D-Phe^6, des-Gly^{10}]-LH-RH ethylamides*	84
Ten analogues of [D-Ala^6, des-Gly^{10}]-LH-RH with substitution at positions 2 and 3	85

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* Cpc=cyclopentane carboxylic acid.

Peptide	*Ref.*
Ten analogues of [D-Phe^2]-LH-RH with either [D-Phe^6] or [D-Trp^6] and substituted at position 3	86
Six analogues substituted in positions 2, 3, and 6	87
Eight [D-Ala^6] or [D-Leu^6]-[des-Gly^{10}]-alkylamides	88
Some Orn analogues including a cyclic analogue	89, 90
Lysozyme	
Hen egg-white-(34—54)-peptide	91
Hen egg-white [Ala^8, Met (O)^{12}]-(1—14)-peptide	92
Mast cell degranulating peptide (MCD)	
Protected -(1—4)-, -(5—7)-, and -(8—11)-peptides	93
Melanocyte-stimulating hormone (MSH)	
Camel β_1 and β_2-MSH	94
Dogfish β-MSH	95
[Ser^{11}]-α-MSH	96
N^{α}-Bromoacetyl and N^{α}-diazoacetyl analogues of α-MSH	97
Melanostatin	
Eleven related peptides	98
Motilin	
Porcine motilin-(1—16)-peptide	99
Myelin protein	
Bovine-(114—122)-peptide	100
A Trp-containing fragment and two analogues	101
Neurotensin	
The (1—12)-peptide	102
Twenty-three analogues with D-residues at positions 3 and 11	103
Oxytocin	56, 104, 105
[Hse^4]-Oxytocin	106

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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.

Coded Amino-acids

Compound	*m.p./°C*	$[\alpha]_D$*	*Conc.*	*Solvent*	*Ref.*
Alanine					
Ala-OPh,TosOH	151—154	+7.5	1.74	H_2O	180
		−4.3	2.22	DMF	
Boc-Ala-OMaq	160—161				18
Boc-Ala-OPh	48	−54	1	EtOH	269
Nps-Ala-NH_2	199—201	−49.0†	1	DMF	268
Voc-Ala-OH,Dcha	152.5—153				235
Z-Ala-OBu^t	oil	−10.18	1	EtOAc	46
Z-Ala-OMpa	oil	−21.71	1	EtOAc	46
Z-Ala-NHNH-Boc	93—95	−48.2	1	MeOH	5
Z-Ala-OPh	96	−46	1	EtOH	269
Z-Ala-OTmse	oil	−23	1	EtOH	270
Z(OMe)-Ala-OQ(5-Cl)	132—133	−24.4‡	0.5	DMF	95
Arginine					
Boc-Arg(Tos)-OBzl	amorphous	+2.81	1	$CHCl_3$	46
TMZ-Arg(NO_2)	176—178	+20.7	1	MeOH	12
		+0.8	1	THF	
Z-Arg-OTmse,HCl	oil	−8	1	DMF	270
Asparagine					
Asn(Mbh)	193—195	+5[c]	1	MeOH	93
Boc-Asn(Xan)	183–184	+2.1‡		DMF	1
Boc-Asn-OBzl	120—122	−17.29	1	DMF	46
Voc-Asn	157.5—158.5				235
Aspartic acid					
Asp(OBu^t)-OBu^t	151—152 (d)	+6.5	1	MeOH	134
Asp(OBu^t)-OMe,TosOH	119—121 (d)	+11.0	1	MeOH	134
Asp[OBzl(Br)]	220—223	+14.1	2	80%AcOH	271
Boc-Asp(NHMe)-OBzl	108—109	−16.1	1	MeOH	242

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* The specific rotations listed were all determined at 20—25 °C and for the D-line except those distinguished by superscripts as follows: [a] 365 nm, [b] 546 nm, [c] 578 nm. The $[\alpha]_D$ values were assumed positive and determined within this temperature range if unspecified.
† At 30 °C.
‡ At 27 °C.

Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Boc-Asp[OBzl(Br)]	128.5—130	−15.5	2.08	DMF	271
Z-Asp(OBu^t)-OBu^t	oil				134
Z-Asp(OBu^t)-OTmse	oil	−13	1	EtOH	270
Cysteine					
Cys(Ac),HCl	122—123	−48.5 [b]	1	MeOH	272
		+7.5 [b]	1	DMSO	272
Cys[Bzl(2-NO_2)]	198—200				46
Cys(Pic)	209.5—211	+34	1.2	1M-NaOH	207
		−11	1	1M-HCl	207
Boc-Cys(Acm)-OTmse	oil	−27	1	EtOH	270
Boc-Cys[Bzl(4-Me)]	74				19
Boc-Cys(Bzl)-OPh	81	−27	1	EtOH	269
Boc-Cys(Pic)	137.5—138.5	−42	1.1	DMF	207
Boc-Cys(Pic)-OEt	solid				207
Boc-Cys(Pic)-OMe	71.5—73	−37.5	1.1	DMF	207
Boc-Cys(Pic)-OPcp	143—145	−41	1	DMF	207
Trt-Cys(Trt)-OTmse	foam	+60	1	EtOH	270
Z-Cys(Pic)	133—134	−47	1	DMF	207
Z-Cys(Pic)-OPcp	152—153.5	−13	1	AcOH	207
Glutamic acid					
Glu(OPac)	163—165	+10.3	1.8	AcOH	243
BMV-Glu(OBu^t),Dcha	139—140 (d)	+20.4	1	MeOH	134
Boc-Glu(NHMe)-OBzl	89—90	−24.2	1	MeOH	242
Boc-Glu(OBzl)-ONSu	106				256
Boc-Glu(OBzl)-O-2-PAOP	112—113	−17.9	0.25	$CHCl_3$	190
Boc-Glu(OPac)	101—104	−19.5	0.8	DMF	243
Nps-Glu(OBu^t)	172—173	−22.6	1	MeOH	134
Voc-Glu(OMe),Dcha	155—156				235
Z-Glu(OBu^t)-OTmse	oil	−16	1	EtOH	270
Z(OMe)-Glu[OBzl(NO_2)]	77—79	−11.7	0.9	DMF	243
Glutamine					
Boc-Gln-OBzl	108—110	−22.69	1	DMF	46
Nps-Gln,Dcha	186—188 (d)				134
Z-Gln-OTmse	78—80	−15	1	EtOH	270
Glycine					
Fmoc-Gly-OTrt	173—175				46
Nps-Gly-NH_2	166—168				268
Voc-Gly	95—95.5				235
Z-Gly-OMpa	oil				46
Z-Gly-OTmse	oil				270
Z(OMe)-Gly-NHNH-Troc	82—83				94
Various alkyl amides					242
Histidine					
BMV-His,Dcha	162—164 (d)	−122	0.5	DMF	134
Boc-His (Trt)	145—150	+10 [c]	1	MeOH	93
TMZ-His(Bzl)	181—183	+34.2	1	MeOH	12
		+12	1	DMF	12
Z-His-OTmse	oil	−12	1	EtOH	270
Isoleucine					
Ile-OPh,Tfa	176	+30.5	2	MeOH	269
Boc-Ile-OPh	oil	−30.4	2	EtOH	269

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Coded Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Leucine					
Leu-OPh,HCl	190	+22	2.03	Pr^iOH	180
Nps-Leu-NH_2	95—98	−24.8†	1	DMF	268
Tos-Leu-OBu^t	131—133	−31.9	0.47	DMF	114
Z-Leu-OTmse	oil	−22	1	EtOH	270
Lysine					
Lys(iNoc)	235—236	−7.32	0.7	2M-AcOH	141
Lys(Z)-NHEt,HCl	151—152	+25[b]	1	DMF	173
Lys(Z)-OBzl,HCl	140—141	−12.4‡	0.5	0.1M-HCl	273
Lys(Z)-OMe	111—113	+14.5[c]	1	MeOH	93
Lys(Z)-OPh,HCl	126—128	+21	1.58	Pr^iOH	180
Lys(Z)-OPh,Tfa	100	+22	2	MeOH	269
Boc-Lys(iNoc)	130—131	+7.49	0.6	2M-AcOH	141
Boc-Lys(Z)-OPh	oil	−22	1	EtOH	269
Boc-Lys(Hmpb)	oil				246
Mfoc-Lys(Mhoc)-ONp	solid				6
Nps-Lys(Boc), Dcha	195—196 (d)	—12.9	0.5	EtOH	134
Nps-Lys(Boc)-ONSu	oil				134
Nps-Lys(Tfa)-ONSu	oil				31
Nps-Lys(Z)-NHEt	126—127	+23.7[b]	1	DMF	173
Nps-Lys(Z)-OPcp	110—111	−59.4[b]	1	DMF	173
Voc-Lys(Boc),Dcha	152—154				235
Voc-Lys(Boc)-OBu^t	62—63.5				235
Z-Lys(Boc)-OTmse	oil	−10	1	EtOH	270
Methionine					
Met-NHNH-Boc	73	+15.5	1.02	MeOH	30
Met-OTmse,HCl	99—101	+14	1	EtOH	270
Boc-Met-OMaq	116—118				18
Boc-Met-OTmse	oil	−29	1	EtOH	270
Boc-Met-OPh	72	−43	1	MeOH	269
Mes-Met,Dcha	200—202	−5.0†	1	EtO	222
Nps-Met-NH_2	135—139				268
Z(NO_2)-Met,Dcha	178—179	+0.05	0.5	MeOH	222
Phenylalanine					
Phe-OPh,Tfa	160	+31.2	2	MeOH	269
Boc-Phe-OPh	91	−12.7	1	EtOH	269
Nps-Phe-NH_2	120—122	+36.6	1	DMF	268
Voc-Phe	56—58				235
Z-Phe-NHNH-Boc	105—110	−27	1	MeOH	274
Z-Phe-OTmse	48—49	−8	1	EtOH	270
Proline					
Boc-Pro-OPh	63	−66	1	EtOH	269
Iboc-Pro-NH_2	75.5—176	−83.7‡	1	EtOH	250
Nps-Pro-NH_2	192—194	−23.4	1	DMF	268
Nps-Pro-OTcp	93—96	−94.2	0.5	MeOH	275
TMZ-Pro,Dcha	165—168	−20	1	MeOH	12
Voc-Pro	93—94.5				235
Z-Pro-OTmse	oil	−48	1	EtOH	270

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† At 27 °C.
‡ At 28 °C.

Coded Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Serine					
Boc-Ser[Bzl(4-Br)],Dcha	147—149	+23	2.2	$CHCl_3$	271
Boc-Ser-OPh	oil	−30	1	EtOH	269
Boc-Ser-OTcp	104—105	−41 [c]	2	DMF	28
Voc-Ser(Bu^t)	97—97.5				235
Threonine					
Thr[Bzl(4-Cl)]-OBzl(4-Cl), hydrogen oxalate	192—193.5	−67	1.18	80% AcOH	271
Boc-Thr[Bzl(4-Cl)],Dcha	150—153	+26.5	2	$CHCl_3$	271
Boc-Thr-OTcp	85—88	−46.2	2	MeOH	159
Boc-Thr(Bzl)-OTcp	80—81	−20	1	DMF + 1% AcOH	159
Z-Thr(Bu^t)-OTmse	33—35	−6	1	EtOH	270
Z-Thr-OMe	88—89				276
Tryptophan					
Trp-OMe,HCl	200—201	+16.3	2	MeOH	277
Boc-Trp-OBzl	140—142	−2.02	1	EtOAc	46
Boc-Trp-OBzl(OMe)	100—102	−4.08	1	EtOAc	46
Boc-Trp-OPh	153	−11.6	1	EtOH	269
Ppt-Trp,Dcha	187—191	+7.5	1	EtOH	264
Z-Trp-OTmse	81—82	−5	1	EtOH	270
Tyrosine					
Tyr(Bzl)-OEt,HCl	190—192	+7.1	1	MeOH	278
Boc-Tyr(Me)	96—98	+29.7	2.1	EtOH	48
	94—95	+38.6	2	$CHCl_3$	279
Boc-Tyr(Z)	85—86	+17.1	4.1	EtOH	39
		+9.2	4	MeOH	39
Z-Tyr(Bu^t)-OTmse	66—67	−5	1	EtOH	270
Valine					
Voc-Val,Dcha	160—161.5				235

Other Amino-acids

L-*erythro*-2-Amino-3-bromobutanoic acid. The Z-,methyl ester	53—54				276
1-Aminocyclopropane-1-carboxylic acid. The Boc-, methyl ester	80.5—81.5				280
The Boc-, methyl ester of the *N*-methyl acid	oil				280
2-Amino-5-(*p*-methoxy)-phenylpentanoic acid (Amp)					
Amp-OBzl,TosOH	129	−27	2	$CHCl_3$	281
4-Aminophenylalanine					
Boc-Phe(NH_2)	126—128	+33.2	1	MeOH	23

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Other Amino-acids (*cont.*)

Compound	*m.p.*/°C	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
D-Aspartic acid					
Boc-D-Asp(OBzl)-NH_2	155—158	−5.32		AcOH	215
α-Aza-amino-acids (carbazic acids)					
Derivatives of azaglycine and azaphenylalanine					259
Derivatives of azaornithine					282
D-Azetidine-2-carboxylic acid (Aze)					
Nps-D-Aze,Dcha	143—148	+28.7	0.5	MeOH	275
Nps-D-Aze-OTcp	110.5—112.5	+110.4	0.5	MeOH	275
allo-4-Bromoproline					
The Z-,methyl ester	oil				276
Capreomycidine (Cpd)					
Cpd (NO_2)	250	+28.0	1	6M-HCl	283
Nps-Cpd(NO_2)	180—181	+31.3	2	DMF	283
γ-Carboxyglutamic acid (Gla)					
Various derivatives of the D- and L-acids					284
Cyclohexylalanine (Cha)					
Cha-OMe,HCl	156—157	−15.6	2	MeOH	132
Dehydroaminobutanoic acid					
The Boc-derivative	133—136				285
The Boc-,methyl ester	70—72				285
Dehydroleucine					
The methyl ester	oil b.p. 78—80/11 mm				286
The Boc-derivative	133—137				285
The Boc-,methyl ester	72—76				285
Dehydrophenylalanine					
The methyl ester	37—38				286
The Boc-derivative	172—174				285
The Boc-,methyl ester	77—79				285
3,4-Dehydroproline					
The Boc-derivative of the L-acid	94—96.5	−272.5	1.04	MeOH	150
The Boc-derivative of the D-acid	94—96.5	+273.8	0.99	MeOH	150
Other derivatives					150
Dehydrovaline					
The methyl ester	oil b.p. 76/11 mm				286
The Boc-derivative	192—194				285
The Boc-,methyl ester	75—77				285
2,4-Diaminobutanoic acid (Dab)					
Boc-Dab(Z),Dcha	102—104	0	0.5	DMF or MeOH	171

[282] C. J. Gray, K. Al-Dulaimi, A. M. Khoujah, and R. C. Parker, *Tetrahedron*, 1977, **33**, 837.
[283] T. Teshima, S. Nomoto, T. Wakamiya, and T. Shiba, *Bull. Chem. Soc. Japan*, 1977, **50**, 3372.
[284] W. Märki, M. Oppliger, P. Thanei, and R. Schwyzer, *Helv. Chim. Acta*, 1977, **60**, 798.
[285] H. Poisel, *Chem. Ber.*, 1977, **110**, 948.
[286] H. Poisel, *Chem. Ber.*, 1977, **110**, 942.

Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
3,4-Diaminobutanoic acid					
The bis-Boc-,ONSu ester	141—142	−34.4	1	DMF	287
2,3-Diaminopropanoic acid (Dap)					
Boc-Dap(Z),Dcha	179—181	0	0.5	MeOH	171
Boc-Dap(Z)-OMe	50—52	−8.8†	1	DMF	283
Boc-Dap(Z)-ONSu	90—92	−30.7‡	1.9	DMF	283
Z-Dap(Z)-ONSu	113—116	−36.2	1	DMF	287
3,4-Dihydroxyphenylalanine (Dopa)					
Several derivatives					217
3,5-Di-iodotyrosine (Dit)					
Boc-Dit	165	+16.1	2	MeOH	97
	187—189	−10.2	2	DMF	4
Boc-Dit[Bzl(3-Br)]	183—185	−10	2	DMF	4
Boc-Dit [Bzl(3-Br)]-OBzl-(3-Br)	114—115	−13	2	DMF	4
Boc-Dit-ONp	102	−16.9	1	MeOH	97
(4-Fluorophenyl)alanine					
Phe(F)-OMe,HCl	174—175	+15.1	3	MeOH	132
D-Glutamic acid					
Boc-D-Glu(OBzl)-NHMe	120—122	+1.8		MeOH	215
4-Guanidinophenylalanine					
The N^{α}-Boc-, N^{ω}-nitro-derivative	179—180	+12.3	1	MeOH	23
Homoserine (Hse)					
Boc-Hse	oil				106
Boc-Hse,Dcha	149—150	−3.5	2	DMF	106
Boc-Hse(Bzl)	oil				106
Boc-Hse(Bzl),Cha	145—147	+3	3	MeOH	106
4-Hydroxyproline (Hyp)					
Voc-Hyp, Dcha	179.5—181.5				235
Z-Hyp-OMe	82				276
A compendium of derivatives					288
allo-4-Hydroxyproline (*a*-Hyp)					
Z-*a*-Hyp	107				276
Z-*a*-Hyp-OMe	oil				276
Isoasparagine					
The Z-derivative	165—166	+4.5	0.7	AcOH	289
		−25.6	0.95	DMF	289
Isoglutamine					
The tosyl derivative	157—158	+25.2	1.5	DMF	289
β-Lysine					
Boc-β-Lys(Z)	89—91	+4.5† [a]	1	DMF	287
Boc-β-Lys(Z)-OMe	69—71	−5.1†	1	DMF	287
N-Methylalanine					
Boc-MeAla	93—94	−30.4	0.5	EtOH	290

[287] T. Wakamiya, T. Teshima, H. Sakakibara, K. Fukukawa, and T. Shiba, *Bull. Chem. Soc. Japan*, 1977, **50**, 1984.
[288] E. Adams, *Internat. J. Peptide Protein Res.*, 1977, **9**, 293.
[289] R. Straka and M. Zaoral, *Coll. Czech. Chem. Comm.*, 1977, **42**, 560.
[290] S. T. Cheung and N. L. Benoiton, *Canad. J. Chem.*, 1977, **55**, 906.

† At 14 °C.
‡ At 29 °C.

Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Z-MeAla	67—68	−25.8	1	EtOH	290
		−31.0	2	AcOH	290
N-Methylarginine					
Tos-MeArg	227—230	−16.1	0.49	5M-HCl	114
Z-MeArg		−15.2	0.71	1M-HCl	114
Z-MeArg(Tos)		−11.2	0.22	DMF	114
N-Methyldehydroalanine					
The Boc-derivative	80				290
3-Methylhistidine					
The methyl ester dihydrochloride	206—208 (d)	+14.97	1	MeOH	150
N-Methylisoleucine					
Boc-MeIle,Dcha	117	−45.6	0.5	MeOH	290
N-Methyl-D-*allo*-isoleucine					
Boc-D-Me-*a*-Ile,Dcha	94	+42.8	0.5	MeOH	290
N-Methylleucine					
Boc-MeLeu	56—57	−24.6	0.5	EtOH	290
Tos-MeLeu-OBu^t	oil	−31.9	0.52	DMF	114
Z-MeLeu	74—75				290
N-Methylornithine					
Tos-MeOrn	180—182	−50.5	0.42	DMF	114
N-Methylphenylalanine					
Boc-MePhe,Dcha	176	−25.5	0.5	MeOH	290
N-Methylserine					
Boc-MeSer(Bzl)	oil				290
N-Methylthreonine					
Boc-MeThr(Bzl)	oil				290
N-Methyltyrosine					
Boc-MeTyr(Bzl)	128—130	−24.9	1.0	90% AcOH	48
	oil				290
N-Methylvaline					
Boc-MeVal	58—59	−90	0.5	EtOH	290
Boc-MeVal,Dcha	113—114	−49.3	0.5	MeOH	290
Z-MeVal	70—71	−86.3	2	EtOH	290
3-(1-Naphthyl)alanine[Nal(1)]					
Z-Nal(1)	143—147	−56.5	1.2	MeOH	55
Z-Nal(1)-ONp	132—133	+1.8	1.1	EtOAc	55
3-(2-Naphthyl)alanine [Nal(2)]					
Z-Nal(2)	105—107	+6.5	1.1	MeOH	55
Z-Nal(2)-ONp	112—113	−4.2	1.1	EtOAc	55
(4-Nitrophenyl)alanine [Phe(NO_2)]					
Boc-Phe(NO_2)-NH_2	187—188	−8	1	DMF	216
Boc-Phe(NO_2)-ONp	187—188	−40	1	DMF	216
Norvaline					
Nva-OTse,HCl	125—126	+1.2	1	MeOH	97
Boc-Nva-OMe	oil	−30	1	MeOH	291
Boc-Nva-OTse	oil	−14.4	1.8	MeOH	97
Ornithine					
Orn(Tfa)	228—231				19
Boc-Orn(Chb),Dcha	170—173	+22	4	MeOH	246
Boc-Orn[Z(NO_2)],Dcha	89—90	+13.6	1	MeOH	292

[291] E. S. Psych and C. Toniolo, *J. Amer. Chem. Soc.*, 1977, **99**, 6211.
[292] K. Sato, H. Abe, T. Kato and N. Izumiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 1999.

Other Amino-acids (*cont.*)

Compound	*m.p./°C*	$[\alpha]_D$	*Conc.*	*Solvent*	*Ref.*
Boc-Orn[Z(NO_2)]-ONSu	87—88	−4.4	1	$CHCl_3$	292
β-Ornithine					
Boc-β-Orn(Boc)-ONSu	130—132	−13.8	1	DMF	287
Other β-amino-acid active esters					287
D-Phenylalanine					
D-Phe-OMe,HCl	157—160	+4.0	5	H_2O	167
D-Proline					
D-Pro-NH_2	95—97				152
Pyroglutamic acid					
Glp,NH_3	138—140	−9.9	0.2	MeOH	148
Tertiary leucine (2-amino-3,3-dimethylbutanoic acid)					
Several useful derivatives					151
1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid					
The Z-derivative	186—188	+56.3	1.0	MeOH	55
The Z-,ONp ester	69—70	+100.2	1.1	EtOAc	55
γ-5-Tetrazolyl-L-α-aminobutanoic acid					
The Z-derivative	83—84	−6.0	1	MeOH	267
The Z-,amide	193—194	+5.0	0.8	MeOH	267
The Z-,methyl ester	145—146	−14.5	1	MeOH	267
Thiazolidine-4-carboxylic acid (Thz)					
Boc-Thz	126—130	−99.0	1	MeOH	107
Boc-Thz-ONSu	130—131	−102.7	1	$CHCl_3$	107
D-Threonine					
Boc-D-Thr(Bzl)	114—116	−16.4	2.25	MeOH	49
D-Tyrosine					
D-Tyr (Z)	208—210 (d)	+9.1	2.14	80% AcOH	39
Boc-D-Tyr(Z)	84—85	−17.9	4.1	EtOH	39

4

Peptides with Structural Features not Typical of Proteins

BY B. W. BYCROFT AND A. E. FARUK

1 Introduction

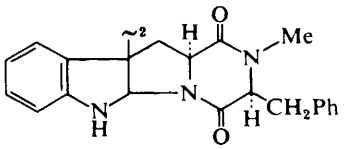
In company with the other chapters in this Report, the area encompassed by this particular title continues to grow at an ever-increasing rate. More new naturally occurring peptides with a multiformity of unusual features have been characterized, and the increasing size and complexity of some of them have led to the introduction of a new section covering large, highly modified, peptides. The majority of new peptides have been isolated from micro-organisms, but the numbers from plant sources, and significantly from marine organisms, are expanding. Although the amount of penicillin and cephalosporin chemistry is still growing at a prodigous rate, there is a detectable switch of emphasis to the new generation of highly active β-lactam antibiotics. In the areas associated with the well-established groups of peptide antibiotics, effort has been aimed at defining structure–activity relationships by the synthesis of various analogues. These, in many cases, have involved an interesting blend of special synthetic skills derived from both peptide and general organic chemistry.

2 Cyclic Peptides (Homodetic Peptides)

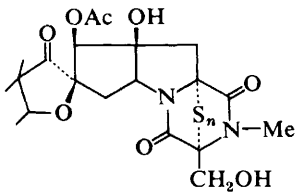
2,5-Dioxopiperazines (Cyclic Dipeptides).—The common occurrence of prolyl-dioxopiperazines, usually with the L,L-configuration, is illustrated by the recent isolation of several from *Streptomyces lavendulae*[1] containing primary amino-acids. *Cyclo*-(L-homoleucine-D-proline) which is clearly an exception has been found[2] in the sclerotia and culture filtrates of an ergot fungus, and is structurally significant in relation to the ergot peptides (see later). The dimeric cyclic dipeptide ditryptophenaline (1) was isolated in the course of an investigation on the mycotoxins of *Aspergillus flavus*.[3] Ditryptophenaline is itself non-toxic and structurally related to the dimeric sulphur-bridged dioxopiperazine chaetomin, first reported last year.

The epipolythiopiperazine system is common to an increasingly large group of fungal metabolites, the latest additions to which are the sirodesmins A, B, C, and G (2) and (3). The structures of these compounds rest upon the comparison of the

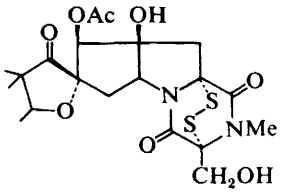
[1] A. Kubo, K. Takahashi, and T. Arai, *Experientia*, 1977, **33**, 12.
[2] S. Ohmomo and M. Abe, *J. Agric. Chem. Soc., Japan*, 1976, **50**, 543.
[3] J. P. Springer, G. Büchi, B. Kobbe, A. L. Demain, and J. Clardy, *Tetrahedron Letters*, 1977, 2403.



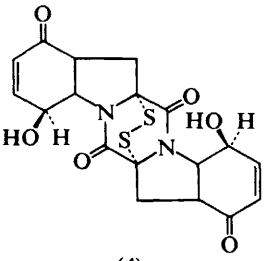
(1)



(2) (A) $n = 2$
(C) $n = 3$
(B) $n = 4$

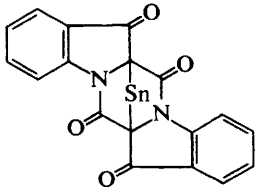


(3) (G)

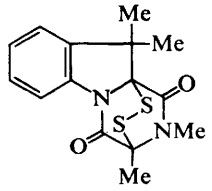


(4)

spectral and chemical properties with sirodesmin A whose structure was established by an *X*-ray analysis of the derived diacetate.[4] A crystallographic analysis has also confirmed [5] the structure of epicorazine A (4) and provides, in common with other analyses in this area, *i.e.* sirodesmin A and (1), detailed conformational information about the dithio bridged system. Synthetic approaches to these compounds and their analogues continue to attract attention and the derivatives (5) and (6) have been prepared.[6] The racemic gliotoxin analogue (7) which is a



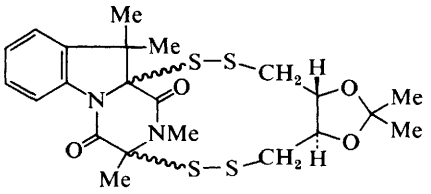
(5) $n = 2$
(6) $n = 3$



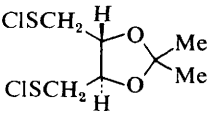
(7)

potent inhibitor of the enzyme reverse transcriptase has been resolved by separation of the diastereoisomers (8) prepared from (9) and the reduced disulphide.[7] Surprisingly both enantiomers, obtained after reductive cleavage of the protecting group and reoxidation, exhibited the same biological activity. This method of resolution probably has general application to synthetic dithio-bridged compounds.

[4] P. J. Curtis, D. Greatbanks, B. Hesp, A. F. Cameron, and A. A. Freer, *J.C.S. Perkin I*, 1977, 180.
[5] G. Deffieux, M. Gadret, J. M. Leger, and A. Carpy, *Acta Cryst.*, 1977, **B33**, 1474.
[6] D. L. Coffen, D. A Katonak, N. R. Nelson, and F. D. Sancilio, *J. Org. Chem.*, 1977, **42**, 948.
[7] H. C. J. Ottenheijm, J. D. M. Herscheid, and R. J. F. Nivard, *J. Org. Chem.*, 1977, **42**, 925.



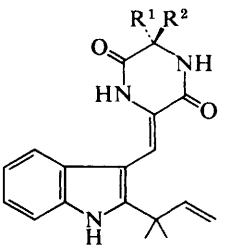
(8)



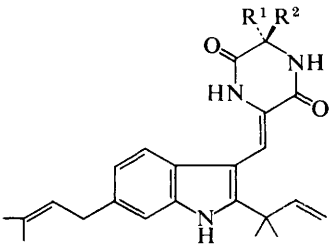
(9)

Five new neochinulin-type metabolites (10)—(14), isolated from *A. amstelodami*,[8] belong to the general group of cyclic dipeptides containing a prenylated tryptophan residue. Also significant is the presence of α,β-didehydroamino-acid residues in these molecules. *Cyclo*-L-Ala-L-Try, the parent compound of this series, has been found in *A. chevalieri* which also produces metabolites related to echinulin.[9] Roquefortine C, present in very small quantities in *Penicillium roqueforti* which is the mould used to produce various Roquefort-type blue cheeses, is identical with the metabolite (15) isolated from a related organism.[10]

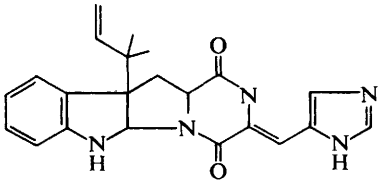
The extensive and successful synthetic programme, initiated some years ago on the ergot peptides, has now encompassed all the natural groups with syntheses of β-ergosine (16) and β-ergoptine (17) employing the now well-established route.[11]



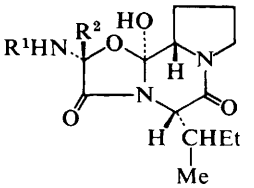
(10) $R^1R^2 = \rangle O$
(11) R^1 = H, R^2 = Me
(12) $R^1R^2 = \rangle CH_2$



(13) R^1 = H, R^2 = Me
(14) $R^1R^2 = \rangle CH_2$



(15)

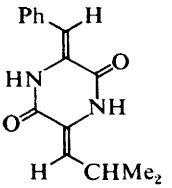


(16) R^1 = Lysergyl
R^2 = Me
(17) R^1 = Lysergyl
R^2 = Et

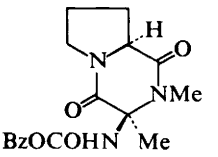
[8] R. Marchelli, A. Dossena, A. Pochini, and E. Dradi, *J.C.S. Perkin I*, 1977, 713.
[9] T. Hamasaki, K. Nagayama, and Y. Hatsuda, *Agric. and Biol. Chem (Japan)*, 1976, **40**, 2487.
[10] S. Ohmomo, T. Utagawa, and M. Abe, *Agric. and Biol. Chem. (Japan)*, 1977, **41**, 2097.
[11] P. A. Stadler, P. Stütz, and E. Stürmer, *Experientia*, 1977, **33**, 1552.

Chemical reduction of dihydroergotamine with lithium in liquid ammonia results in the selective reduction of the amide carbonyl in the dioxopiperazine ring.[12] The biosynthesis of the ergot peptides still continues to puzzle; the di- and tri-peptides corresponding to ergotryptine and ergotoxine are not used by intact mycelium of *Claviceps* for cyclol biosynthesis due, it is claimed, to prior hydrolysis by peptidase enzymes.[13]

Although albonoursin was isolated and synthesized some years ago, there has been renewed interest in this compound because of its antitumour activity. Synthesis of all four geometric isomers has established [14] beyond doubt that the natural material possesses the 3*Z*–6*Z* configuration (18). Catalytic reduction of didehydro-oxopiperazines containing an L-proline residue had previously been shown to afford a convenient asymmetric synthesis of L-amino-acids.[15] The



(18)



(19)

procedure has now been extended employing L-leucine as the chiral reagent.[16] Linear dipeptides containing an $\alpha\beta$-didehydroamino-acid system and L-proline cyclize in the presence of base to give substituted cyclic dipeptides [17] (19). A further synthetic route,[18] as well as mass spectral data [19] of cyclic dipeptides, have been reported.

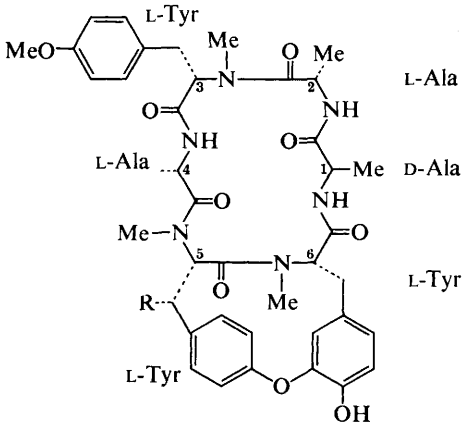
The substantial interest over the past few years on conformational aspects appears to have abated, although some work does continue in this area. A range of proline-containing cyclic dipeptides are claimed to adopt the same bowsprit conformation in both the crystal lattice and solution.[1] Spectral data on *cyclo*-D-Leu-L-Leu and *cyclo*-L-Leu-L-Leu is consistent with a twisted boat conformation,[20] and empirical energy calculations on *cyclo*-L-Pro-L-Pro and *cyclo*-D-Pro-L-Pro have indicated, perhaps not surprisingly, that different conformations are possible for each isomer, a conclusion which is borne out by experimental evidence.[21] The presence of *N*-methylamino-acid residues effects conformation, restricting the mobility of the ring and influencing the side-chain orientations.[22] *cyclo*-Sar-Sar forms a stable crystalline derivative with lithium perchlorate. The crystal structure of the 2 : 1 complex reveals an unusual planar conformation for

[12] L. Bernardi and G. Bosisio, *Experientia*, 1977, **33**, 704.
[13] A. Baumert, D. Gröger, and W. Maier, *Experientia*, 1977, **33**, 881.
[14] C. Shin, M. Hayakawa, K. Mikami, and J. Yoshimura, *Tetrahedron Letters*, 1977, 863.
[15] B. W. Bycroft and G. R. Lee, *J.C.S. Chem. Comm.*, 1975, 988.
[16] N. Izumiya, S. Lee, T. Kanmera, and H. Aoyagi, *J. Amer. Chem. Soc.*, 1977, **99**, 8346.
[17] E. Öhler and U. Schmidt, *Chem. Ber.*, 1977, **110**, 921.
[18] M. Oya and T. Takahashi, *Nippon Nogei Kagaku Kaishi*, 1977, **51**, 259.
[19] S. Eriksen and I. S. Fagerson, *J. Agric. Food Chem.*, 1976, **24**, 1242.
[20] M. M. Exner and R. J. Kostelnik, *Biopolymers*, 1977, **16**, 1387.
[21] D. Ajo, G. Granozzi, and C. Di Bello, *Biopolymers*, 1977, **16**, 707.
[22] B. Liberek, M. Bednarek, A. Kitowska, and A. Macikowska, *Roczniki Chem.*, 1977, **51**, 189.

the dioxopiperazine ring with the lithium at the centre of a tetrahedron co-ordinated to four carbonyl oxygens.[23]

Naturally Occurring Cyclic Peptides.—This section, as in previous years, deals with cyclic peptides other than 2,5-dioxopiperazines or compounds related to major groups of peptides described under the headings below. Biosynthetic aspects are normally only covered in general terms, but for readers interested in more detail relating to the biosynthesis of non-ribosomally formed peptides two excellent reviews have appeared.[24, 25]

In the past, cyclic peptides, with the exception of the so-called peptide alkaloids (see section 4), have been predominantly isolated from micro-organisms. Relatively few are found in plants and consequently the isolation of the novel cyclic hexapeptides (20) and (21) from *Bouvardia ternifola* (Rubiaceae) is of considerable



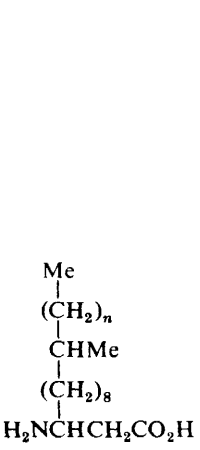
(20) R = OH
(21) R = H

interest, particularly since it exhibits high antitumour activity. The structure (20) was established by an *X*-ray analysis [26] and the molecule is unique among cyclic hexapeptides in that it contains a *cis* peptide bond. This is no doubt because it is part of a relatively strained 14-membered ring, the derivation of which is probably oxidative coupling of the phenolic residues of the adjacent tyrosines.

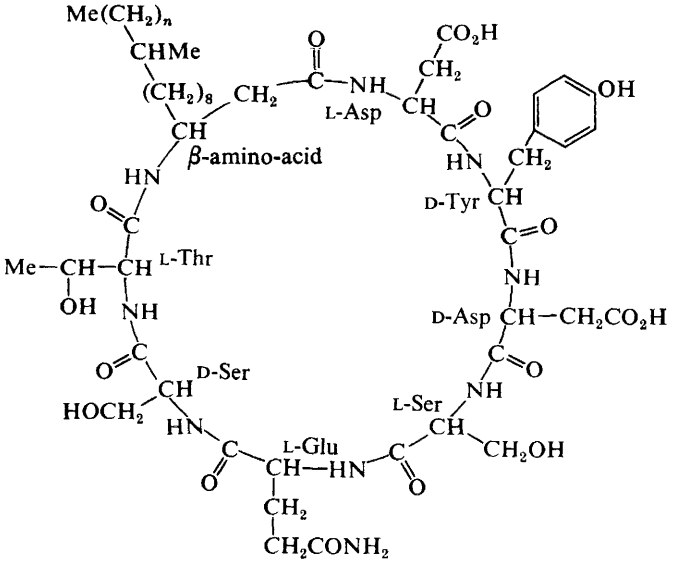
Micro-organisms still continue to provide the majority of new cyclic peptides. Echinocandin B from the fungus *A. rugulosus* is a hexapeptide constructed from L-amino-acids including the unusual 3,4-dihydroxyornithine. The terminal amino group of the latter is involved in the ring structure while the α-amino group is acylated with a linoelic acid residue.[27] The family of bacterial lipopeptides which

[23] N. Takahashi, I. Tanaka, T. Yamane, T. Ashida, T. Sugihara, Y. Imanishi, and T. Higashimura, *Acta Cryst.*, 1977, **B33**, 2132.
[24] E. Katz and A. L. Demain, *Bacteriol. Rev.*, 1977, **41**, 449.
[25] L. C. Vining and J. L. C. Wright, in 'Biosynthesis', Vol. 5, A Specialist Periodical Report, ed. J. D. Bu'lock, The Chemical Society, London, 1977, p. 240.
[26] S. D. Jolad, J. J. Hoffmann, S. J. Torrance, R. M. Wiedhopf, J. R. Cole, S. K. Arora, R. B. Bates, R. L. Gargiulo, and G. R. Kriek, *J. Amer. Chem. Soc.*, 1977, **99**, 8040.
[27] C. Keller-Juslen, M. Kuhn, H. R. Loosli, T. J. Petcher, H. P. Weber, and A. von Wartburg, *Tetrahedron Letters*, 1976, 4147.

includes the mycosubtilins, iturins, and bacillomycins all contain unusual long-chain β-amino-acids designated iturinic acids (22). The structures of bacillomycin L [28] (23) and some further members of the mycosubtilin group [29] together with the chirality of the amino-acids were determined by classical methods coupled with mass spectral analysis. Additional antibiotics [30, 31] of the cyclosporin family have been characterized which possess the same amino-acid sequence as cyclosporin A but differ in the amino-acid residue at the L-α-aminobutyric acid site.



(22)



(23)

Gramicidin S and Related Peptides.—Gramicidin S is probably still the most widely studied of all the cyclic peptide antibiotics. The main endeavour continues to centre around the mapping of structure–activity relationships of synthetic analogues coupled with the effects of amino-acid replacement on conformational aspects. Those syntheses reported [32–36] follow standard procedures and are listed in Table 1. The analogue in which the 5,5′ proline residues are replaced by leucine retains biological activity comparable with the natural antibiotic, and spectral evidence also indicates a similar solution conformation.[34] Other conformational investigations are mainly extensions of existing programmes and

[28] F. Besson, F. Peypoux, G. Michel, and L. Delcambe, *European J. Biochem.*, 1977, **77**, 61.
[29] L. Delcambe, F. Peypoux, M. Guinand, and G. Michel, *Rev. Ferment. Ind. Aliment.*, 1976, **31**, 147.
[30] R. Traber, M. Kuhn, A. Ruegger, H. Lichti, H. R. Loosli, and A. von Wartburg, *Helv. Chim. Acta*, 1977, **60**, 1247.
[31] R. Traber, M. Kuhn, H. R. Loosli, W. Pache, and A. von Wartburg, *Helv. Chim. Acta*, 1977, **60**, 1568.
[32] K. Sato, H. Abe, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 1999.
[33] S. Sofuku, A. Yoshida, H. Baba, and I. Muramatsu, *Bull. Chem. Soc. Japan*, 1977, **50**, 2143.
[34] O. Abe, Y. Utsumi, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 2341.
[35] M. A. El-Maghraby, *Indian J. Chem.*, 1976, **14B**, 988.
[36] K. Okamoto, K. Nonaka, and N. Izumiya, *Bull. Chem. Soc. Japan*, 1977, **50**, 231.

Table 1 *Syntheses of cyclic peptides reported in* 1977 *(derived from linear precursors unless otherwise stated)*

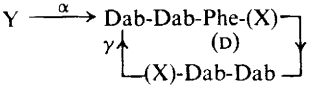
Peptide	*Bond formed in cyclization step*	*Method for cyclization*	*Ref.*
[δ-Aminovaleric acid$^{1'}$, ^{5}]gramicidin S	δ-Ava-Orn	*a*	33
[δ-Aminovaleric acid ^{1}, ^{5}, $^{1'5'}$]gramicidin S	Pro-Val	*a*	33
[Leu ^{5}, $^{5'}$]gramicidin S	Leu-Phe	*b*	34
[4-Fluoro-Pro ^{5}, $^{5'}$]gramicidin S	Pro-Val	*a*	35
[Gly^{6}]tyrocidine A	Leu-Phe	*b*	36
[Gly^{7}]tyrocidine A	Leu-Phe	*b*	36
[Leu^{7}]phalloin	Leu-Ala	*c*	55
D-Ala^{1}]phalloidin	HyLeu-Ala	*c*	56
[Gly^{1}]phalloidin	HyLeu-Gly	*c*	56
[Val^{1}]phalloidin	HyLeu-Val	*c*	56
[Leu^{1}]phalloidin	HyLeu-Leu	*c*	56
[Phe^{1}]phalloidin	HyLeu-Phe	*c*	56
[des-Ala^{1}]phalloidin	HyLeu-Thr	*c*	56
[*endo*-Ala^{1a}]phalloidin	HyLeu-Ala	*c*	56
[Cha^{5}]antamanide*	Phe-Cha	*d*	57
[Cha^{6}]antamanide	Cha-Phe	*d*	57
[Cha^{5}, ^{10}]antamanide	Phe-Cha	*d*	57
[Cha^{6}, ^{9}]antamanide	Cha-Phe	*d*	57
[Ala^{1}, Cha^{6}, ^{9}]antamanide	Cha-Phe	*d*	57
[Cys^{2}, ^{4}]antamanide	Phe-Phe	*d*	58
[Cys^{4}, ^{9}]antamanide	Phe-Phe	*d*	58
[Ala^{3}]tuberactinomycin O	Dea-Cpd†	*a*	64
[Ala^{4}]tuberactinomycin O	Dea-Cpd	*a*	64
[Ala^{3}, ^{4}]tuberactinomycin O	Dea-Cpd	*a*	64
cyclo-(Sar_{10})	Sar-Sar	*e*	80
cyclo-(MeAla-Sar_3)	Sar-MeAla	*e*	81
cyclo-(Ala-Sar_3)	Sar-Ala	*e*	81
cyclo-(D-Ala-Sar-Ala-Sar)	Sar-Ala	*e*	81
cyclo-(Sar-Ala_2-Sar)	Sar-Sar	*e*	81
cyclo-(D-Ala-Sar-D-Ala)	Sar-Ala	*e*	81
cyclo-(Ala_2-Sar_2)	Sar-Ala	*e*	81
cyclo-(Gly-Sar_3)	Sar-Gly	*e*	81
cyclo-(Gly-Sar-Gly-Sar)	Gly-Sar	*e*	81
cyclo-(Gly_2-Sar_2)	Sar-Sar	*e*	81
cyclo-(Gly_3-Sar)	Gly-Sar	*e*	81
cyclo-(MeAla-Sar_3)$_2$‡	Sar-MeAla	*e*	81
cyclo-(Ala-Sar_3)$_2$	Sar-Ala	*e*	81
cyclo-(Ala_2-Sar_2)$_2$	Sar-Ala	*e*	81
cyclo-(Gly-Sar_3)$_2$	Sar-Gly	*e*	81
cyclo-(Gly_2-Sar_2)$_2$	Sar-Gly	*e*	81
cyclo-(Val-Gly-Gly-Pro)$_3$	Pro-Val	*a*	88
cyclo-(Gly-Ala-Leu)$_2$	Leu-Gly	*b*	90
cyclo-(Gly-Ala-D-Leu)$_2$	Leu-Gly	*b*	90
cyclo-(Gly-Val-Leu)$_2$	Leu-Gly	*b*	90
cyclo-(Gly-Val-D-Leu)$_2$	Leu-Gly	*b*	90
cyclo-(Met-Val-Gly-Pro-Asn-Gly)$_2$	Gly-Met	*a*	91
cyclo-(Azet$_3$)§	Azet-Azet	*e*	93
cyclo-(MeAla-Leu-CySMe-Gly)	Gly-MeAla	*e*	94

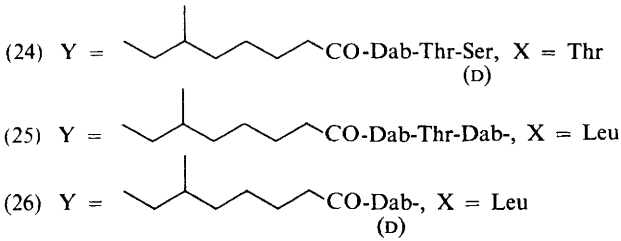
[a] *p*-Nitrophenyl ester. [b] Azide method. [c] Mixed anhydride method. [d] DCC–*N*-hydroxysuccinimide. [e] 2,4,5-Trichlorophenyl ester.

* Cha = cyclohexylalanine. † Dea = diethoxyalanine. Cpd = capreomycidine. ‡ Cyclization dimerization. § Azet = azetidine-2-carboxylic acid.

describe further spectroscopic studies on gramicidin S, analogues,[37–40] and copper complexes.[41] The solution conformations of *N*-methyl-leucine and di-*N*-methyl-leucine gramicidin S which are of considerable interest because they lack some of the intramolecular hydrogen bonds of the native antibiotic have been re-examined in view of the recent conflicting proposals. A further model is suggested with all *trans* peptide bonds based on spectral data and model building.[42] Gramicidin S and those analogues with biological activity form lipid–peptide complexes with phospholipid monolayers. This type of interaction is claimed to result in the destruction of biological membranes and hence is related to the antimicrobial activity.[43]

Polymyxins and Related Antibiotics.—Two new members of the bacterial lipopeptides of the polymyxin family, namely polymyxin, S [44] and T,[45] (24) and (25), together with octapeptin C [46] (26) possess the general structural features of the





group. All three structures were determined by standard peptide procedures coupled with information from spectral data. The amino-acid sequence of polymyxin F is probably similar to polymyxin S_1 with two threonine and the phenylalanine residues replaced by isoleucine and two leucines units.[47] Attempts to modify the biological activity and toxicity by chemical modification [48] and total synthesis of analogues have been described.[49, 50]

[37] S. V. Zenin, L. K. Slobodchikova, N. A. Poddubnaya, and A. A. Akhrem, *Vestsi Akad. Navuk B. SSR*, 1977, 87.
[38] I. D. Rae, E. R. Stimson, and H. A. Scheraga, *Biochem. Biophys. Res. Comm.*, 1977, **77**, 225.
[39] V. P. Golubovich, V. P. Shibut, L. K. Slobodchikova, N. A. Poddubnaya, and A. A. Akhrem, *Vestsi Akad. Navuk B. SSR*, 1977, 76.
[40] A. I. Miroshnikov, L. G. Snezhkova, S. V. Sychev, I. I. Chervin, L. B. Senyavina, V. T. Ivanov, and Yu. A. Ovchinnikov, *Bioorg. Khim.*, 1977, **3**, 180.
[41] A. A. Akhrem, A. S. Tumash, and N. A. Poddubnaya, *Vestsi Akad. Navuk B. SSR*, 1977, 71.
[42] T. Kato and N. Izumiya, *Biochim. Biophys. Acta*, 1977, **493**, 235.
[43] S. N. Semenov, E. I. Melnik, L. G. Snezkova, A. I. Miroshnikov, and V. T. Ivanov, *Bioorg. Khim.*, 1977, **3**, 1055.
[44] J. Shoji, T. Kato, and H. Hinoo, *J. Antibiotics*, 1977, **30**, 427.
[45] J. Shoji, T. Kato, and H. Hinoo, *J. Antibiotics*, 1977, **30**, 1035.
[46] T. Kato and J. Shoji, *J. Antibiotics*, 1976, **29**, 1339.
[47] W. L. Parker, M. L. Rathnum, L. D. Dean, M. W. Nimeck, W. E. Brown, and E. Meyers, *J. Antibiotics*, 1977, **30**, 767.
[48] N. M. Witzke and H. Heding, *J. Antibiotics*, 1976, **29**, 1349.
[49] M. A. Zewail, *Indian J. Chem.*, 1977, **15B**, 128.
[50] M. A. Zewail, *Indian J. Chem.*, 1977, **15B**, 131.

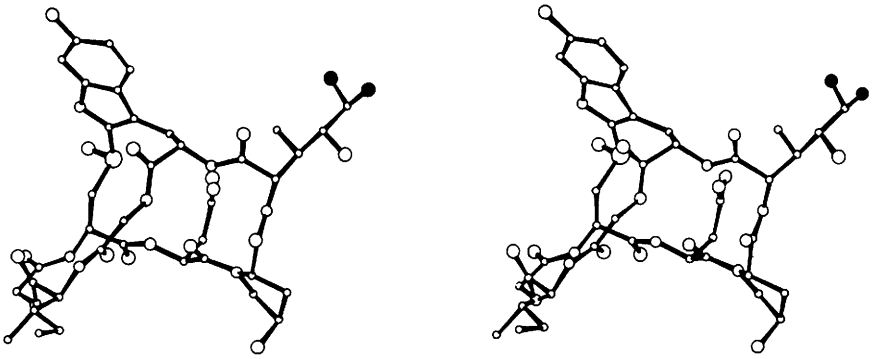
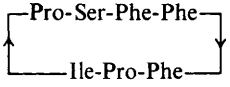


Figure 1 *A stereoscopic view of β-amanitin. The dark atoms represent disordered positions of the terminal hydroxy group. At the right there is a single turn which is very nearly α-helical*
(Reproduced by permission from *J. Amer. Chem. Soc.*, 1977, **99**, 1273)

Cyclic Peptides from *Amanita phalloides*.—Two further reviews cover amatoxins and phallotoxins found in North American Amanita [51] and the chemistry and toxicology of the whole group.[52] A full crystallographic analysis of β-amanitin has confirmed all the structural and stereochemical assignments which were originally made on the basis of spectral and chemical evidence. The noteworthy conformational features are illustrated in Figure 1 and include all *trans* peptide bonds together with three intramolecular hydrogen bonds.[53] *Amanita phalloides* seems to offer a seemingly endless supply of interesting peptides and several new monocyclic peptides (27)—(29), designated cycloamanides, have been isolated. The amino-acid sequences were established by the dansyl modification of the Edman degradation on the corresponding linear peptides.[54]



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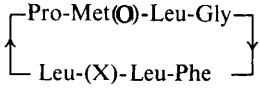
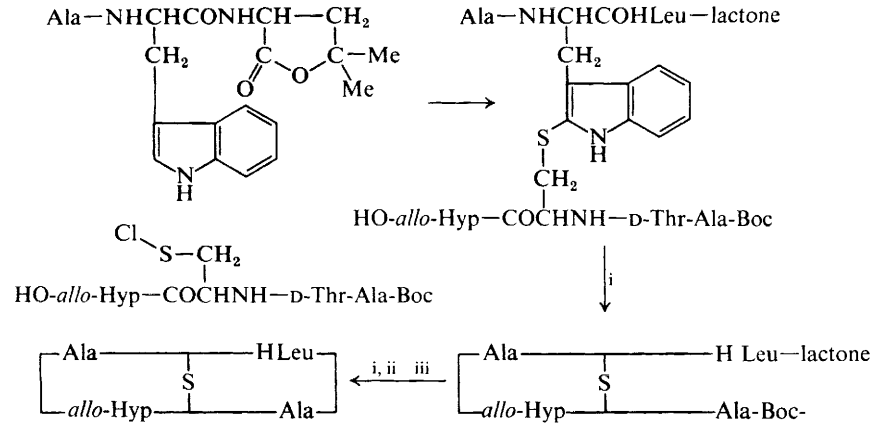


(28) X = Val, (*R*)- and (*S*)-sulphoxides
(29) X = Pro, (*R*)- and (*S*)-sulphoxides

A route developed for the synthesis of phalloin and [Leu^7]phalloin is shown in Scheme 1 [55] and illustrates how total synthesis can be employed to extend structure–activity relationships. An elegant alternative approach which conveniently offers a range of semi-synthetic analogues from natural phalloidin is shown in Scheme 2.[56]

Analogues of the antitoxin antamanide in which the four phenylalanine groups are exchanged in various combinations with L-cyclohexylalanine have been synthesized by the solid phase method (Table 1). The effect on the antitoxic action

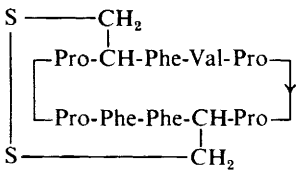
[51] R. R. Yocum and D. M. Simons, *Lloydia*, 1977, **40**, 178.
[52] M. Thévinin, J. R. Claude, and R. Truhaut, *European J. Toxicol.*, 1976, **9**, 197.
[53] E. C. Kostansek, W. N. Lipscomb, R. R. Yocum, and W. E. Thiessen, *J. Amer. Chem. Soc.*, 1977, **99**, 1273.
[54] A. Gauhe and T. Wieland, *Annalen*, 1977, 859.
[55] E. Munekata, H. Faulstich, and T. Wieland, *Annalen*, 1977, 1758.
[56] E. Munekata, H. Faulstich, and T. Wieland, *J. Amer. Chem. Soc.*, 1977, **99**, 6151.



i, $ClCO_2But$-*N*-methylmorpholine; ii, CF_3CO_2H; iii, $\bar{O}H$; iv, $ClCO_2Bu$-*N*-methylmorpholine

Scheme 1

was least altered by an exchange at the 6-position but significantly reduced by alteration at the 10-position.[57] Locking the conformational mobility of the peptide backbone by introducing disulphide bridges offers a further means of exploring the conformation of antamanide and its alkali metal complexes, as well as the molecular basis of the biological activity. Two internal disulphides (30) and (31) and a number of related derivatives have been prepared (Table 1) and the



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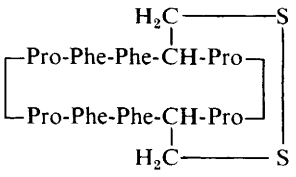
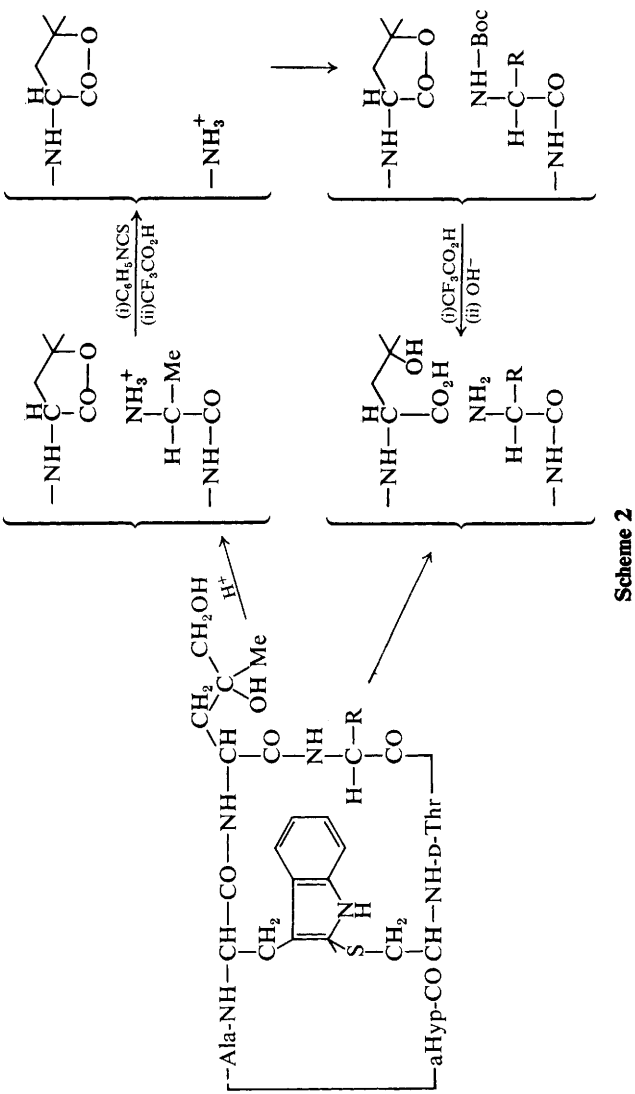


(31)

antitoxic efficacy described.[58] *X*-ray data on [Phe,^{4}Val^6]-antamanide,$12H_2O$ have revealed that some of the water molecules occupy channels within the crystal lattice and the possible significance of this observation in relation to water molecules associated with peptides and biological membranes is developed.[59, 60]

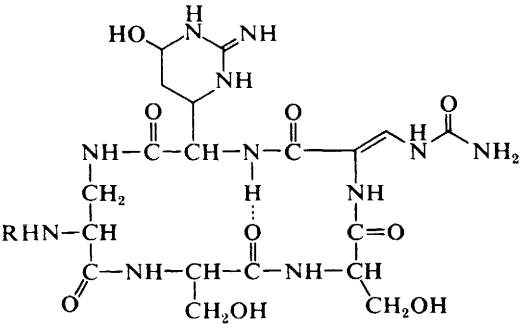
Viomycin, Capreomycin, Tuberactinomycin, and Related Peptides.—A *Nocardia* species has provided two new additions to the viomycin family.[61] Compound (32) possesses the same general ring-structure as viomycin, but the side-chain amino-acid is *N*-methyl-β-arginine as opposed to β-lysine or γ-hydroxy-β-lysine. A further minor component was identified as de-β-lysylviomycin. Last year the structures of the capreomycins were revised on the basis of total syntheses of capreomycin IA and IB. The spectral and chemical data of these antibiotics have

[57] T. Wieland, G. Rohr, H. Faulstich, S. Zobeley, and H. Trischmann, *Annalen*, 1977, 381.
[58] T. Wieland, K. J. Abel, and C. Birr, *Annalen*, 1977, 371.
[59] I. L. Karle and E. Duesler, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 2602.
[60] I. L. Karle, *J. Amer. Chem. Soc.*, 1977, **99**, 5152.
[61] W. J. McGahren, G. O. Morton, M. P. Kunstmann, and G. A. Ellestad, *J. Org. Chem.*, 1977, **42**, 1282.

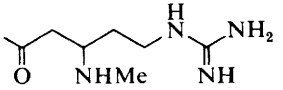


Scheme 2

been reconciled with the new formulations.[62] The experimental details on the synthesis of tuberactinomycin O have appeared [63] and the same general route has been employed to synthesize various analogues.[64] Semi-synthetic analogues with a variety of different side-chains [65] and area [66] groups have also been prepared.



(32) R =



Continuing conformational studies [67–69] on viomycin accord with the view that the predominant solution conformation is similar to that adopted in the crystal lattice. The assignment of all the signals in a high field ^{15}N n.m.r. spectrum of viomycin was possible on the basis of chemical shift differences and ^{1}H coupling constants.[70]

Iron-containing Cyclic Peptides.—More advanced n.m.r. studies including detailed ^{13}C chemical shift analysis [71] and a spin lattice relaxation investigation [72] on both metal-free ferrichromes and the corresponding aluminium chelates have provided more precise data on the overall conformational changes in these peptides induced by the metal binding. An elegant method [73] of assigning the peptide carbonyl resonance, which is normally impossible without specific ^{13}C enrichment of individual amino-acid carboxyl groups, makes use of a ^{15}N–^{13}C double resonance technique on ^{15}N-enriched alumichrome (the aluminium analogue of ferrichrome); the carbon resonance assignments follow from ^{1}H n.m.r. assignments and sequential amide heteronuclear decoupling experiments, *i.e.* ^{1}H–^{15}N and ^{15}N–^{13}C. This technique should be generally applicable for naturally occurring

[62] S. Nomoto, T. Teshima, T. Wakamiya, and T. Shiba, *J. Antibiotics*, 1977, **30**, 955.
[63] T. Teshima, S. Nomoto, T. Wakamiya, and T. Shiba, *J. Antibiotics*, 1977, **30**, 1073.
[64] T. Teshima, S. Nomoto, T. Wakamiya, and T. Shiba, *Bull. Chem. Soc. Japan*, 1977, **50**, 3372.
[65] T. Wakamiya, T. Teshima, H. Sakakibara, K. Fukukawa, and T. Shiba, *Bull. Chem. Soc. Japan*, 1977, **50**, 1984.
[66] S. Nomoto and T. Shiba, *J. Antibiotics*, 1977, **30**, 1008.
[67] E. Brosio, F. Conti, R. Del Giudice, A. Di Nola, and D. Gattegno, *Gazzetta*, 1977, **107**, 139.
[68] T. Kitagawa, T. Miura, K. Mori, H. Taniyama, K. Kawano, and Y. Kyogoku, *Chem. and Pharm. Bull. (Japan)*, 1977, **25**, 280.
[69] T. Kitagawa, T. Miura, and H. Taniyama, *J. Biochem. (Japan)*, 1977, **81**, 1759.
[70] G. E. Hawkes and E. W. Randall, *J.C.S. Chem. Comm.*, 1977, 546.
[71] M. Llinas, D. M. Wilson, and J. B. Neilands, *J. Amer. Chem. Soc.*, 1977, **99**, 3631.
[72] M. Llinas, W. Meier, and K. Wüthrich, *Biochim. Biophys. Acta*, 1977, **492**, 1.
[73] M. Llinas, D. M. Wilson, and M. P. Klein, *J. Amer. Chem. Soc.*, 1977, **99**, 6846.

peptides, provided that it is possible to grow the antibiotic-producing organism under defined conditions with an ^{15}N-enriched nitrogen source.

In vivo biosynthetic studies using a number of strains of *Aspergillus* have resulted in high incorporations of serine and glycine into ferrichrome, ferrichrysin, and ferricroccin. As part of the same investigation, a cell-free system was obtained capable of synthesis of the cyclohexapeptides.[74] In an initial exploratory study, several linear peptides related to the ferrichrome group containing ornithine, serine, and glycine have been synthesized by standard methods.[75]

Conformational Studies on Cyclic Peptides.—The major studies in this area relate to the naturally occurring peptides and these have been reported under the appropriate headings. The work in this section covers conformational studies on synthetic peptides and selected aspects of general interest not conveniently entered elsewhere.

In principle the technique of double resonance on ^{15}N-enriched peptides (see above) clearly offers a relatively convenient means of assigning ^{13}C n.m.r. signals of peptide carbonyls and should find considerable application, particularly in studies on ion-complexing peptides.[73] A new n.m.r. technique termed soft pulse transfer has been developed to measure *cis–trans* rotamer life-times and activation energies, and probably has application to cyclic peptides containing proline and *N*-methylamino-acid residues.[76] There are a variety of n.m.r. methods available for establishing whether or not a peptide NH group is shielded from the solvent, either sterically or through hydrogen bonding. An interesting alternative approach involves differential *N*-methylation with methyliodide and silver oxide in DMF.[77] The results on gramicidin S and destruxin B, the conformations of which are well established, showed extensive methylation of the exposed residues, as determined by amino-acid analysis, while those involved in hydrogen bonds were left unaffected. However, the generality of the method is likely to be limited by side-chain methylation leading to a complexity of hydrolysis products.

In the wake of the considerable amount of *X*-ray data amassed in the past decade, empirical and semi-empirical programmes for predicting conformations are slowly beginning to emerge. Cyclic dipeptides are perhaps exceptional but good agreement was obtained between the parameters obtained from the analysis of eleven crystal structures and classical energy calculations.[78] Computerized empirical valence force-field calculations for *cyclo*-$(Gly)_4$ with various stages of minimization generated low-energy conformations, one of which accorded with the solution conformer observed in most solvents.[79] The results also explained the conformation change observed in trifluoroacetic acid. It is concluded that the conformations of more complex cyclic tetrapeptides can be derived from the calculated *cyclo*-$(Gly)_4$ conformations by introducing appropriate terms for the steric requirements of the side-chains of non-glycyl residues.

[74] H. G. Mueller and H. Dieckmann, *Arch. Microbiol.*, 1977, **113**, 243.
[75] A. M. Islam, A. M. El-Naggar, and A. M. Abdel-Salam, *Egypt. J. Chem.*, 1974, **17**, 593.
[76] J. R. Alger and J. H. Prestegard, *J. Magnetic Res.*, 1977, **27**, 137.
[77] M. Kawai and U. Nagai, *Chem. Letters*, 1977, 1397.
[78] R. Ramani, V. Sasisekharan, and K. Venkatesan, *Internat. J. Peptide Protein Res.*, 1977, **9**, 277.
[79] D. N. J. White and C. Morrow, *Tetrahedron Letters*, 1977, 3385.

The torsion angles and peptide geometry of *cyclo*-(Sar)$_4$ to *cyclo*-(Sar)$_{10}$ from both crystal lattice and solution conformers have been tabulated.[80] The general occurrence of the sequence of *cis* or *trans* amide configuration is, at present, difficult to understand or predict, but this represents an important body of information on peptides unperturbed by hydrogen bonding or α-substituents. A series of cyclic tetrapeptides containing Sar with various combinations of Ala and Gly have been synthesized. Most of them adopt in solution the same conformation as the parent compound *cyclo*-(Sar)$_4$ with the amide sequence *cis*, *trans*, *cis*, *trans*.[81]

Cyclic peptides containing only Pro and Gly are known to complex various cations and the size of the ring determines the ion-binding properties. *cyclo*-(Pro-Gly)$_3$,[82] *cyclo*-(Pro-Gly)$_4$,[83] and *cyclo*-(Pro-Gly-Gly-Pro)$_2$[84] and their metal and amino-acid complexes have been the subjects of extensive spectral investigations. The complexes of the octapeptide *cyclo*-(Pro-Gly)$_4$ and racemic derivatives of amino-acids are diastereoisomers and are chemically distinguishable from one another.[83] Crystallographic data on the *cyclo*-(Pro-Gly)$_4$ RbSCN complex showed the Rb atom at the centre of the peptide ring co-ordinated to the four glycyl carbonyl groups.[85] All the peptide bonds possessed the *trans* geometry. The proline containing nonapeptide, cyclolinopeptide, also binds potassium ions. Evidence has been presented for the solution conformations of the free and complexed peptide, and in both cases one of the proline peptide bonds has the *cis* configuration.[86, 87] The dodecapeptide *cyclo*-(Val-Gly-Gly-Pro)$_3$ forms three different types of complex depending on the size and charge on the cation. Divalent cations such as Ba^{2+} and Ca^{2+} are more strongly bound than K^+. But the stability of the K^+ complex is nevertheless comparable with that of valinomycin.[88]

Synthesis of Homodetic Cyclic Peptides.—The new analogues of naturally occurring, as well as new synthetic cyclic peptides, are listed in Table 1. Cyclic analogues of peptide hormones are excluded from this section. The methodology employed is essentially the same as for linear peptide synthesis (Chapter 3) and here only points of particular relevance to cyclic peptides are reviewed.

The propensity of linear peptides to undergo cyclization is known to depend on the nature and chirality of the amino-acids within the chain. The linear analogue of desthiomalformin (33) readily cyclizes to the corresponding cyclic pentapeptide. It is suggested that this ring-closure is facilitated by intramolecular hydrogen bonding induced by the alternating D- and L-amino-acid residues.[89] A similar study on the cyclization of diastereoisomeric hexapeptides also indicated that the presence of a D-amino-acid unit increased the yield of the ring-closed

[80] J. Dale, P. Groth, and K. Titlestad, *Acta Chem. Scand. B*, 1977, **31**, 523.
[81] K. Titlestad, *Acta Chem. Scand. B*, 1977, **31**, 641.
[82] B. Bartman, C. M. Deber, and E. R. Blout, *J. Amer. Chem. Soc.*, 1977, **99**, 1028.
[83] V. Madison, C. M. Deber, and E. R. Blout, *J. Amer. Chem. Soc.*, 1977, **99**, 4788.
[84] M. Hollosi, L. Radics, and T. Wieland, *Internat. J. Peptide Protein Res.*, 1977, **10**, 286.
[85] Y. H. Chiu, L. D. Brown, and W. N. Lipscomb, *J. Amer. Chem. Soc.*, 1977, **99**, 4799.
[86] I. Z. Siemion, W. A. Klis, A. Sucharda-Sobczyk, and R. Obermeier, *Roczniki Chem.*, 1977, **51**, 1489.
[87] W. A. Klis, R. Obermeier, I. Z. Siemion, A. Sucharda-Sobczyk, and K. Gatner, *Roczniki Chem.*, 1977, **51**, 1499.
[88] D. Baron, L. G. Pease, and E. R. Blout, *J. Amer. Chem. Soc.*, 1977, **99**, 8299.
[89] M. Bodanszky, J. B. Henes, S. Natarajan, and R. L. Foltz, *J. Antibiotics*, 1977, **30**, 856.

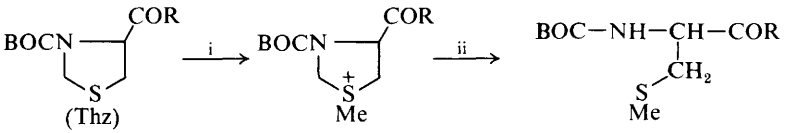
product.[90] The hexapeptide (34), prepared to be the repeating unit in a polypeptide, gave relatively high yields of the cyclic dimer (35) on attempted polymerization of the *p*-nitrophenyl ester. In this case the course of the reaction is controlled by intermolecular hydrogen bonding. Consideration of the conformation of (35) suggests that polymerization would have occurred if the terminal

NH_2-Ile-D-Ala-D-Ala-Val-D-Leu-OH (33)

(Met-Val-Gly-Pro-Asn-Gly)$_n$
(34) $n = 1$
(35) $n = 2$

glycine residue had been replaced by an L-amino-acid with a bulky α-substituent.[91] The results of macrocyclization equilibria calculations on various linear peptides accord in general terms with the above experimental evidence in predicting that ring formation should be easy for the regularly alternating D,L sequence and for chains containing the flexible glycine residue.[92]

Cyclic tripeptides are exceptional from the viewpoint of their unique stereochemistry. All the peptide groups are necessarily *cis* and most probably non-planar. To date only those containing secondary amide groups have been synthesized. The cyclic tripeptide containing three D-2-azetidinecarboxylic acid residues, prepared from the corresponding linear tripeptide, has recently been added to this select group.[93] A method using the thiazolidine derivative of cysteine for introducing a secondary amide bond into small cyclic peptides has been developed and used in the synthesis of (36) and (37) [94] (Scheme 3).



Reagents: i, $MeOSO_2F$; ii, Na_2Co_3

Scheme 3

cyclo(MeAla-Leu-Thz-Gly) (36)

cyclo(MeAla-Leu-Cys(Me)-Gly) (37)

3 Cyclic Depsipeptides and other Cyclic Heterodetic Peptides.

Two valuable review articles [24, 25] which cover the broad aspects of the biosynthesis of non-ribosomally formed peptides include sections on members of the depsipeptide family.

Actinomycins.—The possibility of producing modified actinomycins with broader antitumour activity by the process of directed biosynthesis has been extended. *Streptomyces parvulus* grown in the presence of either *cis*- or *trans*-4-methylproline produced additional actinomycins in which one or both of the proline sites were occupied by methyl proline. ^{1}H n.m.r. studies revealed that the replacement

[90] J. Vičar, A. Trka, and K. Blaha, *Coll. Czech. Chem. Comm.*, 1976, **41**, 3642.
[91] K. D. Kopple and A. Go, *J. Amer. Chem. Soc.*, 1977, **99**, 7698.
[92] M. Mutter, *J. Amer. Chem. Soc.*, 1977, **99**, 8307.
[93] J. Vičar, P. Maloň, A. Trka, J. Smolikova, I. Fric, and K. Blaha, *Coll. Czech. Chem. Comm.*, 1977, **42**, 2701.
[94] D. H. Rich and J. P. Tam, *Tetrahedron Letters*, 1977, 749.

altered the conformation of the actinomycin analogue.[95] 2-Deamino-actinomycin D, prepared by chemical transformation of actinomycin D, effectively binds to DNA but the antitumour activity is markedly reduced.[96] Attempts to produce modified antibiotics with various alterations in the peptide lactone continue to appear.[97, 98]

Spectral evidence has aided the definition of the specific binding of actinomycin D to deoxynucleotides [99, 100] and a synthetic DNA block-polymer.[101] The evidence is consistent with the intercalation model in which the antibiotic is asymmetrically sandwiched between two GC base pairs.

Valinomycin, Enniatins, and Related Compounds.—A paper which covers some of the earlier synthetic work on valinomycin and its analogues has appeared in a Symposium report.[102] This is unfortunately now out of date, but it has the advantage of being published in English. The same group has extended what is already a formidable programme on the synthesis and molecular structure of valinomycin and enniatin derivatives, as well as their metal complexes. The *bis*-derivatives (38) and (39) have been prepared. Each ring of (38) behaved as an independent ion-binding site, whereas (39) formed stable sandwich-complexes.[103] The chirality of the *N*-methylvaline and α-hydroxyisovaleric acid residues in natural enniatin B (40) are respectively D L D L D L. The conformations of the D L L L L L[104] and the rubidium complex of the L D L L D L[105] stereoisomers have been investigated. The former contained one predominant solution conformer

cyclo[-D-Val-L-Lac-L-Glu-D-Hylv-(-D-Val-L-Lac-L-Val-D-Hylv)$_2$]
|
cyclo[-D-Val-L-Lac-L-Lys-D-Hylv-(-D-Val-L-Lac-L-Val-D-Hylv)$_2$]

(38)

cyclo[-L-Glu-D-Hylv-(-L-MeVal-D-Hylv)$_2$]
|
cyclo[-L-Lys-D-Hylv-(-L-MeVal-D-Hylv)$_2$]

(39)

cyclo(-MeVal-Hylv-MeVal-Hylv-MeVal-Hylv)

(40)

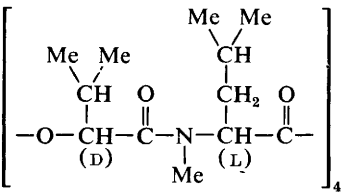
[95] E. Katz, W. K. Williams, K. T. Mason, and A. B. Mauger, *Antimicrob. Agents Chemother.*, 1977, **11**, 1056.
[96] C. W. Mosher, K. F. Kuhlmann, D. G. Kleid, and D. W. Henry, *J. Medicin. Chem.*, 1977, **20**, 1055.
[97] H. Kinoshita and H. Kotake, *Bull. Chem. Soc. Japan*, 1977, **50**, 280.
[98] V. N. Lashkov and G. P. Vlasov, *Zhur. obschei Khim.*, 1977, **47**, 470.
[99] T. R. Krugh, E. S. Mooberry, and Y. C. Chiao, *Biochemistry*, 1977, **16**, 740.
[100] Y. C. Chiao and T. R. Krugh, *Biochemistry*, 1977, **16**, 747.
[101] T. A. Early, D. R. Kearns, J. F. Burd, J. E. Larson, and R. D. Wells, *Biochemistry*, 1977, **16**, 541.
[102] L. A. Fonina, I. S. Savelov, G. Y. Avotina, V. T. Ivanov, and Yu. A. Ovchinnikov, *Proceedings European Peptide Symposium, 14th*, ed. A. Loffet, Brussels University, Belgium, 1976, p. 635.
[103] L. V. Sumskaya, T. A. Balashova, T. S. Chumburidze, E. I. Melnik, I. I. Mikhaleva, V. T. Ivanov, and Yu. A. Ovchinnikov, *Bioorg. Khim.*, 1977, **3**, 5.
[104] T. G. Shishova and V. I. Simonov, *Kristallografiya*, 1977, **22**, 515.
[105] G. N. Tishchenko, Z. Karimov, V. I. Andrianov, B. K. Vainstein, A. V. Evstratov, V. T. Ivanov, and Yu. A. Ovchinnikov, *Bioorg. Khim.*, 1977, **3**, 467.

with one *cis* peptide bond and the remainder of the peptide and ester groups in the *trans*- configuration. In the rubidium complex all the *N*-methylamide and ester groups were in the *trans*- configuration. The analogue in which one of the *N*-methylvaline residues is replaced by an additional α-hydroxyisovaleric acid and with the D L L L L L configuration possessed a similar ring-conformation to that of the corresponding stereoisomer of enniatin B.[106]

The population of the various solution conformers of valinomycin depends on the nature of the solvent and the concentration. Raman spectral data in the solid state and in a variety of solvents suggest that the precise structure of some of these conformers is still open to debate.[107, 108] However, there is further support from a nuclear Overhauser study (NOE) for the recently proposed structure of the predominant conformer in DMSO solution.[109]

Linear polydepsipeptides containing the same basic repeating unit as found in valinomycin gave predominantly products from cyclo-oligomerization when heated in inert solvents. The linear congener of valinomycin appears to be an exception in that the major detectable product was valinomycin.[110] The same factors which control the propensity for cyclization in cyclic homodetic peptides, namely alternating D- and L- units together with the nature of the amino-acids and α-hydroxyacid residues, seem to operate for depsipeptides.[111]

Other Naturally Occurring Cyclic Depsipeptides.—Two independent re-examinations[112, 113] of the entomopathogenic fungus *Beauveria bassiana* have led to the characterization of three new insectidical metabolites, bassianolide (41) and

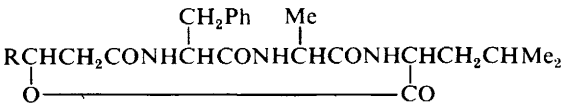


(41)

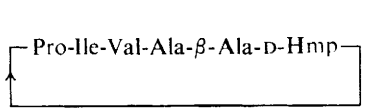
beauverolide H (42) and I (43). Bassianolide (41) was subsequently synthesized from carbobenzoxy L-*N*-methylleucine and t-butyl-D-α-hydroxy-isolvalerate by the procedure employed previously for enniatin B.[114] The insecticidal cyclic depsipeptide protodestruxin (44) has also been synthesized.[115]

[106] T. G. Shishova, V. I. Andrianov, V. I. Simonov, V. Z. Pletnev, A. V. Evstratov, V. T. Ivanov, and Yu. A. Ovchinnikov, *Bioorg. Khim.*, 1977, **3**, 172.
[107] I. M. Asher, K. J. Rothschild, E. Anaestassaskis, and H. E. Stanley, *J. Amer. Chem. Soc.*, 1977, **99**, 2024.
[108] K. J. Rothschild, I. M. Asher, H. E. Stanley, and E. Anatassakis, *J. Amer. Chem. Soc.*, 1977, **99**, 2032.
[109] J. D. Glickson, S. L. Gordon, T. P. Pitner, D. G. Agresti, and R. Walter, *Biochemistry*, 1976, **15**, 5721.
[110] M. Rothe and W. Kreiss, *Angew. Chem.*, 1977, **89**, 117.
[111] G. Boussard, M. Marraud, J. Neel, B. Maigret, and A. Aubry, *Biopolymers*, 1977, **16**, 1033.
[112] A. Suzuki, M. Kanaoka, A. Isogai, S. Murakoshi, M. Ichinoe, and S. Tamura, *Tetrahedron Letters*, 1977, 2167.
[113] J. F. Elsworth, and J. F. Grove, *J.C.S. Perkin I*, 1977, 270.
[114] M. Kanaoka, A. Isogai, and A. Suzuki, *Tetrahedron Letters*, 1977, 4049.
[115] S. Lee and N. Izumiya, *Internat. J. Peptide Protein Res.*, 1977, **10**, 206.

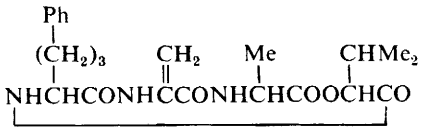
The toxin (45), produced by *Alternaria mali* which causes necrosis on apple leaves, has been the subject of a wide synthetic programme. The toxin,[116] its dihydro derivative,[117] and various analogues[118] have been prepared. The didehydroalanine unit was introduced by means of the dehydration of a serine



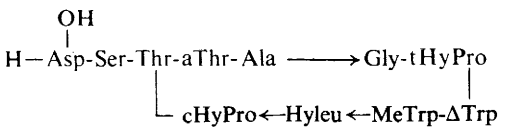
(42) R = $Me(CH_2)_5$
(43) R = $Me(CH_2)_7$



(44)



(45)



(46)

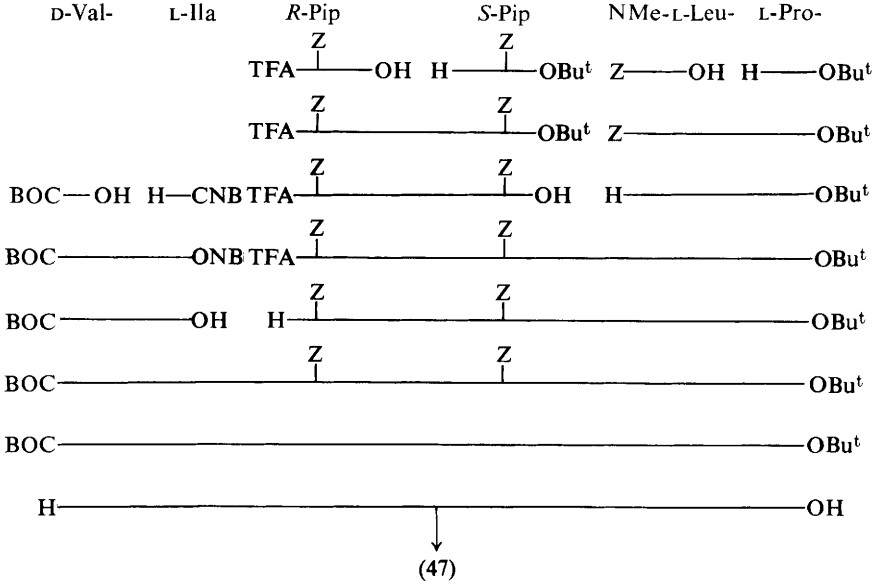
aThr = L-*allo*-threonine; t and c HyPro = *trans*- and *cis*-3-hydroproline; Δ Trp = α, β-dehydrotryptophan; Hyleu = *erythro*-β-hydroxyleucine.

residue. Mass spectroscopy can be used diagnostically for the identification of (45) and related compounds.[119] The antibiotic A-128-HYP contains a dehydrotryptophan unit and is identical with telomycin (46).[120]

The synthesis of a congener (47) of the cyclodepsipeptide monamycin is outlined in Scheme 4.[121] The cyclization step was achieved in acceptable yield with *N*-hydroxysuccinimide and DCC and the route should allow the synthesis of a range of further analogues. Evidence, derived largely from studies of monamycins D_1 (48) and H_1 (49) in solution using ^1H and ^{13}C n.m.r. spectroscopy, supported the presence of a single conformer for each congener, the major feature of which is a β-loop with hydrogen bonding between the NH group of the valine and the carbonyl of the hydroxypiperazic acid residues.[122]

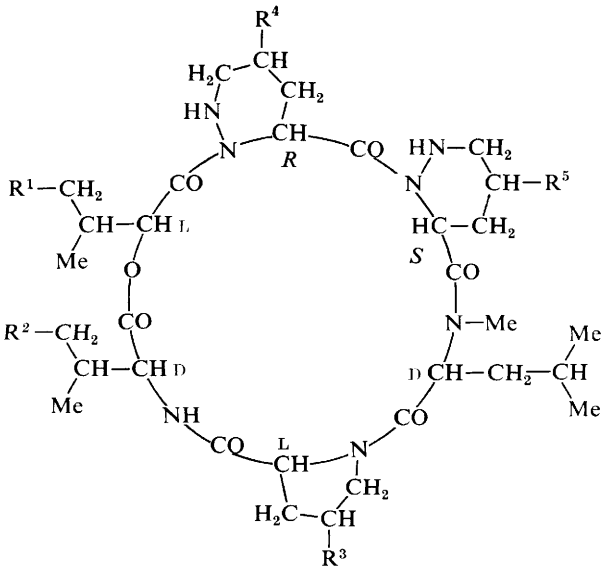
Triostin A (50) and related bicyclic octadepsipeptides have attracted considerable interest lately, due to their ability to act as bifunctional intercalating agents with DNA. The first synthesis of a close relative of the quinoxaline antibiotics,

[116] Y. Shimohigashi, S. Lee, T. Kato, N. Izumiya, T. Ueno, and H. Fukami, *Chem. Letters*, 1977, 1411.
[117] Y. Shimohigashi, S. Lee, T. Kato, N. Izumiya, T. Ueno, and H. Fukami, *Agric. and Biol. Chem. (Japan)*, 1977, **41**, 1533.
[118] Y. Shimohigashi, S. Lee, H. Aoyagi, T. Kato, and N. Izumiya, *Internat. J. Peptide Protein Res.*, 1977, **10**, 197.
[119] T. Ueno, T. Nakashima, M. Uemoto, H. Fukami, S. Lee, and N. Izumiya, *Biomed. Spectrom.*, 1977, **4**, 134.
[120] G. S. Katrukha, S. N. Maevskaya, and A. B. Silaev, *Bioorg. Khim.*, 1977, **3**, 422.
[121] C. H. Hassall, W. H. Johnson, and C. J. Theobald, *J.C.S. Chem. Comm.*, 1977, 635.
[122] C. H. Hassall, W. A. Thomas, and M. C. Moschidis, *J.C.S. Perkin I*, 1977, 2369.



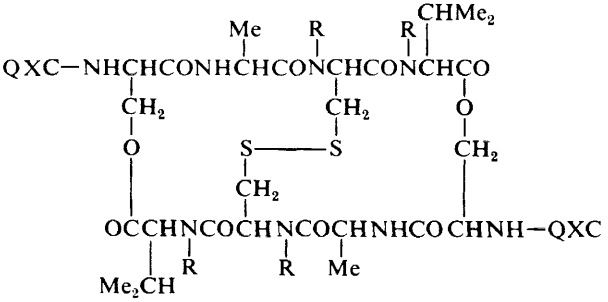
Pip = Hexahydropiperazic acid; Ila = isoleucic acid [Me-$CH_2CH(Me)CH(OH)CO_2H$]; Z = benzyloxycarbonyl-; BOC = t-butyloxycarbonyl-; TFA = trifluoroacetyl-; ONB = 4-nitrobenzyl-.

Scheme 4



(47) R^1 = Me, R^2 = R^3 = R^4 = R^5 = H
(48) R^1 = Me, R^2 = H, R^3 = Me, R^4 = H, R^5 = OH
(49) R^1 = Me, R^2 = H, R^3 = Me, R^4 = Cl, R^5 = OH

des-*N*-tetramethyl triostin A (51), has been described.[123] The two halves of the triostin A molecule are related by a two-fold axis of symmetry. The data from n.m.r. spectroscopy in DMSO accord with this symmetry. However, the data from $CDCl_3$ had previously been interpreted in terms of a conformation in which the two halves are non-equivalent. Re-examination of this evidence excludes the presence of an unsymmetrical conformer and suggests two slowly interconverting symmetrical conformers to account for the spectral data.[124]



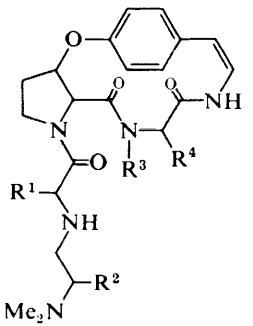
QXC = Quinoxaline-2-carbonyl

(50) R = Me
(51) R = H

An antibiotic, isolated from *Actinoplanes philippinensis* and designated A2315A, appears to be identical with madumycin II which was described in last year's Report.[125] A detailed study on both the crystal and solution conformations of several members of this group of highly modified cyclic depsipeptides concludes that for each compound studied, one predominant solution conformer exists which is essentially the same as that adopted in the crystal lattice.[126]

4 Peptide Alkaloids

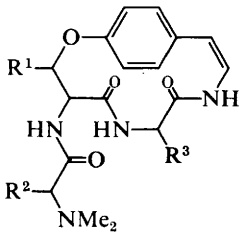
Several new peptide alkaloids have been isolated from bark extracts of members of the Rhamnaceae family and add to the considerable number of these peptides



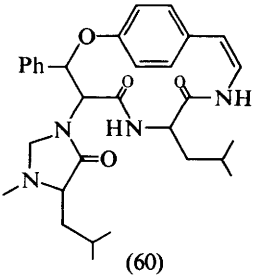
(52) R^1 = $PhCH_2$, R^2 = EtMeCH, R^3,R^4 = $-(CH_2)_3-$
(53) R^1 = Me_2CHCH_2, R^2 = Me, R^3 = H, R^4 = $PhCH_2$

[123] T. L. Ciardelli and R. K. Olsen, *J. Amer. Chem. Soc.*, 1977, **99**, 2806.
[124] T. J. Blake, J. R. Kalman, and D. H. Williams, *Tetrahedron Letters*, 1977, 2621.
[125] J. W. Chamberlin and S. Chen, *J. Antibiotics*, 1977, **30**, 197.
[126] B. W. Bycroft, *J.C.S. Perkin I*, 1977, 2464.

already characterized. With one exception, all contain the 14-membered ring-system. Hysodricanine A (52) and mauritin H (53), isolated from *Ziziphus hysodrica* and *Z. mauritiana* respectively, belong to the amphibine B type.[127] Whereas scutianine F [127] and scutianine G [128] from *Scutia buxifolia* are new members of the frangulanine class. The latter (54) is a diastereoisomer of the previously reported scutianines D and E, while scutianine F is similar to scutianine B (55) but with the *NN*-dimethylphenylalanyl residue replaced by an *N*-methylphenylalanylprolyl residue. Aralionin C (56) from *Araliorhammus vaginata* is a new member of the integerrine class.[127] *Z. nummularia* has yielded three new alkaloids, nummularine G, H, and K.[129] Nummularine K (57) possesses a terminal *NN*-dimethyl tryptophan which has not previously been encountered in these peptides. Nummularine H is the only new addition to the 13-membered cyclopeptides and was identified



(54) $R^1 = Me_2CH$, $R^2 = PhCH_2$, $R^3 = PhCHOH$
(55) $R^1 = Me_2CH$, $R^2 = R^3 = PhCH_2$
(56) $R^1 = Ph$, $R^2 = EtMeCH$, $R^3 = PhCHOH$
(57) $R^1 = Me_2CH$, $R^2 = Indolyl$, $R^3 = Me_2CHCH_2$
(58) $R^1 = R^2 = Me_2CH$, $R^3 = Me_2CHCH_2$
(59) $R^1 = R^2 = Me_2CH$, $R^3 = p\text{-}HO\cdot C_6H_4\cdot CH_2$



(60)

as the *N*-demethyl derivative of jubanin A described in last year's Report. Nummularine G (60) is the first peptide alkaloid to be isolated in which the terminal *N*-methyl-amino-acid unit is linked to the neighbouring amide by a methylene group.

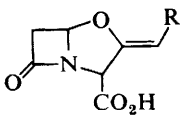
Finally, two new alkaloids melonovine A (58) and B (59) have been isolated from the roots of *Melochia tomentosa*, a member of the Sterculiaceae family.[130]

5 Penicillins, Cephalosporins, and Related Antibiotics

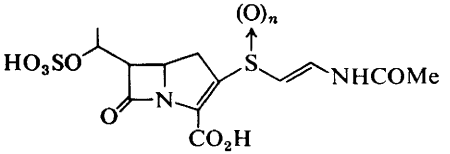
The policy adopted in previous years of outlining only the more significant developments relating to the chemistry and origin of these important antibiotics has been maintained. For a valuable summary of the present state of this area, readers are referred to the proceedings of a recent symposium on β-lactam antibiotics.[131] In addition, a valuable survey of the current evidence and theories relating to the biosynthesis of these highly modified peptides has appeared.[132]

[127] R. Tschesche, D. Hillebrand, H. Wilhem, E. Ammermann, and G. Eckhardt, *Phytochemistry*, 1977, **16**, 1025.
[128] R. Tschesche and D. Hillebrand, *Phytochemistry*, 1977, **16**, 1817.
[129] R. Tschesche, M. Elgamal, and G. Eckhardt, *Chem. Ber.*, 1977, **110**, 2649.
[130] G. J. Kapadia, Y. N. Shukla, J. F. Morton, and H. A. Lloyd, *Phytochemistry*, 1977, **16**, 1431.
[131] D. J. Aberhart, *Tetrahedron*, 1977, **33**, 1545.
[132] 'Recent Advances in the Chemistry of β-Lactam Antibiotics', ed. J. Elks, Chemical Society, London, 1977.

Much interest has been generated by the recent discoveries of new β-lactam antibiotics which are also potent inhibitors of the enzymes β-lactamase. Clavulanic acid (61) was the first of these to be characterized [133] and a synthetic programme [134–136] has resulted in total synthesis of the racemic compound and its *Z*-isomer.[137] Analogues of clavulanic acid exhibiting β-lactamase inhibition have also been prepared,[138, 139] the phenyl derivative (62) showing potent activity. Two further fused β-lactams displaying similar biological activity have been isolated



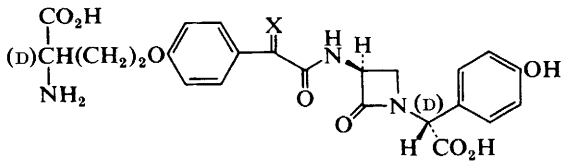
(61) R = CH_2OH
(62) R = Ph



(63) *n* = 0
(64) *n* = 1

from *Streptomyces olivaceus* for which structures (63) and (64) were proposed.[140] Mild degradation studies confirmed (64) and also led to the elucidation of the structure of a minor co-occurring metabolite in which the exocyclic double bond has been reduced.[141] Compound (64) has been independently identified in *S. fulvoviridis*.[142]

The characterization of nocardicins A (65) and B (66), new antibiotics produced by a strain of *Nocardia* and containing a unique monocyclic β-lactam, has been



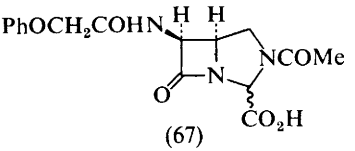
(65) X = NOH (*syn* with respect to the acylamino group)
(66) X = NOH (*anti* with respect to the acylamino group)

followed by the isolation of five new relateda ntibiotics nocardicin C, D, E, F, and G.[143] The structures of these metabolites represent minor variations and they display weaker biological activity than the parent compounds. A biosynthetic study on nocardicin A has established, very much as expected from structural analysis, that the molecule is derived from two molecules of tyrosine and one

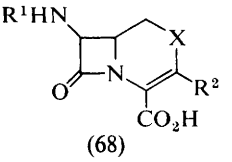
[133] C. Reading and M. Cole, *Antimicrob. Agents Chemother.*, 1977, **11**, 852.
[134] A. G. Brown, D. F. Corbett, and T. T. Howarth, *J.C.S. Chem. Comm.*, 1977, 359.
[135] R. G. Alexander and R. Southgate, *J.C.S. Chem. Comm.*, 1977, 405.
[136] A. J. Eglington, *J.C.S. Chem. Comm.*, 1977, 720.
[137] P. H. Bentley, P. D. Berry, G. Brooks, M. L. Gilpin, E. Hunt, and I. I. Zomaya, *J.C.S. Chem. Comm.*, 1977, 748.
[138] P. H. Bentley, G. Brooks, M. L. Gilpin, and E. Hunt, *J.C.S. Chem. Comm.*, 1977, 905.
[139] E. Hunt, P. H. Bentley, G. Brooks, and M. L. Gilpin, *J.C.S. Chem. Comm.*, 1977, 906.
[140] A. G. Brown, D. F. Corbett, A. J. Eglington, and T. T. Howarth, *J.C.S. Chem. Comm.*, 1977, 523.
[141] D. F. Corbett, A. J. Eglington, and T. T. Howarth, *J.C.S. Chem. Comm.*, 1977, 953.
[142] K. Maeda, S. Takahashi, M. Sezaki, K. Inuma, H. Naganawa, S. Kondo, M. Ohno, and H. Umezawa, *J. Antibiotics*, 1977, **30**, 770.
[143] J. Hosoda, T. Konomi, N. Tani, H. Aoki, and H. Imanaka, *Agric. Biol. Chem.*, 1977, **41**, 2013.

molecule each of serine and homoserine.[144] No new naturally occurring antibiotics with the penicillin or cephalosporin skeleton have been reported during the year.

The synthesis of nuclear analogues of penicillin and cephalosporin continues to dominate the search for β-lactam derivatives with improved antibacterial activity. A 1-oxadethiapenicillin derivative [136] and the nitrogen analogue [145] (67) have been prepared, the latter displaying modest antibacterial activity. Much of the work



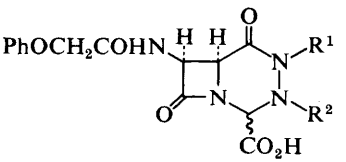
(67)



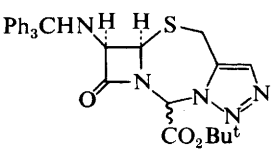
(68)

documented in this area relates to the modification of the cephalosporin skeleton. In an extended study on the isocephem system (68), oxygen,[146–150] nitrogen,[151] and the parent sulphur [152] analogues have been synthesized, commencing from a monocyclic β-lactam intermediate. Two further independent investigations [153, 154] have also culminated in successful syntheses of isocephem systems (68; X = S).

The possibility that cephalosporin and penicillin derivatives with increased ring-strain may exhibit enhanced activity has been explored. The tricyclic hydrazine derivatives (69) and (70) demonstrated modest activity against *S. aureus*, whereas the related bicyclic compound (71) was devoid of activity.[155] Other tricyclic systems including the triazole [156] (72), cephalosporin 3,4-lactones,[157]



(69) R^1, R^2 = -$(CH_2)_4$-
(70) R^1, R^2 = -$(CH_2)_3$-
(71) R^1 = H, R^2 = CO_2Me

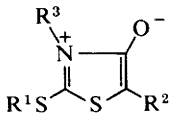


(72)

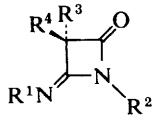
[144] J. Hosoda, N. Tani, T. Konomi, S. Ohsawa, H. Aoki, and H. Imanaka, *Agric. Biol. Chem.*, 1977, **41**, 2007.
[145] W. F. Huffman, K. G. Holden, T. F. Buckley, J. G. Gleason, and L. Wu, *J. Amer. Chem. Soc.*, 1977, **99**, 2352.
[146] T. W. Doyle, B. Belleau, B.-Yu. Luh, C. F. Ferrari, and M. P. Cunningham, *Canad. J. Chem.*, 1977, **55**, 468.
[147] T. W. Doyle, B. Belleau, B.-Yu. Luh, T. T. Conway, M. Menard, J. L. Douglas, D. T. Chu, G. Lim, L. R. Morris, P. Rivest, and M. Casey, *Canad. J. Chem.*, 1977, **55**, 484.
[148] T. W. Doyle, B.-Yu. Luh, and A. Martel, *Canad. J. Chem.*, 1977, **55**, 2700.
[149] T. W. Doyle, A. Martel, and B.-Yu. Luh, *Canad. J. Chem.*, 1977, **55**, 2708.
[150] T. W. Doyle, *Canad. J. Chem.*, 1977, **55**, 2714.
[151] T. W. Doyle, B.-Yu. Luh, D. T. Chu, and B. Belleau, *Canad. J. Chem.*, 1977, **55**, 2719.
[152] T. W. Doyle, J. L. Douglas, B. Belleau, J. Meunier, and B.-Yu. Luh, *Canad. J. Chem.*, 1977, **55**, 2873.
[153] J. E. T. Corrie, J. R. Hlubucek, and G. Lowe, *J.C.S. Perkin I*, 1977, 1421.
[154] D. B. Bryan, R. F. Hall, K. G. Holden, W. F. Huffmann, and J. G. Gleason, *J. Amer. Chem. Soc.*, 1977, **99**, 2353.
[155] J. Finkelstein, K. G. Holden, R. Sneed, and C. D. Perchonock, *Tetrahedron Letters*, 1977, 1855.
[156] M. J. Pearson, *J.C.S. Perkin I*, 1977, 189.
[157] T. Petrzilka, K. K. Prasad, and G. Schmid, *Helv. Chim. Acta*, 1977, **60**, 2911.

and rearrangement products of penicillin derived diazo-ketones [158] showed little or no biological activity. Compounds (69) and (70) are of interest since it had previously been thought that the Δ^3 double bond was critical for activity in cephalosporin analogues.

Several new routes to the basic β-lactam ring-system have been elaborated. Interesting photochemical methods involve the cyclizations of *NN*-dialkyl α,β-unsaturated amides [159] and the photolytic ring contraction of mesoionic 2-alkylthiazol-4-ones [160] (73). The extensively employed ketene method has been refined to yield optically active 2-iminoazetidin-4-ones [161] (74). Cycloaddition of



(73)



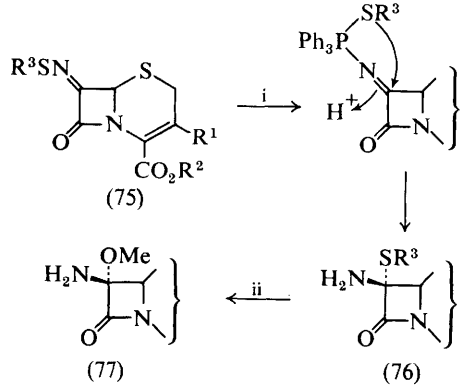
(74)

chiral carbodi-imides to prochiral ketenes gives diastereoisomeric mixtures of (74). Alternatively, ketene silyl acetals may be condensed with Schiff bases in the presence of titantium tetrachloride.[162]

Rearrangement and modification of the basic skeletons of the natural antibiotics still continue to be of importance. The now well-established ring expansion of penam *S*-oxides has once again been exploited to produce novel cephams and cephems.[163–167] The past five years have witnessed considerable efforts directed towards the conversion of cephalosporins into general analogues of the naturally occurring cephamycin group (7α-methoxycephalosporins). Two elegant routes, both of which employ a thioxime intermediate and which have the added advantage of subsequently allowing the addition of various side-chains, have been developed independently,[168, 169] and one of them is outlined in Scheme 5. The thioxime (75), prepared by treating 7-aminocephalosporanate with *p*-toluenesulphenyl chloride, rearranged on treating with triphenylphosphine to give (76) which with methanol and mercuric acetate afforded the methoxylamine (77) in high yield. The methods are equally applicable to the penicillin skeleton.

Unusual rearrangements of 7α-methoxycephalosporins [170] have been reported including an interesting ring-contraction to a penam structure.[171] 6-Diazopenicil-

[158] I. Ernest, *Tetrahedron*, 1977, **33**, 547.
[159] T. Hasegawa, M. Watabe, H. Aoyama, and Y. Omote, *Tetrahedron*, 1977, **33**, 385.
[160] D. H. R. Barton, E. Buschmann, J. Haüsler, C. W. Holzapfel, T. Sheradsky, and D. A. Taylor, *J.C.S. Perkin I*, 1977, 1107.
[161] C. Belzecki and Z. Krawczyx, *J.C.S. Chem. Comm.*, 1977, 302.
[162] I. Ijima, S. Inaba, and K. Yoshida, *Tetrahedron Letters*, 1977, 3643.
[163] E. M. Gordon and C. M. Cimarusti, *Tetrahedron Letters*, 1977, 3425.
[164] G. A. Koppel, M. D. Kinnick, and L. J. Nummy, *J. Amer. Chem. Soc.*, 1977, **99**, 2822.
[165] A. Nudelman and R. J. McCaully, *J. Org. Chem.*, 1977, **42**, 2887.
[166] G. Franceschi, M. Foglio, P. Masi, A. Suarato, G. Palamidessi, L. Bernardi, F. Arcamone, and G. Cainelli, *J. Amer. Chem. Soc.*, 1977, **99**, 248.
[167] M. J. V. de Oliveira Baptista, A. G. M. Barrett, D. H. R. Barton, M. Girijavallabhan, R. C. Jennings, J. Kelly, V. J. Papadimitriou, J. V. Turner, and N. A. Usher, *J.C.S. Perkin I*, 1977, 1477.
[168] E. M. Gordon, H. W. Chang, and C. M. Cimarusti, *J. Amer. Chem. Soc.*, 1977, **99**, 5504.
[169] T. Kobayashi, K. Iino, and T. Hiraoka, *J. Amer. Chem. Soc.*, 1977, **99**, 5505.
[170] Y. Sugimura, Y. Iwano, K. Iino, T. Saito, and T. Hiraoka, *Tetrahedron Letters*, 1977, 2947.
[171] T. Kobayashi and T. Hiraoka, *Chem. Letters*, 1977, 1399.

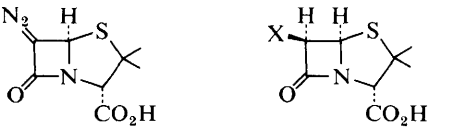


Reagents: i, Ph_3P; ii, $Hg(OAC)_2$–MeOH

Scheme 5

lin (78) has been used to synthesise the homlogous 6β-methylamides [172] (79) and 6β-thioesters [173] (80), the sulphoxides of which were rearranged into the corresponding cephem analogues.

The presence of an hydroxyethyl side-chain in the recently characterized and highly active antibiotic thienomycin has led to the synthesis of penicillin and cephalosporin analogues with this group in the 6- and 7-positions respectively.[174] The procedure elaborated for the penicillin analogues (81) which were found to possess reasonable activity is shown in Scheme 6.

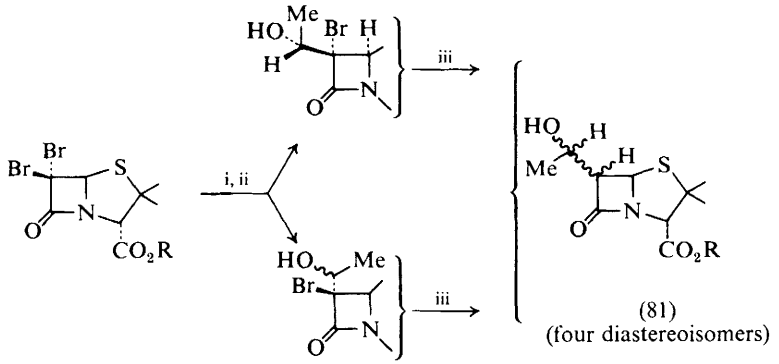


(78)

(79) X = $RCONHCH_2$
(80) X = RCOS

Other transformations conducted on the cephalosporin skeleton, possibly of general interest, are the syntheses of 7-hydrazino-,[175] 6-thiomethyl-,[176] 3-trifluoromethyl-,[177] 2,2-spiroaziridino,[178] and other 2,2-substituted derivatives.[179] Additional modifications of the penam skeleton *via* 1,2-secopenicillin intermediates are also noteworthy.[180, 181]

[172] J. C. Sheehan, A. Buku, E. Chacko, T. J. Commons, Y. S. Lo, D. R. Ponzi, and W. C. Schwarzel, *J. Org. Chem.*, 1977, **42**, 4045.
[173] J. C. Sheehan, T. J. Commons, and Y. S. Lo, *J. Org. Chem.*, 1977, **42**, 2224.
[174] F. DiNinno, T. R. Beattie, and B. G. Christensen, *J. Org. Chem.*, 1977, **42**, 2960.
[175] J. C. Sheehan, Y. S. Lo, and D. R. Ponzi, *J. Org. Chem.*, 1977, **42**, 1012.
[176] J. L. Fahey, B. C. Lange, J. M. Van der Veen, G. R. Young, and A. K. Bose, *J.C.S. Perkin I*, 1977, 1117.
[177] T. Watanabe, Y. Kawano, T. Tanaka, T. Hashimoto, M. Nagano, and T. Miyadera, *Tetrahedron Letters*, 1977, 3053.
[178] D. O. Spry, *Tetrahedron Letters*, 1977, 3611.
[179] D. H. Bremner and M. M. Campbell, *J.C.S. Perkin I*, 1977, 2298.
[180] M. D. Bachi, N. Frydman, S. Sasson, C. Stern, and J. Vaya, *Tetrahedron Letters*, 1977, 641.
[181] E. G. Brain, A. J. Eglington, J. H. C. Nayler, N. F. Osborne, R. Southgate, and P. Tolliday, *J.C.S. Perkin I*, 1977, 2479.

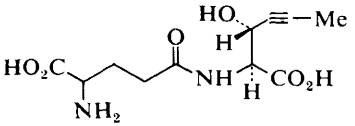


Reagents: i, MeMgBr or BuLi; ii, MeCHO–H^+; iii, Zn(Ag)–MeOH

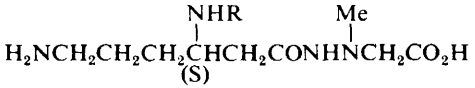
Scheme 6

6 Linear Peptides containing Unusual Structural Features

The widespread distribution of γ-glutamyl peptides testifies to their importance in general metabolism and physiological functions. In recent years a variety of di- and tripeptides containing unusual amino-acids and possessing a γ-glutamyl linkage have been isolated. γ-L-Glutamyl-L-2-aminohex-4-ynoic acid and γ-L-glutamyl-*erythro*-2-amino-3-hydroxyhex-4-ynoic acid (82) occur in the fruit bodies of the fungus *Tricholomopsis rutilans*, as yet no specific function has been assigned to these compounds.[182] A claim that mass spectrometry can differentiate



(82)

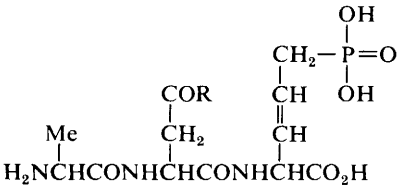


(83) R = H
(84) R = —COCH(NH_2)CH_2CHMe_2
(S)

between α- and γ-linked glutamyl residues, if substantiated for a wide range of derivatives, could provide a valuable alternative to the existing methods.[183] Compounds (83) and (84) are newly identified members of the negamycin family.[184] Peptides containing hydrazino groups are of some interest at the present time and considerable synthetic effort has been directed towards producing analogues of this type. A review on naturally occurring compounds with a nitrogen–nitrogen bond which includes a section on hydrazino peptides is a valuable contribution to

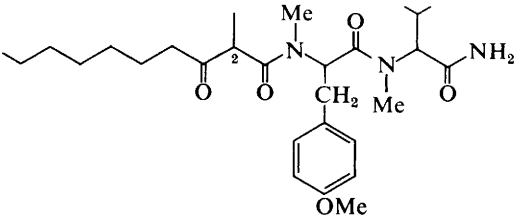
[182] Y. Niimura and S. Hataraka, *Phytochemistry*, 1977, **16**, 1435.
[183] K. Okada and M. Kawase, *Chem. Pharm. Bull. (Japan)*, 1977, **25**, 1497.
[184] S. Kondo, K. Yoshida, T. Ikeda, K. Iinuma, Y. Honma, M. Hamada, and H. Umezawa, *J. Antibiotics*, 1977, **30**, 1137.

this area.[185] The tripeptides plumbemycin A (85) and B (86) produced by *S. plumbeus* are specific threonine antagonists.[186] The phosphoric acid residue is increasingly common among the so-called anti-metabolites, and peptide analogues in which a carboxyl function is replaced by a phosphono group are becoming targets for synthetic chemists.[187]



(85) R = OH
(86) R = NH_2

Peptides conjugated with a fatty acid are common to all the classes of unusual peptides. The major lipophilic constituents of the blue-green alga *Lyngbya majuscula* are majusculamide A (87) and B (88) and it is claimed that their presence in algae can be used for taxonomical identification. The structures followed from spectral analysis, chemical degradation, and an *X*-ray analysis.[188] The *NO*-dimethyltyrosine in both compounds has the D-configuration. The



(87) 2*R*
(88) 2*S*

RCO-Asn-Val-Val-Asn-Asn-Hyl-aThr-Ser-Trp-aIle
(D) (D) (D) (D) (D) (D) (D)

γHyl = L-*threo*-γ-hydroxylysine

(89)

bacterial antibiotics of the cerexin family (89) appear to differ only in the nature of the fatty acid residue. The decapeptide entity of this molecule is remarkable in that seven of the constituent amino-acids are of the D-configuration.[189–191]

An alternating sequence of L- and D-amino-acids, as found in gramicidin A, results in a well-defined helical structure and these have been studied for simple

[185] T. A. La Rue, *Lloydia*, 1977, **40**, 307.
[186] B. K. Park, A. Hirota, and H. Sakai, *Agric. Biol. Chem.* (*Japan*), 1977, **41**, 573.
[187] P. Kafarski and P. Mastalerz, *Roczniki Chem.*, 1977, **51**, 433.
[188] F.-J. Marner, R. E. Moore, K. Hirotsu, and J. Clardy, *J. Org. Chem.*, 1977, **42**, 2815.
[189] J. Shoji, T. Kato, and R. Sakazaki, *J. Antibiotics*, 1976, **29**, 1268.
[190] J. Shoji and T. Kato, *J. Antibiotics*, 1976, **29**, 1275.
[191] J. Shoji, T. Kato, K. Matsumoto, Y. Takahashi, and M. Mayama, *J. Antibiotics*, 1976, **29**, 1281.

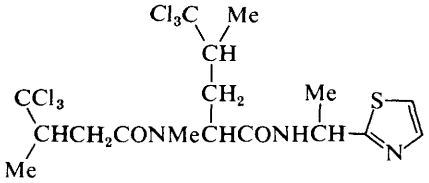
Table 2 *Structures of some peptaibophols*

Compound	*Structure*
(90) Antiamoebin	**1** **5** Ac → L-Phe → Aib → Aib → Aib → L-*Iva* → Gly → L-Leu → **10** Aib → Aib → L-Hyp → L-Gln → L-Iva → L-Hyp → *Aib* → L-Pro → L-Phol
(91) Emerimicin	**1** **5** Ac → L-Phe → Aib → Aib → Aib → L-*Val* → Gly → L-Leu → **10** Aib → Aib → L-Hyp → L-Gln → L-Iva → L-Hyp → L-*Ala* → (*Aib*) **15** L-Phol
(92) Alamethicin (previous structure)	**1** **5** Ac → Aib → L-Pro → Aib → L-Ala → Aib → L-Ala → L-Gln → **10** **15** Aib → L-Val → Aib → Gly → L-Leu → Aib → L-Pro → L-Val Aib → Aib → L-Gluα → L-Gln → OH γ↓ Phol **20**
(93) Alamethicin (current structure)	**1** **5** Ac → Aib → L-Pro → Aib → L-Ala → Aib → L-Ala → (Aib) **10** L-Gln → Aib → L-Val → Aib → Gly → L-Leu → Aib → L-Pro → **15** **20** L-Val → Aib → Aib → L-Gluα → L-Gln → L-Phol ↓γ OH
(94) Suzukacillin (previous structure)	**1** **5** Ac → Aib → L-Pro → L-Val → Aib → L-Val → L-Ala → Aib → **10** **15** L-Ala → Aib → Aib → L-Gln → Aib → L-Leu → Aib → Gly → **20** L-Leu → Aib → L-Pro → L-Val → Aib → Aib → L-Gluα → γ↓ L-Gln → OH Phol **24**
(95) Suzukacillin (current structure)	**20** → Aib → Aib → L-Gluα→ L-Gln → L-Phol ↓γ OH *d*

linear peptides.[192, 193] The ability of gramicidin A to alter the ionic conductance or permeability of biological membranes by forming conductance channels makes it a valuable tool in molecular biology. Further syntheses by the Russian school have provided a considerable number of analogues but relatively few possess biological activity.[194] Recent years have seen the emergence of a number of peptide antibiotics with similar biological properties to those of gramicidin A of which alamethicin is perhaps the best known. In extensive investigations, a number of new members (90) and (91) of this class, which are now termed peptaibophols, have been characterized,[195, 196] and the recently proposed structure (92) of alamethicin revised yet again [197] to (93). A similar revision (95) is made to the previously proposed structure (94) of suzukacillin [197] (see Table 2). The members of the group are characterized by having several α-aminoisobutyric acid units (Aib) and a terminal amino alcohol phenylalaninol. All the amino-acids have the L-configuration.

A compound with the chain sequence shown in (92) has been synthesized and was found not be identical with alamethicin, although it did display some channel conductance.[198]

Although marine natural product chemistry has turned up a wide variety of halogenated compounds, the number of peptides among these is relatively small. The isolation and characterization of (96) from the sponge *Dysidea herbacea* is an encouraging development [199] and also noteworthy is the presence of the thiazole

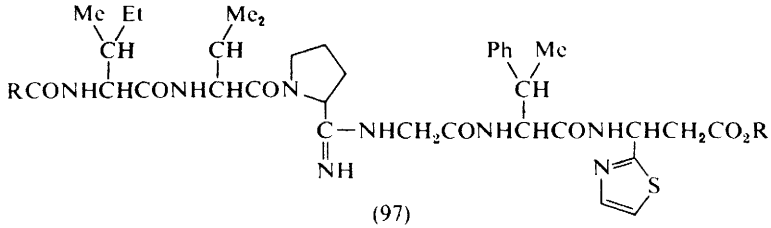


(96)

unit which is presumably derived from cysteine by an oxidation and decarboxylation process. The same structural feature also occurs in the microbial antibiotics of the bottromycin group (97). A number of model systems of these linear peptides which also contain an iminopeptide group have been synthesized.[200, 201]

The topochemical approach for studying structure–activity relationships has, in the past, been extensively explored for cyclic peptides. However, the same

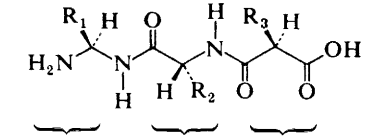
[192] B. V. V. Prasad and R. Chandrasekaran, *Internat. J. Peptide Protein Res.*, 1977, **10**, 129.
[193] G. P. Lorenzi and T. Paganetti, *J. Amer. Chem. Soc.*, 1977, **99**, 1282.
[194] E. N. Shepel, S. Iordanov, I. D. Ryabova, A. I. Miroshnikov, V. T. Ivanov, and Yu A. Ovchinnikov, *Bioorg. Khim.* 1976, **2**, 581.
[195] R. C. Pandey, H. Meng, J. C. Cook, jun., and K. L. Rinehart, jun., *J. Amer. Chem. Soc.*, 1977, **99**, 5203.
[196] R. C. Pandey, J. C. Cook, jun., and K. L. Rinehart, jun., *J. Amer. Chem. Soc.*, 1977, **99**, 5205.
[197] R. C. Pandey, J. C. Cook, jun., and K. L. Rinehart, jun., *J. Amer. Chem. Soc.*, 1977, **99**, 8469.
[198] B. F. Gisin, S. Kobayashi, and J. E. Hall, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 115.
[199] R. Kazlauskas, R. O. Lidgard, R. J. Wells, and W. Vetter, *Tetrahedron Letters*, 1977, 3183.
[200] T. Yamada, K. Suegane, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1977, **50**, 1088.
[201] T. Yamada, T. Miyazawa, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Japan*, 1977, **50**, 1827.



(97)

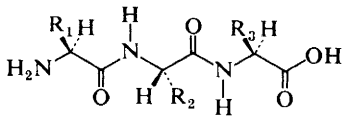
conceptual approach for linear peptides has not been widely developed. Retro-isomers of biologically active peptides may be resistant to inactivation by proteolytic enzymes and are therefore of potential significance. A general method for the synthesis of retroisomers of the type (98) in which the amino-acids have the opposite chirality and the peptide backbone is reversed from the parent compound (99) has been developed.[202]

Other areas of general interest include the conformational [203] and configurational [204] effects of incorporating didehydroamino-acids in linear peptides and the synthesis of *N*-hydroxy peptides.[205]



α,α-diamino residue — D-amino-acid retro-isomer — malonic acid residue

(98)



parent peptide

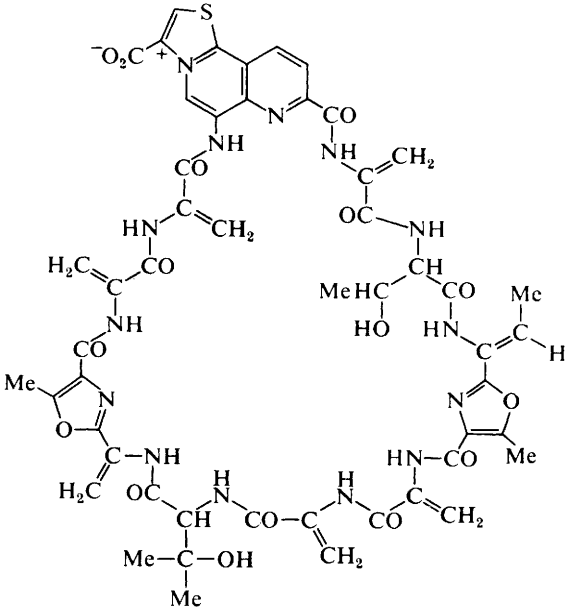
(99)

7 Large Highly Modified Peptides

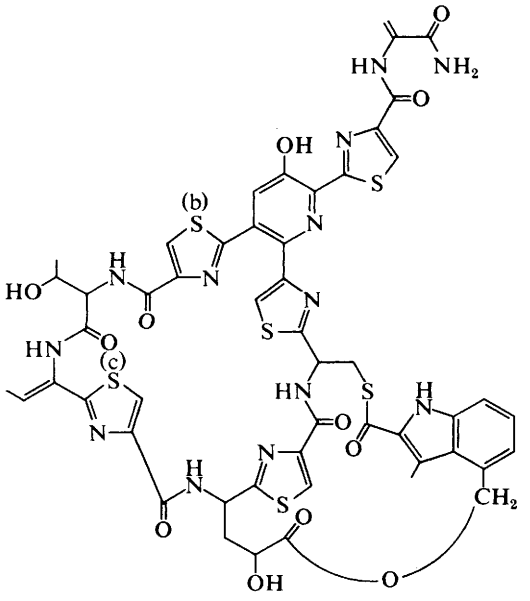
It has been considered necessary to introduce this rather loosely defined section to highlight the increasing number of relatively large peptide antibiotics, the predominant structural features of which are apparently derived by extensive modification of the individual amino-acid residues. Berninamycin A (100), a potent inhibitor of bacterial protein synthesis, contains several such units.[206] Particularly noteworthy are the didehydroalanine and butyrine entities presumably derived by dehydration of serine and threonine respectively, and the oxazole structures which can formally be considered as cyclized forms of didehydrothreonine residues.

The structure of nosiheptide (101) followed from ^{13}C [207] and ^{15}N [208] n.m.r. data,

[202] M. Chorev, C. G. Willson, and M. Goodman, *J. Amer. Chem. Soc.*, 1977, **99**, 8075.
[203] O. Pieroni, A. Fissi, G. Montagnoli, and F. Ciardelli, *Biopolymers*, 1977, **16**, 1677.
[204] J. S. Davies and M. N. Ibrahim, *Tetrahedron Letters*, 1977, 1453.
[205] U. Kolasa and A. Chimiak, *Tetrahedron*, 1977, **33**, 3285.
[206] J. M. Liesch and K. L. Rinehart, jun., *J. Amer. Chem. Soc.*, 1977, **99**, 1645.
[207] H. Depaire, J. P. Thomas, A. Brun, A. Olesker, and G. Lukacs, *Tetrahedron Letters*, 1977, 1397.
[208] H. Depaire, J. P. Thomas, A. Brun, W. E. Hull, A. Olesker, and G. Lukacs, *Tetrahedron Letters*, 1977, 1401.

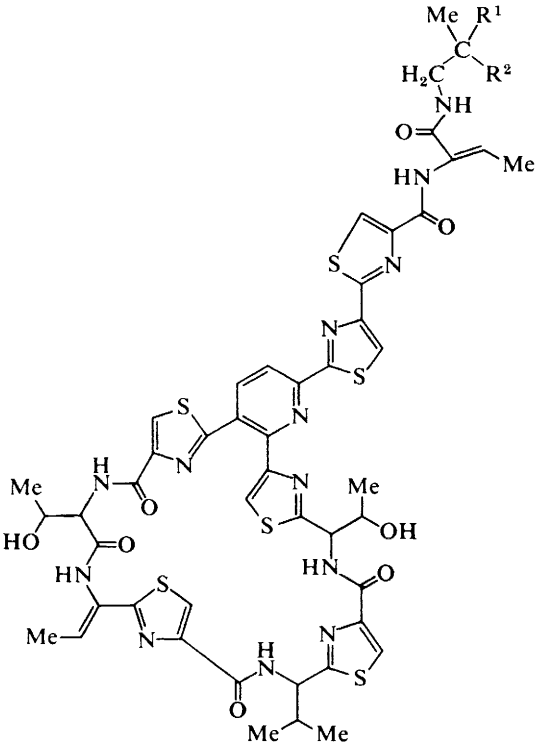


(100)



(101)

chemical degradation,[209, 210] and *X*-ray crystallographic evidence,[211] and once again the presence of substantially transformed amino-acid units is immediately apparent. Micrococcin P_1 (102) and P_2 (103) are clearly closely related to (101). Two independent investigations [212, 213] have come to similar conclusions concerning the structural units but propose slightly different sequences. A general hypothetical scheme for the derivation of thiostrepton, micrococcin, and related antibiotics has been outlined.[213]



(102) R^1 = H, R^2 = OH
(103) R^1, R = >O

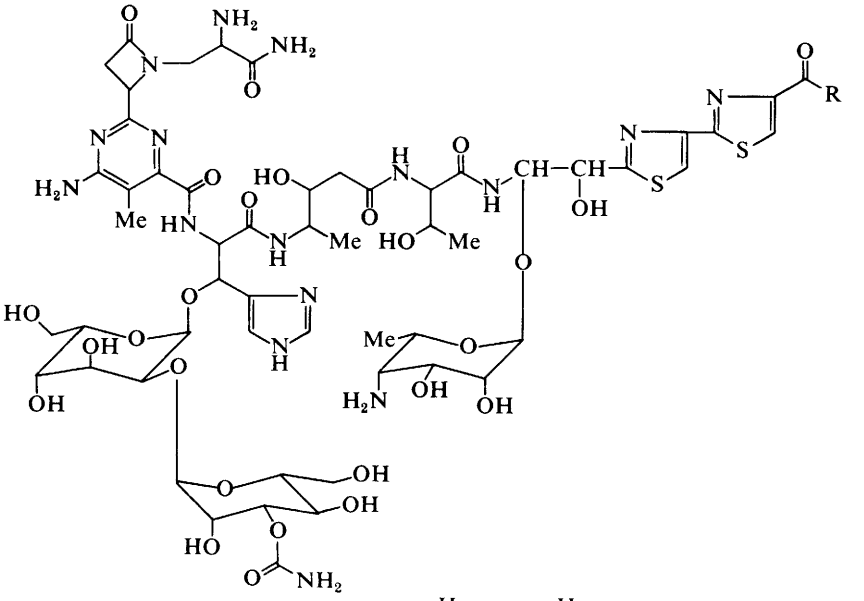
8 Peptides Linked to Carbohydrates

Glycopeptide Antibiotics.—An excellent review on the bleomycin family covers all aspects relating to the chemistry and biology of this important antitumour agent.[214] Tallysomycin A (104) and B (105) are new metabolites [215] closely related

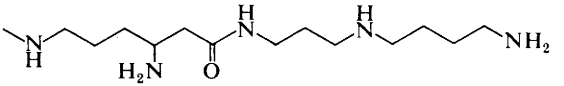
[209] H. Depaire, J. P. Thomas, A. Brun and G. Lukacs, *Tetrahedron Letters*, 1977, 1395.
[210] H. Depaire, J. P. Thomas, A. Brun, A. Olesker, and G. Lukacs, *Tetrahedron Letters*, 1977, 1403.
[211] C. Pascard, A. Ducruix, J. Lunel, and T. Prangé, *J. Amer. Chem. Soc.*, 1977, **99**, 6418.
[212] J. Walker, A. Olesker, L. Valente, R. Rabanal, and G. Lukacs, *J.C.S. Chem. Comm.*, 1977, 706.
[213] B. W. Bycroft and M. S. Gowland, *J.C.S. Chem. Comm.*, 1978, 256.
[214] H. Umezawa, *Lloydia*, 1977, **40**, 67.
[215] M. Konishi, K. Saito, K. Numata, T. Tsuno, K. Asama, H. Tsukiura, T. Naito, and H. Kawaguchi, *J. Antibiotics*, 1977, **30**, 789.

to the bleomycins. They contain two new amino-acids and the unique amino-sugar 4-amino-4,6-dideoxy-L-talose. Tallysomycin A has an additional amino-acid, L-β-lysine which had previously only been observed in the viomycin (see Section 2) and streptothricin (see below) groups.

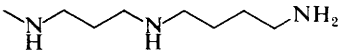
All the resonances have been assigned to specific hydrogens in the two most abundant congeners of bleomycin on the basis of homonuclear spin-decoupling experiments.[216] It is anticipated that this will serve as a basis for subsequent



(104) R =



(105) R =



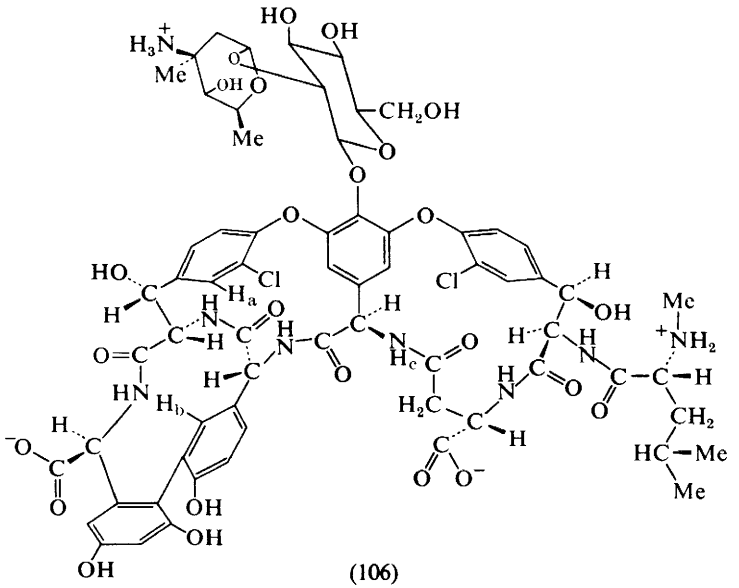
studies on the conformation of the molecule and its interaction with metals and nucleic acids. Substituted thiazole systems of the type found in bleomycin are common to many microbial peptides, and undoubtedly are derived from cysteine by cyclization and oxidation processes. An interesting biomimetic synthesis of the dithiazole entity of bleomycin offers a useful general route to these systems.[217]

A comprehensive investigation on the broad spectrum antibiotic vancomycin has culminated in the assignment of structure (106), based on spectral data [218] and an *X*-ray analysis [219] of a degradation product. The similarity of the structures derived, presumably by oxidative coupling of the phenolic moiety of

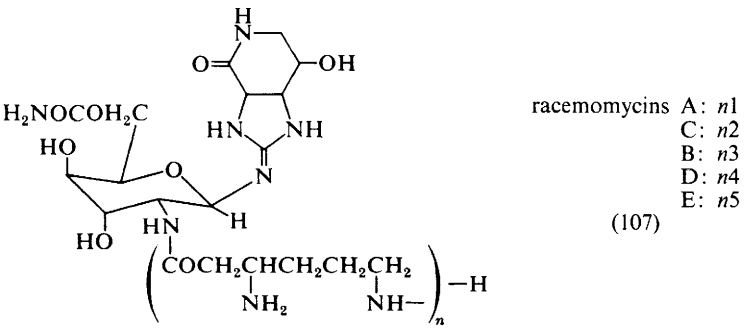
[216] D. M. Chen, B. L. Hawkins, and J. D. Glickson, *Biochemistry*, 1977, **16**, 2731.
[217] D. A. McGowan, U. Jordis, D. K. Minster, and S. M. Hecht, *J. Amer. Chem. Soc.*, 1977, **99**, 8078.
[218] D. H. Williams and J. R. Kalman, *J. Amer. Chem. Soc.*, 1977, **99**, 2768.
[219] G. M. Sheldrick, P. G. Jones, O. Kennard, D. H. Williams, and G. A. Smith, *Nature*, 1978, **271**, 223.

tyrosine units, and that found in (20) is noteworthy. The antibiotic inhibits the growth of cell walls by forming complexes with peptides terminating in D-alanyl-D-alanine, and a model for the complexes based on the spectral evidence has been advanced.[219]

The raceomycins (107) belong to the highly basic streptothricin family which contain varying numbers of β-lysine residues. Semisynthetic derivatives of this series may be prepared by condensing raceomycin A with *NN*-dicarbobenzoxy-L-

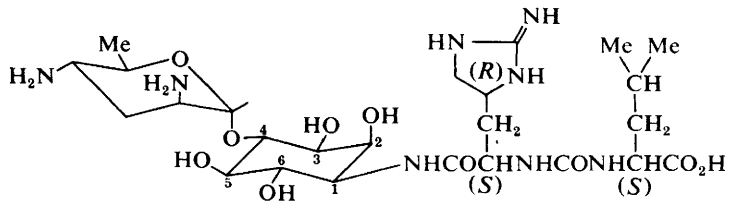


(106)



racemomycins A: *n*1
C: *n*2
B: *n*3
D: *n*4
E: *n*5

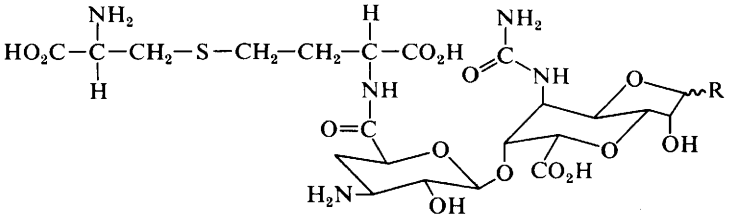
(107)



(108)

β-lysine or with similarly protected lysine peptides.[220] Biosynthetic studies suggest that raceomycin A is also the basic precursor of the whole group *in vivo*,[221] and, contrary to an earlier report, the streptolidine residue is derived from arginine.[222, 223] The details of a synthesis of the related guanidine containing antibiotic minosaminomysin (108) have appeared.[224]

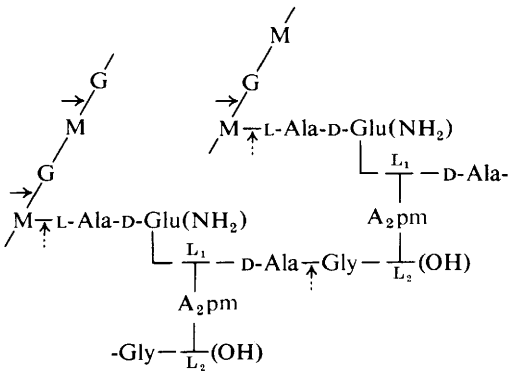
Several new derivatives of the important peptide nucleosides of the polyoxin family have been prepared [225] and the structures (109) and (110) established for the two peptide members of the ezomycins.[226]



(109) R = β-5-uracil
(110) R = α-5-uracil

Glycopeptides in Bacterial Cell Walls.—Due to the pressure on space and because this area comes under the general auspices of carbohydrate chemistry, this particular section has been curtailed and only highlights relating to the peptide fragments are covered.

Examination of the cell walls of several Streptomyces species related to *S. roseochromogenes* suggests that the structure of the main part of the peptidoglycan is as shown (111).[227, 228] *X*-ray results on the amorphous scattering of whole



(111)

[220] Y. Sawada and H. Taniyama, *Chem. Pharm. Bull. (Japan)*, 1977, **25**, 1302.
[221] Y. Sawada, S. Nakashima, and H. Taniyama, *Chem. Pharm. Bull. (Japan)*, 1977, **25**, 3210.
[222] U. Grafe, G. Reinhardt, H. Bocker, and H. Tkrum, *J. Antibiotics*, 1977, **30**, 106.
[223] Y. Sawada, S. Nakashima, H. Taniyama, Y. Inamori, S. Sunagawa, and M. Tsuruga, *Chem. Pharm. Bull. (Japan)*, 1977, **25**, 1161.
[224] F. Iinuma, S. Kondo, K. Maeda, and H. Umezawa, *Bull. Chem. Soc. Japan*, 1977, **50**, 1850.
[225] K. Sakata, A. Sakurai, and S. Tamura, *Agric. Biol. Chem. (Japan)*, 1977, **41**, 2033.
[226] T. Azuma, T. Saita, and K. Isono, *Chem. Pharm. Bull. (Japan)*, 1977, **25**, 1740.
[227] T. Nakamura, G. Tamura, and K. Arima, *Agric. Biol. Chem. (Japan)*, 1977, **41**, 763.
[228] T. Nakamura, G. Tamura, and K. Arima, *Agric. Biol. Chem. (Japan)*, 1977, **41**, 769.

bacterial cell wall and extracted peptidoglycans support the proposal that the peptide has proportions of residues in both helical and hydrogen-bonded β-sheet conformations.[229] Membrane preparations from *Gaffkya homari* catalysed the *in vitro* biosynthesis of soluble uncrossed-linked spin-labelled peptidoglycan from the spin-labelled nucleotide UDP-MurNAc-Ala-D-Glu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carbonyl-D-Ala-D-Ala and UDP-GlcNAc.[230] The complexation of the soluble spin-labelled peptidoglycan with vancomycin (106) (see ref. 219) resulted in both pronounced free-radical immobilization and a decrease in spin–spin exchange. These and other exchange effects are consistent with distance measurements in molecular models for peptidoglycans. A similar fluorescent labelled UDP-MurNAc-pentapeptide has been used as a specific reporter group to monitor the membrane-associated peptidoglycan synthesis [231] in *Staphylococcus aureus*.

Bacterial cell wall has a unique immuno-potentiating ability. It is now established that MurNAc-Ala-D-Gln, a common component of peptidoglycan in many bacteria, is the smallest structure which will invoke antibody production. Several analogues [232] have been synthesized as well as radio-labelled derivatives.[233]

[229] R. E. Burge, R. Adams, H. H. M. Balyuzi, and D. A. Reaveley, *J. Mol. Biol.*, 1977, **117**, 955.
[230] L. S. Johnston and F. C. Neuhaus, *Biochemistry*, 1977, **16**, 1251.
[231] W. A. Weppner and F. C. Neuhaus, *J. Biol. Chem.*, 1977, **252**, 2296.
[232] P. Lefrancier, J. Choay, M. Derrien, and I. Lederman, *Internat. J. Peptide Protein Res.*, 1977, **9**, 249.
[233] S. Kusumoto, K. Ikenaka, and T. Shiba, *Tetrahedron Letters*, 1977, 4055.

5

Chemical Structure and Biological Activity of Hormones and Related Compounds

With contributions by
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1 Anterior Pituitary Hormones

Contributed by R. Schwyzer and colleagues

This section is a continuation of last year's review [1] and mainly comprises of the literature of 1977 and early 1978. The nomenclature is according to the recommendations of the Commission on Biochemical Nomenclature IUPAC–IUB,[2] and the source designation is: h = human, b = bovine, c = canine, e = equine, o = ovine, and p = porcine.

The following compounds are treated (with the exception of the endorphines, but including chorionic hormones).

A. The Corticotropin–Melanotropin–Lipotropin–Endorphin Family (The Opiocortin [3] Family)
   Subfamily 1: Corticotropin (ACTH), α-melanotropin (α-MSH)
   Subfamily 2: Lipotropin (LPH), β-melanotropin (β-MSH), endorphins (including enkephalins)

B. The Glycoprotein Hormone Family
   Subfamily 1: Thyrotropin (TSH), follitropin (FSH)
   Subfamily 2: Lutropin (LH, ICSH), choriogonadotropin (CG)

C. The Single-chain Protein Hormone Family
   Subfamily 1: Prolactin (PRL)
   Subfamily 2: Somatotropin (STH), choriomammotropin (CS)

A number of general articles on polypeptide hormone receptors has appeared, covering: principles and techniques,[4] their functions and pitfalls in their study,[5] symmetrical features in polypeptide hormone–receptor interactions,[6] and receptor diseases.[7] The cytochemical bioassay of hormones like corticotropin, lutropin, thyrotropin, thyroliberin, and gastrin has also been reviewed.[8]

[1] R. Schwyzer, in 'Amino-acids, Peptides, and Proteins', Vol. 9, A Specialist Periodical Report, ed. R. C. Sheppard, The Chemical Society, London, 1978, p. 445.
[2] *J. Biol. Chem.*, 1975, **250**, 3215; *Bioorg. Khim.*, 1975, **2**, 129.
[3] M. Rubinstein, S. Stein, and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 669.
[4] M. D. Hollenberg and P. Cuatrecasas, *Methods Cancer Res.*, 1976, **12**, 317.
[5] P. Cuatrecasas, *Adv. Cyclic Nucleotide Res.*, 1975, **5**, 79.
[6] C. R. Beddell, G. C. Sheppey, T. L. Blundell, K. Sadaki, S. Dockerill, and P. J. Goodford, *Internat. J. Peptide Protein Res.*, 1977, **9**, 161.
[7] K. Megyesi, R. S. Bar, R. C. Eastman, and J. S. Flier, *Ann. Intern. Med.*, 1977, **86**, 205.
[8] J. Chayen, J. R. Daly, N. Loveridge, and L. Bitensky, *Recent Prog. Horm. Res.*, 1976, **32**, 33.

The Opiocortin Family.—The group of neural hormones: ACTH, MSH, LPH, and the endorphins, is becoming increasingly important because of the central nervous effects, not only of the endorphins, but also of peptide sequences contained in the more 'classical' members, ACTH and MSH. New evidence has been provided that all of these hormones are derived from one large glycoprotein precursor (originally containing a 'signal' [9] sequence ?) with mol. wt. ~30 000.[3, 10, 11, 12] The precursor was dubbed 'pro-opiocortin,[3] from which the reviewer gratefully derives the name '*Opiocortin Family*'.

Corticotropin (ACTH).—*Isolation.* Contradictory reports have again appeared about the properties, in particular the molecular weight, of the corticotropin liberin(s) (releasing factors, CRF): > 15 000 [13] and < 1500.[14] ACTH was isolated for the first time from the avian species *Struthio camelus;* it contains 39 amino-acid residues and has 8.3 < p*I* < 8.7.[15] ACTH from a thymic tumour associated with ectopic hormone production was shown to be hACTH-(2—38)-heptatriacontapeptide, suggesting a different mode of cleavage from the precursor.[16]

Synthetic Work. New syntheses in solution of h- and p-ACTH have been reported: in one case, the protecting groups Z, Tos, NO_2, and Bzl were removed with HF in the final step,[17] in the other, N(α)-protection was effected with Z, whereas a new group, 1-methyl cyclohexyloxycarbonyl (Mhoc), was used for Lys side-chain protection; it was removed by trifluoroacetic acid.[18] Purity was ascertained chemically and biologically. ACTH-(1—24)-tetracosapeptide was prepared on a solid support from '6 synthetic fragments by *C*-terminal chain elongation', using the so-called oxidation–reduction condensation method (triphenylphosphine, 2-pyridyldisulfide).[19]

Structure–Activity Relations. A new approach to immobilized ACTH has been described:[20] it is based on the formation of very stable complexes with $K_{ass} = 10^{15}$ between [biocytin [25]]ACTH-(1—25)-pentacosapeptide amide (a biologically fully active ACTH derivative) and an avidin–Sepharose conjugate. The synthesis of ACTH analogues lacking the *C*-terminal sequence 25—39 and various *N*-terminal segments (1—4, 1—5, 1—6, and 1—7) that showed a dissociation of adenylate cyclase-stimulating and steroidogenic effects [1] has been reported in

[9] G. Blobel and B. Dobberstein, *J. Cell Biol.*, 1975, **67**, 835.
[10] D. N. Orth and W. E. Nicholson, *J. Clin. Endocrinol. Metabol.*, 1977, **44**, 214.
[11] R. E. Mains, B. A. Eipper, and N. Ling, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3014.
[12] T. H. Lee and M. S. Lee, *Biochemistry*, 1977, **16**, 2824.
[13] J. W. Kendall, D. K. Gray, and N. D. Gaudette, *Proc. Soc. Exp. Biol. Med.*, 1976, **152**, 691.
[14] D. M. F. Cooper, D. Synetos, R. B. Christie, and D. Schulster, *J. Endocrinol.*, 1976, **71**, 171.
[15] R. J. Naudé and W. Oelofsen, *Biochem. J.*, 1977, **165**, 519.
[16] P. J. Lowry, L. H. Rees, S. Tomlin, G. Gillies, and J. Landon. *J. Clin. Endocrinol. Mebatol.*, 1976, **43**, 831.
[17] K. Koyama, H. Watanabe, H. Kawatani, J. Iwai, and H. Yajima, *Chem. Pharm. Bull. (Tokyo)*, 1976, **24**, 2558.
[18] K. Watanabe and K. Inouye, *Bull. Chem. Soc. Japan*, 1977, **50**, 201; K. Inouye and K. Watanabe, *Bull. Chem. Soc. Japan*, 1977, **50**, 207; K. Inouye, K. Watanabe, and H. Otsuka, *Bull. Chem. Soc. Japan*, 1977, **50**, 211.
[19] H. Maruyama, R. Matsueda, E. Kitazawa, H. Takahagi, and T. Mukaiyama, *Bull. Chem. Soc. Japan*, 1976, **49**, 2259.
[20] K. Hofmann and Y. Kiso, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3516.

detail.[21] Replacement of arginine-(8) by its lower homologue, norarginine (Nar), as in [Nar^{8},Lys^{17,18}]ACTH-(1—18)-octadecapeptide amide and [Nar^{8}]ACTH-(1—24)-tetracosapeptide, lead to biologically active compounds.[22] In contrast, the substitution of tryptophan-(9) by phenylalanine or *ar*-pentamethyl-phenylalanine reduced the steroidogenic and lipolytic activity rather drastically without affecting the melanophore-stimulating potency.[23] The latter observation is in keeping with the findings of the Zürich group [1] on the organization of information in the ACTH and α-MSH molecules.

Interesting results were reported by the Riga group.[24] [Lys^{17,18}]ACTH-(11—18)-octadecapeptide amide (1) was found to stimulate steroidogenesis in isolated rat adrenal cells at high doses (1—100 μg ml^{-1}). Compound (1) and a structurally somewhat related peptide, wasp kinin-(4—12)-nonapeptide (2), were found to potentiate ACTH-induced lipolysis and steroidogenesis in isolated cells at low doses (10^{-8} to 10^{-3} μg ml^{-1}) but to suppress the myotropic effect of bradykinin on guinea-pig ileum.

Lys-Pro-Val-Gly-Lys-Lys-Lys-Lys

(1)

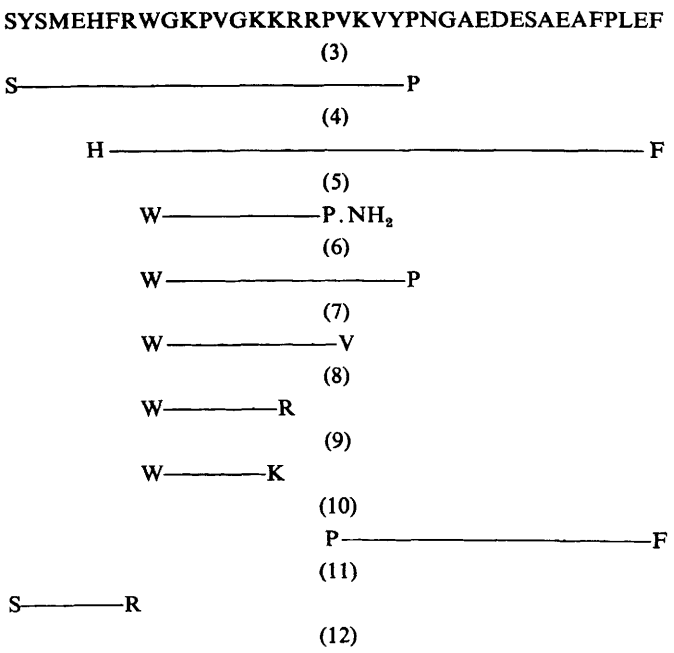
Lys-Lys-Lys-Leu-Arg-Gly-Arg-Pro-Pro

(2)

Agonistic and antagonistic effects of h-ACTH and synthetic fragments on rat adrenal cell membrane adenylate cyclase were determined and correlated with the binding of the peptides to the membrane preparation.[25] Only synthetic h-ACTH (3) and ACTH-(1—24)-tetracosapeptide (4) behaved as agonists, shorter fragments, (5)—(9) were [in contrast to (10)—(12)] inhibitors of ACTH-stimulated adenylate cyclase activity and of the binding of iodinated (8) to membranes. That (5) does not stimulate adenylate cyclase is an apparent contradiction to the findings of Lang [26] and Bonnafous [27] (reviewed in ref. 1).

An alkylpropyl derivative of ACTH-(1—19)-nonadecapeptide was reported to induce histamine release under a variety of experimental conditions and to be more active than mellitin in this respect.[28] *In vivo* 11β-hydroxylase activity of the adrenal cortex appears to parallel the ACTH secretion, but was found to be independent of the angiotensin II level.[29] A model of ACTH-induced steroidogenesis in rat

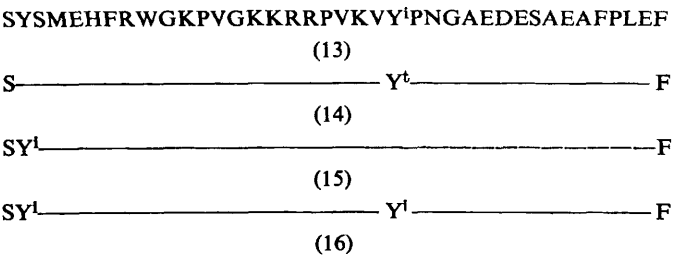
[21] J. L. Fauchère and C. Petermann, *Helv. Chim. Acta*, 1978, **61**, 1186.
[22] J. W. van Nispen, G. I. Tesser, and R. J. F. Nivard, *Internat. J. Peptide Protein Res.*, 1977, **9**, 193.
[23] J. W. van Nispen, G. I. Tesser, P. L. Barthe, R. Maier, and L. Schenkel-Hulliger, *Acta Endocrinol.*, 1977, **84**, 470; J. W. van Nispen, P. J. Smeets, E. H. Poll, and G. I. Tesser, *Internat. J. Peptide Protein Res.*, 1977, **9**, 203.
[24] Ye. A. Porunkevich, G. G. Kublis, A. A. Skujinsh, and G. I. Chipens, *Biokhimiya*, 1977, **42**, 267; G. G. Kublis, Ye. A. Porunkevich, A. A. Skujinsh, N. A. Makarova, I. P. Misinish, V. E. Klusha, and G. I. Chipens, *Biokhimiya*, 1977, **42**, 616.
[25] D. K. Ways, C. F. Zimmerman, and D. A. Ontjes, *Mol. Pharmacol.*, 1976, **12**, 789.
[26] U. Lang, J. L. Fauchère, G. M. Pelican, G. Karlaganis, and R. Schwyzer, *F.E.B.S. Letters*, 1976, **66**, 246.
[27] J. C. Bonnafous, J. L. Fauchère, W. Schlegel, and R. Schwyzer, *F.E.B.S. Letters*, 1977, **78**, 247.
[28] M. Rüegg and R. Jaques, *Pharmacology*, 1977, **15**, 134.
[29] A. Canguly, A. W. Meikle, F. H. Tyler, and C. D. West, *J. Clin. Endocrinol. Metabol.*, 1977, **44**, 560.



For a key to the one-letter abbreviation of amino-acids see these Reports, Vol. 5, p. 487.

adrenal cortex cells was proposed [30] in which calcium ion serves as a direct messenger, and cAMP would be a subserving factor maintaining full steroidogenesis.

Labelled Compounds. Synthetic [Tyr(3,5-I_2)[23]]h-ACTH (13) was catalytically tritiated to give biologically fully active [Tyr(3,5-$^3H_2$)[23]]h-ACTH (14) with specific activities of 46 ci mmol^{-1} [31] and 25.2 Ci mmol^{-1}.[32] The steroidogenic activity of (13) was found to be 64% of h-ACTH, that of [Tyr (3,5-I_2)[2]]h-ACTH (15) only about 2.4%, and of [Tyr(3,5-I_2)[2, 23]]h-ACTH (16) about 2.2%,[33] entirely



Y^i = 3,5-di-iodotyrosine, Y^t = 3,5-ditritiotyrosine

[30] R. Neher and A. Milani, *Clin. Endocrinol.*, 1976, **5** (Suppl.), 29s.
[31] J. Ramachandran and C. Behrens, *Biochim. Biophys. Acta*, 1977, **496**, 321.
[32] D. E. Brundish and R. Wade, *Biochem. J.*, 1977, **165**, 169.
[33] S. Lemaire, D. Yamashiro, C. Behrens, and C. H. Li, *J. Amer. Chem. Soc.*, 1977, **99**, 1577.

in keeping with our knowledge on the organization of information in the ACTH molecule.[34]

An introduction of the tritium label into compound (16) might prove to be rather useful for degradation studies.

α- and β-Melanotropins.—*Isolation and Structure.* Eight new peptides with melanotropic activity were isolated from incubated neurointermediate lobes of *Xenopus laevus* using a radioactive pulse-chase paradigm; their release was inhibited by dopamine.[35] The immunoreactive 'β-MSH-like' substances of human plasma are most likely lipotropin(s).[36] From *Bombyx mori* larvae a melanizing compound (MRCH = melanization and reddish coloration hormone) that might prove to be a peptide was isolated.[37]

Chemical Synthesis. A new synthesis of desacetyl α-MSH was described and its specific acetylation *via* an enzyme from calf lens studied.[38] New syntheses of camel β-MSH I and II with melanotropic activites of 5×10^9 and 1×10^9 U g^{-1}, respectively, were also described.[39]

Biosynthesis, Localization, Storage, and Release. Ectopic production of MSH by two tumours was reported.[40] Immunohistochemical localization studies of α-MSH in the brain,[41] and of both ACTH and MSH in the adenohypophysis (human and rat,[42] *Eliomys quercinus* [43]) were performed. In a study of the subcellular localization of α-MSH in the rat hypothalamus, it was suggested that α-MSH is stored in synaptosomes, supporting the idea of a neurotransmitter function of the hormone.[44] During dark adaptation of *Xenopus laevis*, a stored precursor in the *pars intermedia* is converted to α-MSH, which is then released; the precursor being resynthesized in an accelerated fashion.[45] The effects on the release of MSH from the *pars intermedia* of *Rana pipiens* by apomorphine and ergocrystine,[46] and by dopamine receptor blockers in the rat [47] were studied.

Biological Effects. Besides acting on its 'classical' target, the melanocyte, MSH appears to influence the CNS, the sebaceous, preputial, and foetal adrenal glands,

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[35] Y. P. Loh and H. Gainer, *J. Gen. Physiol.*, 1977, **70**, 37; *Brain Research*, 1977, **130**, 169.
[36] X. Bertagna, M. Donnadieu, M. Binoux, and F. Girard, *Ann. Endocrinol. (Paris)*, 1976, **37**, 455.
[37] A. Suzuki, S. Matsumoto, N. Ogura, A. Isogai, and S. Tamura, *Agric. Biol. Chem.*, 1976, **40**, 2307.
[38] P. Smeets, M. Granger, J. W. Van Nispen, H. Bloemendal, and G. I. Tesser, *Internat. J. Peptide Protein Res.*, 1977, **9**, 52.
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[40] K. Abe, I. Adashi, S. Miyakawa, M. Tanaka, K. Yamaguchi, N. Tanaka, T. Kameya, and Y. Shimosato, *Cancer Res.*, 1977, **37**, 4190; H. L. Waldum, P. G. Burhol, J. A. Johnson, and A. G. Smith, *Acta Hepato-gastroenterologica*, 1977, **24**, 386.
[41] G. Pelletier and D. Dube, *Amer. J. Anat.*, 1977, **150**, 201.
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and perhaps other target organs: for this reason its biological effects are receiving much attention. The conversion of tyrosine to catecholamines,[48] the induction of melanogenesis in melanoblasts,[49] the effects on human melanocytes *in vitro*,[50] and the enhancement of tyrosinase activity in melanoma cells [51] were studied. It is likely that the influence of MSH on growth-stimulation of melanoma cell cultures is due to enhancement of cAMP levels and that growth inhibition is caused by the increase of tyrosinase activity.[52] In the field of MSH action on the CNS, papers have appeared on its influence on learning (attentiveness) in laboratory animals [53] and in man,[54] on various innate behavioural effects,[55] and on pharmacologically measurable effects: response of mesencephalic and hypothalamic dopamine neurones to α-MSH (mediated by *area postrema* ?),[56] effect on α-motoneurones of cat spinal cord.[57] α-MSH effects on preputial [58] and sebaceous glands [59] have been reported. In mammals, α-MSH possibly plays its main role during foetal life (sebaceous glands [59]): it now appears that it might also be a foetal trophic hormone to adrenal function in contrast to ACTH that is ineffective in the foetus.[60] In addition, a number of other studies on various (including clinical) aspects have appeared.[61]

Receptor Binding. Cultured *Cloudman* melanoma cells exposed either to dibutyryl cAMP and theophyllin or to cholera toxin bind significantly more [^{125}I]-β-MSH or fluorescein-labelled β-MSH than untreated cells. The effect is most marked in the G2 phase of the cell cycle. The affinity is not affected. The significance of this positive feedback phenomenon remains unclear.[62]

Structure–Activity Relationships. [11-Serine]-α-MSH was synthesized and reported to have 40% activity of α-MSH (1.7×10^{10} U g^{-1}) in three assay systems (frog skin *in vitro*, light-adapted frogs, and hypophysectomized frogs).[63] The syntheses and activities of [9-phenylalanine]-α-MSH and [9-pentamethylphenylalanine]-α-MSH were described: the melanotropic activity of the second compound is now

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[49] T. Hirobe and T. Takeuchi, *In Vitro*, 1977, **13**, 311.
[50] Y. Kitano, *Arch. Dermatol. Res.*, 1976, **255**, 163.
[51] T. H. Lee and M. S. Lee, *Acta Endocrinol.*, 1977, **84**, 663.
[52] R. Halaban and A. B. Lerner, *Exp. Cell Res.*, 1977, **108**, 111.
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[60] J. R. G. Challis and J. D. Torosis, *Nature*, 1977, **269**, 818.
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[62] A. DiPasquale, J. McGuire, and J. M. Varga, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 601.
[63] R. Geiger, J. Sandow, and A. J. Kastin, *Hoppe-Seyler's Z. Physiol. Chem.*, 1977, **358**, 1475.

reported to be 4—5 times greater than that of the first; the relative potencies are reversed for *in vivo* lipolysis in rabbits.[64] A report on the synthesis and biological activity of camel β-MSH and analogues has appeared.[65] The following compounds were prepared:

DGGPYKMQHFRWGSPPKD (camel β-MSH)

(17)

D——————W^f———D (W^f = [formyl]Trp)

(18)

D——————F———D

(19)

D——————G———D

(20)

D————R——————D

(21)

The results support findings obtained for ACTH and α-MSH:[1] integrity of the message-sequence Trp is essential for lipolysis, much less so for melanotropy (18, 19); message-sequence Phe is necessary for both activities (20); in contrast to earlier findings,[66] replacement of potentiator-sequence Glu/Gln by Arg (21) does not enhance, but decreases rabbit lipolytic potency (and melanotropic potency as well).

Labelled Compounds. [N^{α}-Bromoacetyl-D-alanine[1], 3′,5′-ditritio-L-tyrosine[2], glycine[3], L-norvaline[4]]-α-MSH (22), [N^{α}-diazoacetylglycine[1], 3′,5′-ditritio-L-tyrosine[2], L-norvaline[4]]-α-MSH (23),[67] and [3′,5′-ditritiotyrosine[2]]-α-MSH (24)[68] were prepared (A^d = D-alanine; Y^t = 3′,5′-ditritiotyrosine; V^n = L-norvaline):

$Br.CH_2CO.A^dY^tGV^nEHFRWGKPV.NH_2$ (22)	2×10^9 U mmol^{-1} (determined for the compound with Br replaced by H); 19.7 Ci mmol^{-1}
$N_2{=}CHCO.GY^tSV^nEHFRWGKPV.NH_2$ (23)	5×10^9 U mmol^{-1}; 36 Ci mmol^{-1}
$Ac.SY^tSMEHRFWGKPV.NH_2$ (24)	$1—2 \times 10^{10}$ U mmol^{-1}; 2.6—4 Ci mmol^{-1}

Furthermore, three biologically active, covalent α-MSH–protein conjugates were described. One contained seven molecules of (22) attached through their bromoacetyl moieties with mercaptosuccinyl human serum albumin (9×10^8 U mmol^{-1} = 45% of the activity of 22),[69] another contained 500 molecules of 22 similarly

[64] J. W. van Nispen, P. J. H. Smeets, E. H. A. Poll, and G. I. Tesser, *Internat. J. Peptide Protein Res.*, 1977, **9**, 203.
[65] S. Lemaire, D. Yamashiro, A. J. Rao, and C. H. Li, *J. Med. Chem.*, 1977, **20**, 155.
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[68] K. Nikolics, I. Teplán, and K. Medzihradszky, *J. Labelled Compd. Radiopharmaceuticals*, 1976, **12**, 163.
[69] A. Eberle, V. M. Kriwaczek, and R. Schwyzer, *F.E.B.S. Letters*, 1977, **80**, 246.

attached to tobacco mosaic virus (8×10^8 U mmol^{-1} = 40%).[70] The latter served for electron-microscopic identification of receptor-bearing vesicles by *co-operative affinity labelling* and their separation by density gradient centrifugation. A third conjugate was that of five molecules of α-MSH (bound through the N^{ε}-group of lysine) with ferritin that was labelled with six molecules of fluorescein (4×10^9 U mmol^{-1} = 10% of α-MSH).[71] This compound served the purpose of electron-microscopic and fluorescence-microscopic visualization of receptors.

Assay. A reflectance bioassay for MSH using the skin of the American chameleon *Anolis carolinensis* was developed to give a rapid (20 min per sample) assay with an index of precision of 0.1.[72] Methods for preparing ACTH and MSH from plasma samples for immunoassay are reported: they are based on silicate adsorption and elution by different eluants.[73]

Lipotropin.—The analgesic effect of β-LPH and its fragments administered intracerebroventricularly to rats was studied: whereas β-LPH proved to be a specific, morphine-like analgesic with about half of its potency, several smaller fragments, including β-LPH-(61—65)-pentapeptide (enkephalin), were found to be inactive, in contrast to their behaviour in the longitudinal muscle strip of guinea-pig ileum.[74] A similar study was carried out with o-β-LPH and its fragments using the mouse *vas deferens* assay: the 'active core' appears to be the enkephalin sequence.[75]

The Glycoprotein Hormone Family.—*Isolation.* Human pituitary *thyrotropin* was separated by preparative gel electrophoresis into five chemically very closely related, equally active, glycoproteins.[76] Human pituitary tissue appears to contain a large molecular weight protein with *follitropin*-like biological and immunological activity, but which is distinct from native h-FSH (a precursor?).[77] A reproducible method (involving a radioligand-receptor assay) was reported for the purification of b-FSH from frozen b pituitary glands; potency in the Steelman–Pohley bioassay (164 × NIH-FSH-S1 standard) and amino-acid analysis are given.[78] The isolations of a 'mini *lutropin*' from human serum[79] and of four whale lutropins[80] were described. Sedimentation velocity and equilibrium experiments demonstrated a complex system of association–dissociation for h-lutropin: only at $I = 0.1$, pH 5.8, was there a clear indication of $\alpha_1\beta_1$ composition mol. wt. = 32 000 over a range of concentrations.[81]

[70] V. M. Kriwaczek, A. N. Eberle, M. Müller, and R. Schwyzer, *Helv. Chim. Acta*, 1978, **61**, 1232; V. M. Kriwaczek, J. C. Bonnafous, M. Müller, and R. Schwyzer, *ibid.*, 1241.
[71] A. DiPasquale, J. M. Varga, G. Moellmann, and J. McGuire, *Analyt. Biochem.*, 1978, **84**, 37.
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[78] K.-W. Cheng, *Biochem. J.*, 1976, **159**, 651.
[79] F. A. Leidenberger, D. Graesslin, H. J. Scheel, N. Hess, V. Lichtenberg, and G. Bettendorf, *J. Clin. Endocrinol. Metabol.*, 1976, **43**, 1410.
[80] H. Tamura-Takahashi and N. Ui, *J. Biochem.*, 1977, **81**, 1155.
[81] A. Nureddin and P. Johnson, *Biochemistry*, 1977, **16**, 1730.

Structure. Evidence was provided that the *N*-terminal 40-residue sequence of *choleratoxin β-chain* is significantly homologous with the same region of TSH, FSH, LH, and CG, although the overall similarity of the toxin β-chain with the hormone β-chains was not statistically different from random.[82] Studies of disulphide bond location in o-*lutropin* β-subunit[83] and of the optical activity of ionized tyrosyl residues in o-LH[84] were reported. Native and recombinant h-*choriogonadotropins* are fully active in tests of follicle and interstitial cell stimulation whereas the isolated α- and β-subunits are completely inactive.[85]

Biological Activity. In cytosols from rat thyroid glands, *thyrotropin* was observed to stimulate selectively the activities of cAMP-dependent and cAMP-independent protein phosphokinases;[86] TSH and dibutyryl cAMP promote specifically the phosphorylation of serine residues situated in the *N*-terminal region of thyroid H1 histones.[87] Further evidence was produced indicating that – at low hormone concentrations – *lutropin* and *follitropin* might act through a pathway independent of cAMP synthesis and activation of protein kinase to activate steroidogenesis in dispersed rat testis interstitial cells.[88] Human TSH and h-CG stimulate lipolysis in isolated rat adipocytes: the isolated subunits were inactive, reconstitution regenerated 60—100% activity (the h-LH α-chain + h-TSH β-chain recombinant was considerably less active).[89] TSH and CG inhibit the antiviral action of interferon probably by competing for cell surface binding-sites.[90]

Receptors.—*Thyrotropin.* The NIH group working with TSH receptors was able to separate a protein receptor component from a ganglioside component of thyroid plasma membranes upon solubilization with lithium di-iodosalicylate;[91] it appears that artificial liposomes containing mixed brain gangliosides specifically bind the ^{125}I-labelled hormone.[92] A good correlation was found between p-thyroid membrane receptor occupancy by TSH (K_D = 0.28 nm) and adenylate cyclase activation.[93] Studies with thyroid cell cultures revealed a single type of high-affinity low-capacity TSH binding-site and an excellent correlation between K_D, half-stimulation of adenylate cyclase, and iodide transport mechanism activation; furthermore, the morphogenetic action of TSH and its ability to maintain the specific metabolic properties of thyroid cells in culture were shown to be mediated by cAMP *via* new RNA and protein synthesis.[94] Similar observations on TSH binding, cAMP and iodine release were made with isolated p-

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[94] S. Lissitzky, G. Fayet, and B. Verrier, *Adv. Cyclic Nucleotide Res.*, 1975, **5**, 133.

thyroid cells; large doses of TSH resulted in a *reduction* of iodine release [95] (for similar inhibitory actions of corticotropin in high doses, see ref. 27). Although in b-thyroid plasma membrane preparations, the receptors for TSH linked to adenylate cyclase functioned independently from the β-adrenergic receptors,[96] the β-blocker propranolol doubled the amount of TSH binding-sites in h-thyroid membranes, probably by a general membrane effect of the drug (not β-receptor-mediated),[97] reminiscent of the increase of corticotropin receptor-sites of fat cells caused by actinocin and actinomycin.[98]

Follitropin. During photoperiodically induced testicular growth of sparrow testes, the amount of specific FSH-binding-sites per mg of tissue homogenate was found to decrease, although the total amount increases and reaches a plateau after three weeks despite further growth of the glands.[99] The FSH receptor of calf testis (an unusually rich source) was solubilized with Triton X100 and characterized as containing one binding-site class with K_D = 0.48 nm at pH 7.5, 24 °C, and 3 h incubation; stokes radius 47 Å, S = 6.3 (with bound hormone: 50 Å and 7.4, respectively).[100]

Lutropin. The h-CG-induced regulation of testicular LH receptors and cAMP and testosterone response was studied [101] and the properties of detergent-solubilized adenylate cyclase and gonadotropin receptors of testes and ovaries described.[102] A negative regulation of LH receptors in rat testes by LH and h-CG, but not FSH, prolactin, and somatotropin was observed: the method made use of 4M-$MgCl_2$ to dissociate the LH–receptor complex without altering either capacity or affinity.[103] It was shown that the species specificity of h-ovarian binding-sites for LH and CG is greatest in fresh ovaries and strongly deteriorates in frozen glands.[104]

A method for the differential labelling of b-*corpus luteum* plasma membrane h-CG or LH receptor with ^{131}I was described; its solubilization and purification were achieved with Sepharose–concanavalin and Sepharose–h-CG conjugates, followed by preparative polyacrylamide gel electrophoresis. The fairly stable compound retained its hormonal specificity, binding affinity, and pH optimum.[105] The b-*corpus luteum* cell membrane [^3H]prostaglandin F_{2a} receptor was shown to be a protein with a critical SH group and with two classes of saturable binding-site (K_D = 1.6 nm and 24 nm).[106] Because many binding studies suffer from the

[95] P. M. Povey, B. Rees Smith, T. F. Davies, and R. Hall, *F.E.B.S. Letters*, 1977, **78**, 251.
[96] N. J. Marshall, S. von Borcke, and R. P. Ekins, *Nature*, 1976, **261**, 603.
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[106] C. V. Rao, *Mol. Cell. Endocrinol.*, 1976, **6**, 1.

use of iodine-labelled LH with reduced specificity (see ref. 107 where structure–binding relationships are also reported), studies with non-labelled hormone would be very welcome: Factors influencing binding and response in the LH/r ovary system for LH and LH β-chain were studied using a radioimmunoassay to measure bound LH.[108] The labelling of LH by tritium using reductive methylation[109] and biosynthesis in the presence of labelled leucine or glucosamine[110] as well as pertinent binding studies were also reported.

Human Chorionic Gonadotropin. Gangliosides inhibit the binding of [^{125}I]h-CG to rat testes membranes: this is the result of an interaction between the hormone and the inhibitor rather than of an interaction between the membrane and the ganglioside (see also the interaction of ganglioside-containing liposomes with thyrotropin [92]).[111]

Modification of h-Follitropin with $^{125}I_2$ was examined[112] and *radioimmunoassays* for TSH[113] and h-CG/h-LH[114] reported; antigenic similarities in the unique carboxy-terminal peptide of CG β-chains of primates were investigated.[115]

The Single-chain Protein Hormone Family.—*Prolactin.* A partial amino-acid sequence of rat pre-prolactin from cell-free biosynthesis was reported: the precursor contains 20 additional residues at the *N*-terminus of the hormone with considerable similarity with other precursors.[116] Pituitary prolactin from dogs[117] and chorioprolactin from human amniotic fluid[118] were isolated and partially characterized. High molecular weight aggregates, dimeric and monomeric forms of o- and h-prolactin were demonstrated in iodinated hormone preparations (lactoperoxidase); the polymers appear to contain disulphide bonds between the monomers.[119] In mammary-gland homogenates, phospholipase A activity is quite specifically stimulated by prolactin (somatotropin is without effect);[120] in tissue cultures from pregnant mouse mammary glands, a mixture of insulin, cortisol, and prolactin was found to activate both lactogenesis and glutathione–insulin transhydrogenase.[121]

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[120] J. A. Rillema and E. A. Wild, *Endocrinology*, 1977, **100**, 1219.
[121] B. M. Ferrier, J. M. Hendrie, and C. A. Cardy, *Canad. J. Biochem.*, 1977, **55**, 340.

Prolactin Receptors or Binding-sites were the subject of a number of studies. Hepatocytes from female rats bind both somatotropin and prolactin with about 40 000 sites per cell for each hormone and apparent affinity constants of 1×10^9 and 0.4×10^9 M^{-1}, respectively; male hepatocytes bind only somatotropin; only the PRL binding-sites are lost after hypophysectomy.[122] About 70% of the prolactin binding in female rat livers can be inhibited by concanavalin A;[123] the number of sites in male rat liver membranes is enhanced by castration and reduced by testosterone.[124] Apparent affinity constants for prolactin of about 10^9 M^{-1} were found for foetal rhesus cell membrane fractions from placenta, liver, lung, and myocardium, but not from brain.[125] Similar constants were found for fluorescent-labelled prolactin (and somatotropin) with subcellular fractions from rabbit mammary tissue by fluorescence polarization.[126]

Chemical Modification of prolactin with iodine and tetranitromethane gave the following sequence of reactivity for the seven tyrosine residues: 44, 125 highly reactive, 147, 169, 185 partially so, and 96, 28 unreactive; limited modification hardly affects biological activity on the pigeon crop sac.[127]

Structure–Activity Relationships for o-prolactin were considered under the aspect that the *C*-terminal undecapeptide bears sequence similarities with protease inhibitors.[128] A *radioimmunoassay* for prolactin in the plasma of domestic fowl[129] and a bioassay distinguishing prolactins and various somatotropins[130] were described. The latter uses mouse mammary gland casein synthesis and teleost urinary bladder water permeability.

Somatotropin. An excellent *review* on the chemistry of human pituitary growth hormone[131] should be recalled; it covers aspects like isolation and characterization, molecular properties, structure–function relationship, and chemical synthesis of peptides with somatotropin-like activity. *High molecular weight forms* of STH from pituitaries were described that were or were not cleaved to smaller molecules by mercaptans.[132] Somatotropins have been *isolated and purified* from teleosts,[133] reptiles, and amphibia,[134] and a *growth-modulating peptide* from h-serum and plasma was identified as Gly-His-Lys.[135] The *primary structure* of sei whale,

[122] M. B. Ranke, C. A. Stanley, A. Tenore, D. Rodbard, A. M. Bongiovanni, and J. S. Parks, *Endocrinology*, 1976, **99**, 1033.
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[135] D. H. Schlesinger, L. Pickart, and M. M. Thaler, *Experientia*, 1977, **33**, 324.

Balaenoptera borealis, STH was elucidated.[136] The *conformation* and conformational transitions of biologically active STH fragments [137] and the *configuration* of the disulphide bridges [138] were studied and predictions made. The tissue-specific stimulation of *ornithine decarboxylase* by pituitary factors immunologically related to STH [139] and antiserum effects [140] were examined. STH was reported to bind to *prolactin binding-sites* (see also ref. 122) of male and female rat liver membranes, the number of which can be regulated by pretreatment with oestradiol (increase) and testosterone (decrease).[141]

Structure–Activity Relationships were examined by chemical modification of individual amino-acids (oxidation of methionine,[142] trinitrophenylation,[143] nitration,[144] and polyalanination [145]), and the investigation of fragments (from p-pituitary,[146] by plasmin modification,[147] and diabetogenic peptide from peptic digest,[148] as well as non-covalent interaction of a natural and a synthetic fragment of the hormone [149]).

Choriomammotropin. The *precursor* of h-choriomammotropin (h-CS) from cell-free synthesis contains an additional 20 amino-acids at the *N*-terminal end with a high percentage of leucine (see also pre-prolactin, ref. 116); it is cleaved to h-CS by a membrane-associated enzyme.[150] Rabbit [151] and goat [152] CS were isolated, purified, and characterized. An interesting *modification* of h-CS [153] contains h-CS covalently linked to diphtheria toxin A chain: biological activity of the toxin moiety (ADP–ribosyltransferase activity) and the specific binding to mammary gland plasma membrane lactogenic receptors were partially retained, but the compound failed to inhibit protein synthesis in organ-cultured mammary gland explants. Five out of six methionine residues (Met 170 does not react) were converted to the sulphoxides by H_2O_2 in the undenatured hormone (all are modified in 8-M urea): biological activity was strongly decreased.[154] Circular

[136] Y. A. Pankov, A. A. Bulatov, T. A. Osipova, Y. M. Keda, and A. L. Sinitsyna, *Biokhimiya*, 1976, **41**, 2047.
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[143] A. Cascone, M. Biscoglio de Jimenez Bonino, A. C. Paladini, and J. A. Santomé, *Arch. Biochem. Biophys.*, 1977, **180**, 303.
[144] S. T. Daurat-Larroque, M. E. Moya Portuguez, and J. A. Santomé, *Internat. J. Peptide Protein Res.*, 1977, **9**, 119.
[145] K. Hara and M. Sonenberg, *Biochim. Biophys. Acta*, 1977, **492**, 95.
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[151] F. F. Bolander, jun. and R. E. Fellows, *Biochem. J.*, 1976, **159**, 775.
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[154] R. A. Houghten and C. H. Li, *European J. Biochem.*, 1977, **77**, 119.

dichroism and fluorescence spectra as a function of pH were carefully investigated and *structural transitions* in the alkaline region observed.[155] Two *radioimmunoassay methods* for the quantitative determination of plasma levels of h-CS in normal pregnancy were presented in detail.[156]

2 Posterior Pituitary Hormones

Contributed by M. Manning, K. Bankowski, and W. H. Sawyer

Studies on the posterior pituitary peptides continue in a state of high activity. An International Conference on the Neurohypophysis was held in 1976.[157] The Proceedings of the 4th [158] and 5th [159] American Peptide Symposia and of the 14th European Peptide Symposium [160] contain numerous presentations on the neurohypophysial peptides. A number of reviews have also appeared.[161–165] This report covers the literature for 1976, 1977, and early 1978. Its primary focus is on structure–activity studies reported during this period. Over 100 analogues of oxytocin and of vasopressin have been reported during this period. The vast majority of these were synthesized by the solid-phase method.[166–168] Many of these have resulted from attempts to design analogues in a rational fashion through (i) analysis of structure–activity data [161, 162] and (ii) by the correlation of conformational analysis and structure–activity data.[163, 164] The essence of the first approach is based on (a) ascertaining how individual structural modifications affect the characteristic activities and selectivities of oxytocin and of vasopressin and (b) combining in one molecule those changes which affect agonistic or antagonistic activities and selectivities in a given manner. The basis of the second approach has been described previously (see Vol. 5, p. 409; Vol. 6, p. 444). Examples of peptides designed by both approaches will be illustrated below. For ease of reading we have grouped all of the analogues in tables under the following categories.

1. Analogues more active and selective than oxytocin.
2. Analogues more active and selective than arginine vasopressin (AVP).

[155] H. Kawauchi, T. A. Bewley, and C. H. Li, *Biochim. Biophys. Acta*, 1976, **446**, 525.
[156] J. C. Prieto Villapun, M. Serrano Rios, I. Cifuentes, I. Romero, L. Montoya, and F. Garcia Albertos, *Rev. Clin. Esp.*, 1977, **145**, 17.
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[158] 'Peptides: Chemistry, Structure, and Biology', Proceedings of the 4th American Peptide Symposium, New York, 1975, ed. R. Walter and J. Meienhofer, Ann Arbor Science, Ann Arbor, 1975.
[159] 'Peptides', Proceedings of the 5th American Peptide Symposium, University of California, San Diego, 1977, ed. M. Goodman and J. Meinhofer, Halstead Press, 1977.
[160] 'Peptides 1976', Proceedings of the 14th Peptide Symposium, Wepion, Belgium, 1976, ed. A. Loffet, L'Université de Bruxelles, Brussels, 1976.
[161] M. Manning, J. Lowbridge, J. Haldar, and W. H. Sawyer, *Fed. Proc.*, 1977, **36**, 1848.
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[165] V. Pliska in ref. 160, p. 33.
[166] R. B. Merrifield, *J. Amer. Chem. Soc.*, 1963, **85**, 2149.
[167] R. B. Merrifield, *Biochemistry*, 1964, **3**, 1385.
[168] B. W. Erickson and R. B. Merrifield, in 'The Proteins', 3rd ed., ed. H. Neurath, R. L. Hill, and C. L. Boeder, Academic Press, New York, 1976, Vol. 2, p. 257.

3. Antagonists of the oxytocic response to oxytocin and of the vasopressor response to AVP.
4. Miscellaneous analogues.
5. (a) Iodinated, (b) deuteriated/tritiated, and (c) ^{13}C-enriched analogues of oxytocin and AVP.
6. New syntheses of oxytocin, of AVP, and of their known analogues.

Space considerations do not permit a thorough discussion of all of the data presented in these tables. We will touch briefly on the highlights and refer the interested reader to the original literature. No attempt will be made to cover the vast literature on studies dealing with the physiology, metabolism, and mechanism of action of these peptides, and only a brief mention of some of the more interesting work in these and in related areas will be mentioned.

Analogues More Active and Selective than Oxytocin I (Table 1).—*More Active Analogues.* For ten years deamino-oxytocin [169] stood alone as the only analogue, among many hundreds, which was more active in the rat uterus assay than oxytocin. Then in 1970 [4-threonine]oxytocin,[170] followed in 1971 by hydroxy-oxytocin,[171] were also shown to be more active than oxytocin. In the last two years alone six additional analogues all more potent than oxytocin have been reported (Table 1). These highly potent analogues have resulted from individual structural changes at positions 1, 4, and 7 and in some cases from combinations of changes in these positions. Thus (i) replacement of the α NH_2 group at position 1 by a hydrogen atom, *i.e.* deamination, (ii) replacement of the α NH_2 group at position 1 by an hydroxy group, (iii) threonine substitution at position 4, (iv) thiazolidine-4-carboxylic acid substitution at position 7, and (v) 3,4-dehydroproline substitution at position 7 all lead to enhanced oxytocic activity.

Effect of Combined Changes on Oxytocic Activity. The combination of hydroxy and threonine substitutions were additive in bringing about a dramatic enhancement of oxytocic potency in the resulting analogue hydroxy[Thr^4]oxytocin.[1b]* This contrasts with the lack of additivity of the hydroxy and de-hydroproline substitutions as demonstrated by the diminished oxytocic potency of hydroxy[Δ^3-Pro^7]-oxytocin.[1e] This decrease is analogous to the lack of additivity of deamination and threonine substitutions observed with deamino[Thr^4]oxytocin.[1a] The effects of the combinations of (1) deamination and de-hydroproline and of (b) deamination and thiazolidine-4-carboxylic acid substitutions reveal an interesting contrast. Thus, the oxytocic potency of [Δ^3-Pro^7]oxytocin was virtually unchanged by deamination,[1e] whereas the oxytocic potency of [Thz^7]oxytocin was appreciably increased by deamination.[1c] No explanations have been offered for these puzzling findings. It is thus clear from these data that it is not always possible to predict additivity or non-additivity either from structure–activity data or from conformational considerations.

More Selective Analogues. Changes in only two positions have to date been

[169] V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, jun., *J. Biol. Chem.*, 1960, **235**, 64.
[170] M. Manning, E. Coy, and W. H. Sawyer, *Biochemistry*, 1970, **9**, 3925.
[171] D. B. Hope and M. Walti, *Biochem. J.* 1971, **125**, 909.

* Refs. 1*a*—1*i* appear in Table 1, refs. 2*a*—2*m* in Table 2 etc.

Table 1 *Analogues more active and more selective than oxytocin*

1 5 9

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

Oxytocin

Compound number	*Peptide*	*Biological activities*[a] *Rat uterus oxytocic (O)*†	*Rat milk ejection (M.E.)*	*Rat antidiuretic (A)*	*Rat pressor (P)*	*O/A Selectivity*	*Ref.*[a]
25	Oxytocin	520 ± 12	474 ± 16[c]	4.0 ± 0.8	4.3 ± 0.12	130	1*a*
		486 ± 15 (Mg^{2+})	533 ± 62[b]				
26	Hydroxy[Thr[4]]oxytocin	4179 ± 222	808 ± 19	5.3 ± 0.5	4.92 ± 0.09	790	1*b*
		937 ± 55 (Mg^{2+})	378 ± 10[b]				
27	Deamino[Thz[7]]oxytocin[d]	1538 ± 45			1.69 ± 0.08		1*c*, 1*d*, 1*e*
28	[Thz[7]]oxytocin	1180 ± 47			3.46 ± 0.05		
29	[Δ^3-Pro[7]]oxytocin[f]	1071 ± 59		5.9 ± 0.2	*g*	180	1*e*
30	Deamino[Δ^3-Pro[7]]oxytocin	1066 ± 95		23.3 ± 1.1	*g*	45	1*e*
31	Hydroxy[Δ^3-Pro[7]]oxytocin	880 ± 180		76.7 ± 2.3	*g*	11	1*e*
32	[Thr[4],Gly[7]]oxytocin	166 ± 4	802 ± 23	0.002 ± 0.0004	< 0.01	83 000	1*f*
		857 ± 26 (Mg^{2+})	490 ± 25[b]				
		270 ± 10		0.002 ± 0.0008	depressor/pressor	135 000	1*g*
33	Hydroxy[Thr[4],Gly[7]]oxytocin	218 ± 8	950 ± 32	0.0040 ± 0.0005	0.01	54 500	1*f*
		1002 ± 29 (Mg^{2+})	623 ± 13[b]				
		337 ± 23		0.048 ± 0.005	depressor/pressor	7000	1*g*

34	Deamino[Gly^7]oxytocin	355 ± 3		0.062 ± 0.006	0.5	5725	1*h*
35	Deamino[Ala^7]oxytocin	123 ± 4		0.17 ± 0.01	0.5	725	1*h*
36	[Ala^7]oxytocin	22 ± 1		0.08 ± 0.01	0.4	275	1*h*
37	[Hse^4]oxytocin[h]	125 ± 13 380 ± 28 (Mg^{2+})	357 ± 16	0.24 ± 0.03	0.021 ± 0.001	520	1*i*

(*a*) Activities expressed in I.U. $mg^{-1} \pm$ S.E.; (*b*) rat mammary strip; (*c*) rabbit; (*d*) Thz = Thiazolidine-4-carboxylic acid $\overbrace{CH_2—S—CH_2—NH}—CH—CO_2H$; (*e*) an improved synthesis; (*f*) Δ^3-Pro = 3,4-dehydroproline $\overbrace{CH=CH—CH_2—NH}—CH—CO_2H$; (*g*) difficulties in bioassay; (*h*) Hse = homoserine $HOCH_2CH_2CH(NH_2)CO_2H$; †Mg^{2+} indicates that assays were carried out in the presence of 0.5 mM Mg^{2+}.

1*a* M. Manning, E. J. Coy, and W. H. Sawyer, *Experientia*, 1971, **27**, 1372.
1*b* M. Manning, J. Lowbridge, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1976, **19**, 376.
1*c* J. D. Rosamond and M. F. Ferger, *J. Med. Chem.*, 1976, **19**, 873.
1*d* M. Barber and J. H. Jones, *Internat. J. Peptide Protein Res.*, 1977, **9**, 269.
1*e* S. Moore, A. M. Felix, J. Meienhofer, C. W. Smith, and R. Walter, *J. Med. Chem.*, 1977, **20**, 495.
1*f* J. Lowbridge, M. Manning, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1977, **20**, 120.
1*g* G. L. Stahl and R. Walter, *J. Med. Chem.*, 1977, **20**, 492.
1*h* R. Walter, C. W. Smith, J. Roy, and A. Formento, *J. Med. Chem.*, 1976, **19**, 822.
1*i* A. Turan, M. Manning, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1977, **20**, 1169.

shown to enhance oxytocic–antidiuretic (O/A) selectivity relative to oxytocin. These are positions 4 and 7. O/A selectivity is increased by a variety of position four substituents and by glycine substitution at position 7.[172] 4-Threonine substitution alone also gives rise to enhanced oxytocic potency.[170] It was thus predicted that the combination of Thr[4] and Gly[7] substitutions might lead to greater selectivity than either substitution alone.[172] [Thr[4], Gly[7]]oxytocin exhibited a markedly enhanced O/A selectivity. This high O/A selectivity was subsequently confirmed by an independent synthesis in another laboratory.[1g] The enhanced oxytocic potency of these Gly[7]-containing peptides in the presence of Mg^{2+} [1f] is also noteworthy. It seems interesting to note how closely the highly active oxytocins seem to bunch when assayed in the more 'physiological' medium containing Mg^{2+}. The 7-glycine analogues increase in relative potency to about 1000 and the hydroxy analogues drop to about 1000.

Analogues More Active and Selective than Arginine Vasopressin (AVP) (Table 2).—*More Active Analogues.* To date, four individual structural modifications of AVP have been shown to give rise to analogues with enhanced antidiuretic activity. These are (i) replacement of the α NH_2 group at position 1 with hydrogen, *i.e.* deamination,[173] (ii) replacement of the α NH_2 group at position 1 with an hydroxy group,[2e] (iii) valine substitution at position 4 [174] and (iv) 3,4-dehydroproline substitution at position 7.[2b, 2c] Deamination is by far the most effective of these individual changes and hydroxy substitution is the least effective. Deamino-AVP has an antidiuretic potency of 1745 ± 385 units mg^{-1} whereas hydroxy-AVP has an antidiuretic potency of 467 ± 44 units mg^{-1} (Table 2). Until 1977 deamino-AVP, first reported in 1966,[173] was the most potent analogue of AVP. Also, prior to 1977, no analogue which contained combinations of the first three of these structural modifications was more active than deamino-AVP. In 1977, and in early 1978, two analogues of deamino-AVP which have a 3,4-dehydroproline residue at position 7 were reported to exhibit strikingly enhanced antidiuretic activities. These were deamino[Δ^3-Pro[7]]AVP[2c] with an antidiuretic potency of 3310 ± 240 and deamino[Phe[2]-Δ^3-Pro[7]]AVP[2b] with an antiduretic potency of 13 000 ± 1250 (Table 2). Thus in AVP the combination of deamination and 3,4-dehydroproline substitution were additive in enhancing antidiuretic activity. This contrasts with the non-additive effects on oxytocic activity of these same modifications in oxytocin (Table 1). The amazingly high antidiuretic activity of deamino-[Phe[2], Δ^3-Pro[7]]AVP[2b] is even more difficult to explain on the basis of known structure–activity correlations. Thus phenylalanine substitution has not previously been shown to enhance the antidiuretic activity of AVP analogues.[173, 175] For example, the substitution of phenylalanine at position 2 in deamino-AVP led to a decrease in antidiuretic activity of over one-third.[173] Yet the substitution of phenylalanine in deamino[Δ^3-Pro[7]]AVP has effected a four-fold enhancement in antidiuretic activity. The high *in vivo* potencies of the hydroxy and deamino analogues may result in part from their resistance to metabolic destruction. The

[172] M. Manning, J. Lowbridge, and W. H. Sawyer, in ref. 158, p. 737.
[173] R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, 1966, **49**, 695.
[174] W. H. Sawyer, M. Acosta, L. Balaspiri, J. Judd, and M. Manning, *Endocrinology*, 1974, **94**, 1106.
[175] R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, 1962, **45**, 1629.

more dramatic effect of adding [Δ^3-Pro^7], however, suggests that other factors (increased affinity, intrinsic activity, or both) may be involved. The synthesis and study of other [Δ^3-Pro^7]-containing analogues will undoubtedly shed light on this striking but puzzling finding.

More Selective Analogues. AVP has an antidiuretic/pressor (A/P) ratio of ~ 1. Among the changes which have been shown to enhance A/P selectivity are: (i) D-Arg^8 substitution, (ii) substitution of hydrophobic amino-acids at position four, (iii) deamination, (iv) Phe^2 substitution (see Vol. 6, p. 449). To these can now be added (v) dehydroproline^7 substitution. Hydroxy[Val^4, D-Arg^8]vasopressin and deamino[Thr^4, D-Arg^8]vasopressin are both highly active and highly selective antidiuretic peptides. The substitution of Phe^2 in deamino[Δ^3-Pro^7]AVP in addition to enhancing its antidiuretic activity has brought about a marked reduction in its pressor activity, the latter being described as 'negligible'. Thus deamino[Phe^2, Δ^3-Pro^7]AVP besides being the most active antidiuretic analogue reported to date is also among the most selective.

Antagonists of the Oxytocic Response to Oxytocin and of the Vasopressor Response to AVP (Table 3).—Since the original synthesis of oxytocin by du Vigneaud in 1954,[176] much effort has been expended in attempts to make *in vivo* antagonists of the oxytocic response to oxytocin and of the vasopressor and antidiuretic responses to AVP. While there has been no success in designing an anti-antidiuretic agent, and very little success in designing good *in vivo* oxytocin antagonists, there has been much success in designing oxytocic antagonists to the oxytocic response *in vitro* (see Vol. 8, p. 381), and of antagonists to the vasopressor response to AVP. The penicillamine-type substitution[177] at position 1 in oxytocin and in vasopressin analogues, *i.e.* the replacement of the two hydrogens on the β-carbon atom with alkyl groups converts agonists into antagonists, *i.e.* binding is permitted but is unaccompanied by the appropriate biological response. [Deamino-penicillamine-Thr^4]oxytocin is one of the most potent antagonists of the *in vitro* oxytocin response reported to date.[3a] [Deaminopenicillamine, Val^4, D-Arg^8]vasopressin is by far the most potent antivasopressor peptide reported to date.[3f] Cort *et al.*[178] have shown that increasing the hydrophobic character of a series of amino-acid substituents at position 4 in [deamino]-8-D-arginine vasopressin gave rise to increased pressor antagonism. The substitution of leucine or of isoleucine in [1-deaminopenicillamine, 4-valine, 8-D-arginine]vasopressin (dPVDAVP) might also increase its antagonistic potency. Meraldi *et al.*,[179] using proton and ^{13}C n.m.r. techniques, have studied the relative conformational rigidity of oxytocin and of [1-penicillamine]oxytocin and have found that the latter is less flexible. They conclude that the inhibitor activity of [1-penicillamine]-oxytocin may be related to the relative rigidity of the molecule. It is of interest in this regard that the tocinoic and tocinamide rings, both virtually inactive

[176] V. du Vigneaud, C. Ressler, J. M. Swan, P. G. Katsoyannis, and C. W. Roberts, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.
[177] H. Schulz and V. du Vigneaud, *J. Med. Chem.*, 1966, **9**, 647.
[178] J. H. Cort, I. Fric, L. Carlsson, D. Gillessen, S. Bystricky, J. Skopkova, V. Gut, R. Studer, J. L. Mulder, and K. Blaha, *Mol. Pharmacol.* 1976, **12**, 313.
[179] J. P. Meraldi, V. J. Hruby and A. I. R. Brewster, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 1373.

Table 2 *Analogues more active and more selective than arginine vasopressin*

1 5 9

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

Arginine vasopressin

Compound number	*Peptide*	*Biological activity*[a] (*O*)	(*A*)	(*P*)	*A/P Selectivity*	*Ref.*
38	Arginine vasopressin	15 ± 1 31 ± 2 (Mg^{2+})	332 ± 20	376 ± 6	0.9	2*a*
39	Deamino[Phe[2],Δ^3-Pro[7]]arginine vasopressin		$13\,000 \pm 1250$	negligible		2*b*
40	Deamino[Δ^3-Pro[7]]arginine vasopressin		3310 ± 240	160	20	2*c*
41	[Δ^3-Pro[7]]arginine vasopressin		930	180	5	2*c*
42	Deamino[Dab[8]]vasopressin[b]	24.7 ± 7	1373 ± 128	450 ± 30	3	2*d*
43	Hydroxy[Val[4], D-Arg[7]]vasopressin	1.1 ± 0.1 2.8 ± 0.2 (Mg^{2+})	892 ± 45	< 0.02[c]	$> 45\,000$	2*e*
44	Deamino[Thr[4], D-Arg[8]]vasopressin	1.02 ± 0.14 0.98 ± 0.35 (Mg^{2+})	793 ± 95	< 0.02[c]	$> 39\,000$	2*f*
45	Deamino[Asn[4], D-Arg[8]]vasopressin	0.007	10 500–11 000[d]			2*g*
46	Hydroxy[D-Arg[8]]vasopressin	4.3 ± 0.7 2.6 ± 0.6 (Mg^{2+})	886 ± 105	0.86 ± 0.03	1030	2*e*
47	Hydroxy-arginine vasopressin	31 ± 0.3 96 ± 9 (Mg^{2+})	467 ± 44	549 ± 16	0.9	2*e*

48	Deamino[Val^4, D-Har^8]vasopressin[d]		31 ± 5	< 0.01	> 3100	2*h*
49	Deamino[Val^4, D-Hls^8]vasopressin[e]		21 ± 4	~ 0.01	~ 2100	2*h*
50	Deamino[Leu^4, D-Arg^8]vasotocin	0.005	130	0.171 ± 0.04	760	2*i*
51	[Leu^4, D-Arg^8]vasotocin	0.24 ± 0.04	100	0.146 ± 0.05	685	2*i*

2*a* M. Manning, L. Balaspiri, M. Acosta, and W. H. Sawyer, *J. Med. Chem.*, 1973, **16**, 975.
2*b* C. W. Smith and R. Walter, *Science*, 1978, **199**, 297.
2*c* C. W. Smith, C. R. Botos, and R. Walter, in ref. 159, p. 161.
2*d* M. Zaoral, F. Brtnik, T. Barth, and A. Machova, *Coll. Czech. Chem. Comm.*, 1976, **41**, 2088.
2*e* J. Lowbridge, M. Manning, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1977, **20**, 1173.
2*f* M. Manning, L. Balaspiri, J. Moehring, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1976, **19**, 842.
2*g* M. Zaoral and J. Blaha, *Coll. Czech. Chem. Comm.*, 1977, **42**, 3500.
2*h* G. Lindenberg, S. M. Karlsson, and P. Melin, *Internat. J. Peptide Protein Res.*, 1977, **10**, 240.
2*i* M. Zaoral, F. Brtnik, T. Barth, and A. Machova, *Endocrinol. Exp.*, 1976, **10**, 183.

(*a*) Activities expressed in I.U. $mg^{-1} \pm$ S.E.; abbreviations are used as in Table 1; (*b*) Dab = α,γ-diaminobutyric acid; (*c*) antagonist of the vasopressor response to arginine-vasopressin; (*d*) Har = homoarginine $NH_2CH([CH_2]_4NH\ C[NH_2]{=}NH)CO_2H$; (*e*) Hls = homolysine $NH_2CH([CH_2]_5NH_2)CO_2H$; (*f*) this value was obtained by a modified Burn assay.[2j] Similar high values for antidiuretic activities by this assay method have been found (for deamino-AVP analogues and shown to be due to the prolongation of activity caused by deamination.[2k] Thus deamino[D-Arg^8])vasopressin (dDAVP) had an antidiuretic activity of 50 445 U mg^{-1} [2l] by the Burn assay whereas in the standard antidiuretic assay [2m] it had an activity of 1200 ± 126 U mg^{-1}.[2f]

2*j* J. H. Burn, *Quart. J. Parmacol.*, 1931, **4**, 517.
2*k* W. H. Sawyer, M. Acosta, L. Balaspiri, J. Judd, and M. Manning, *Endocrinology*, 1974, **34**, 1106.
2*l* J. Vavra, A. Machova, and J. Krejci, *J. Pharmacol. Exp. Ther.*, 1974, **188**, 241.
2*m* W. H. Sawyer in 'The Pituitary Gland', Vol. 3, ed. G. W. Harris and B. T. Donovan, Butterworths, London, 1966, p. 288.

Table 3 *Antagonists of the oxytocic response to oxytocin and of the vasopressor response to AVP*

Compound number	*Peptide*	*Antioxytocic* pA_2[a]	*Antivasopressor* pA_2[a]	*Antidiuretic activity*[c]	*Ref.*
52	[Deaminopenicillamine[1], Thr[4]]oxytocin	7.46 ± 0.04 7.59 ± 0.08[b]	6.67 ± 0.09[h]	~ 0.02	3*a*
53	*N*-bromoacetyl[Tyr(Me)[2]]oxytocin	7.33	*d*	0.002	3*b*
54	*N*-maleoylglycine[Tyr(Me)[2]]oxytocin	7.27	*d*	0.001	3*b*
55	[Tyr-3-I)[2]]oxytocin	7.05 ± 0.08	*d*	~ 0.01[i]	3*c*
56	[Tyr(3-Me)[2]]oxytocin	6.79 ± 0.15	—	~ 0.01[i]	3*c*
57	[Hcy[1,6]]oxytocin[e]	6.02	—		3*d*
58	[γ-Mba[1],Hcy[6]]oxytocin[f]	6.74 (0.3—0.5 U mg^{-1}) as agonist	5.17[g]		3*d*
59	[β-Mpa(β-Et_2)]tocinoic acid	6.48	weak[h]		3*e*
60	[β-Mpa(β-Et_2)]tocinamide	6.68	weak[h]		3*e*
61	[β-Mpa(β-$(CH_2)_5$)]tocinoic acid	6.37	no inhibition[h]		3*e*
62	[β-Mpa(β-$(CH_2)_5$)]tocinamide	6.74	no inhibition[h]		3*e*
63	[Deaminopenicillamine[1], Val[4], D-Arg[8]]vasopressin	7.23 ± 0.04[h]	7.82 ± 0.05[h]	123 ± 22	3*f*

(*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; (*b*) material from the repeat synthesis; (*c*) U mg^{-1}; (*d*) slightly inhibitory effect on preadministered LVP; (*e*) Hcy = homocysteine $HS(CH_2)_2CH(NH_2)CO_2H$; (*f*) Mba = γ-mercaptobutyric acid $HS(CH_2)_3CO_2H$; (*g*) LVP used as an agonist; (*h*) USP Posterior Pituitary Reference Standard used as an agonist; (*i*) I.U. $\mu mole^{-1}$.

3*a* M. Manning, J. Lowbridge, J. Seto, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1978, **21**, 179.
3*b* T. Barth, M. Krojidlo, and K. Jost, in ref. 160, p. 491.
3*c* V. Pliska, P. Marbach, J. Vasak, and J. Rudinger, *Experientia*, 1977, **33**, 367.
3*d* C. W. Smith and M. F. Ferger, *J. Med. Chem.*, 1976, **19**, 250.
3*e* J. J. Nestor, jun., M. F. Ferger, and V. du Vigneaud, in ref. 158, p. 755.
3*f* M. Manning, J. Lowbridge, C. T. Stier, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, 1977, **20**, 1228.

agonists, were converted into antagonists, albeit weak ones, of the *in vitro* oxytocic response when substituted at their respective position 1 β carbons with diethyl [180] and cyclopentamethylene [181] groups. Substitutions at position 2 of oxytocin and of its analogues also give rise to analogues which can inhibit the *in in vitro* oxytocic response.[182] Kaurov *et al.*[183] have shown that substitution of D-amino-acids at position 2 of a series of oxytocin analogues enhances oxytocic antagonism *in vitro*. Thus, modifications of oxytocin at position 2 involving the use of *O*-alkylated L- and D-amino-acids combined with $\beta\beta$ dialkyl substitutions at position 1 might give analogues with potent antioxytocic activity *in vivo*. Barth *et al.*[184] have examined the anti-antidiuretic properties of a number of oxytocin analogues modified in positions 1 and 2 which had previously been shown to be antagonists of the *in vitro* oxytocic responses and in some instances weaker antagonists of the *in vivo* oxytocin responses to oxytocin. In spite of earlier findings that some of them were able to compete with ^{3}H[8-lysine]vasopressin for receptors from pig kidney medulla and to inhibit the hormone sensitive adenylate cyclase, they found no inhibition of the antidiuretic action of oxytocin or of vasopressin in the rat. Thus attempts to obtain antagonists of the antidiuretic response have proved very elusive. Success in this area will probably depend on luck as much as on rational design.

Comments on Table 4.—A very interesting analogue [Dopa^2]oxytocin was reported to have a low biological activity.[4b, 4c] However, under the conditions required for the bioassay, the rapid oxidation of the Dopa residue to the quinone could have taken place causing some loss of biological activity. Thus the authors wrote '. . . the figures we quote are minimum potencies'.[4b] [MeLeu^8]oxytocin and [N^{α}-Me-Arg^8]vasopressin were designed to obtain analogues with prolonged half-lives compared to the native hormones.[4g] Alkylation of the α nitrogen resulted in moderately to marked reductions in some of the biological activities of the parent hormones. [MeArg^8]vasopressin, however, has the same natriuretic activity as AVP but the half-life of its effect was prolonged by a factor of 3.5. The prolonged duration provides evidence that the 8-N^{α}-methylation does in fact increase the metabolic stability of the AVP analogue to enzymic degradation. The coupling of oxytocin and analogues to polymeric supports may facilitate the isolation and characterization of receptors for oxytocin. [Lys^8]oxytocin which has an oxytocic activity of 100 U mg^{-1} was coupled through the Lys^8 ε-amino group to the carboxyl groups of carboxymethylated dextrans (mol. wt. 40 000, 500 000) and *carboxypropionyl-gelatin* (mol. wt. 40 000). The macromolecular oxytocins retained significant oxytocic activity.[4h] The activity observed implies that at least some myometrial receptors are accessible to large molecules and are on the surface of the plasma membrane.

Comments on Table 5.—A number of iodinated oxytocin and vasopressin compounds were prepared in the hope that they might act as specific ligands for the

[180] R. J. Vavrek, M. F. Ferger, G. A. Allen, D. H. Rich, A. T. Blomquist, and V. du Vigneaud, *J. Med. Chem.*, 1972, **15**, 123.
[181] J. J. Nestor, jun., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, 1975, **18**, 284.
[182] J. Rudinger, V. Pliska, and I. Krejci, *Rec. Prog. Hormone Res.*, 1972, **28**, 131.
[183] O. V. A. Kaurov and M. P. Smirnova, *Khim. prirod. Soedinenii*, 1977, 392.
[184] T. Barth, M. Krojidlo, and J. Jost, *Endocrin. Experimentalis*, 1977, **11**, 57.

Table 4 *Miscellaneous analogues*

Compound number	Peptide	Biological activity[a] (O)	(M.E.)	(A)	(P)	Ref.
	A. OXYTOCIN					
64	[Phe(F_5)^2]oxytocin[b]	0.01				4a
65	[D-Phe(F_5)^2]oxytocin	antagonist				4a
66	[Dopa^2]oxytocin[c]	26 ±4	54±9[d]			4b, 4c
67	[Thi^4]oxytocin[e]	0.51±0.03		<0.05	<0.05	4d
68	[Dab^4]oxytocin[f]					4e
69	[Dab^5]oxytocin					4e
70	[Hcy^6]oxytocin[g]	12.0 ±0.5			*h*	3d
71	[Pro(4F)^7]isotocin[i]					4f
72	[Pro(4F)^7]mesotocin					4f
73	[MeLeu^8]oxytocin[j]	45		0.27		4g
74	[ε-3-Carboxypropionyl-Lys^8]oxytocin	55 ±4[k]				4h
75	[ε-3-Carboxybutyryl-Lys^8]oxytocin	50 ±3.5[k]				4h
76	[Lys^8]oxytocin coupled to geletin	0.93±0.1[k]				4h
77	[Lys^8]oxytocin coupled to T500 dextran	2.4 ±0.5[k]				4h
78	[Lys^8]oxytocin coupled to 400 dextran	0.24±0.01[l]				4h
79	[Pro^3, Gly^4]oxytocin				0.004±0.001	4i
80	[Phe(NH_2)^2, Glu^8]oxytocin[l]	1.1				4j
81	Deamino[Phe(NH_2)^2, Glu^4]oxytocin	0.025				4j
82	[Cys^5, Asn^6]oxytocin	*h*	*h*	<0.001	*h*	4k
83	Cyclo[Asp^1, Dapa^6]oxytocin[m]	16 ±2		5.6 ±3.8		4l
84	[Maa^1, Hcy^6]oxytocin[h]	37 ±1			*n*	3d
85	[Phe^{3,4}, Met^8]oxytocin			0.43±0.12	*o*	4m
86	[Phe^3, Leu^4, Met^8]oxytocin	0.62±0.07		0.33±0.09	0.92±0.03	4m
87	[des-(9-glycinamide)]oxytocin	2.4 ±0.07		0.08±0.03	*p*	4n
88	[Des(8-leucine, 8-glycinamide)]oxytocin	2.7 ±0.5		0.06±0.006	*p*	4n
89	[D-aIle^3]retro-D-deaminotocinamide	*r*				4o
90	Retro-L-deaminotocinamide	*h*				4o
91	*N*-formyl-[D-aIle^3]-retro-D-deaminotocinamide	*h*				4o
92	*N*-formyl-retro-L-deaminotocinamide	*h*				4o
93	[D-aIle^3, Gly^7]-retro-D-deamino-oxytocin	*s*				4o
94	[D-aIle^3, Gly^7, Malonamide^3]-retro-D-deamino-oxytocin	*h*				4o

95	[Gly^7]-retro-L-deamino-oxytocin		*h*					4*o*
	B. VASOPRESSIN							
96	Deamino[D-Orn^8]vasopressin	1.9	±0.25		29	4.8	±0.9	2*d*
97	[Har^8]vasotocin	320	±38	340	±46	300	±21	4*p*
98	Deamino[Har^8]vasotocin	149	±10	125	±12	180	±8	4*p*
99	Deamino[MeArg^8]vasopressin[t]				50		*u*	4*g*
100	[Pro(4-F)^7]vasotocin							4*f*
101	[Pro(4-F)^7]lysine vasopressin							4*f*

(*a*) Activities expressed in I.U. mg^{-1} ± S.E., abbreviations are used as in Table 1; (*b*) Phe(F_5) = pentafluorophenylalanine; (*c*) DOPA = 3,4-dihydroxyphenylalanine; (*d*) mouse mammary gland; (*e*) Thi = β-(2-thienyl)alanine  —$CH_2CH(NH_2)CO_2H$; (*f*) Dab = α,γ-diaminobutyric acid; (*g*) Hcy = homocysteine $HS(CH_2)_2CH(NH_2)CO_2H$; (*h*) not active; (*i*) Pro(4-F) = 4-fluoroproline; (*j*) MeLeu = N-methyl-leucine; (*k*) I.U. μmole^{-1}; (*l*) Phe(NH_2) = *p*-aminophenylalanine; (*m*) Dapa = α,β-diaminopropionic acid; (*n*) Maa = α-mercaptoacetic acid; (*o*) could not be determined; (*p*) negligible; (*r*) weak inhibitor; (*s*) partial agonist; (*t*) MeArg = N$^{\alpha}$-methyl-arginine; (*u*) very low.

4*a* O. A. Kaurov and M. P. Smirnova, *Khim. prirod. Soedinenii*, 1977, 392.
4*b* B. M. Ferrier and L. A. Branda, *Canad. J. Biochem.*, 1976, **54**, 507.
4*c* S. Drabarek and T. Wachal, *Roczniki Chem.*, 1977, **51**, 1119.
4*d* C. W. Smith, G. Skala, and R. Walter, *J. Med. Chem.*, 1978, **21**, 115.
4*e* M. A. El-Maghraby, *J. Indian Chem. Soc.*, 1976, **53**, 673 (*Chem. Abs.*, 1977, **86**, 140 449d).
4*f* M. A. El-Maghraby, *J. Indian Chem. Soc.*, 1976, **53**, 670 (*Chem. Abs.*, 1977, **86**, 140 448c).
4*g* J. Hlavack, K. Poduska, K. Jost, J. Fric, T. Barth, J. H. Cort, K. Blaha, and F. Sorm, *Coll. Czech. Chem. Comm.*, 1977, **42**, 1233.
4*h* C. R. Snell and D. G. Smyth, *Biochem. J.* 1977, **165**, 43.
4*i* H. R. Wyssbrod, A. Ballardin, W. A. Gibbons, J. Roy, J. L. Schwartz, and R. Walter in ref. 158, p. 815.
4*j* O. A. Kaurov, V. F. Martynov, and E. J. Grygorev, *Khim. prirod. Soedinenii*, 1977, 433.
4*k* D. Theodoropoulos, M. Liakopoulou, and C. W. Smith, in ref. 160, p. 505.
4*l* C. W. Smith, R. Walter, S. Moore, R. C. Makofse, and J. Meienhofer, *J. Med. Chem.*, 1978, **21**, 117.
4*m* C. W. Smith, C. R. Botos, G. Skala, and R. Walter, *J. Med. Chem.*, 1977, **20**, 1457.
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4*o* S. H. Nakagawa, F. Yang, T. Kato, G. Flouret, and O. Hechter, *Internat. J. Peptide Protein Res.*, 1976, **8**, 465.
4*p* L. E. Larsson, E. G. G. Lindeberg, and P. Melin, *Internat. J. Peptide Protein Res.*, 1977, **9**, 182.

Table 5 *Iodinated, deuteriated, tritiated, and ^{13}C-enriched analogues of oxytocin and vasopressin*

Compound number	*Peptide*	*Ref.*
55	[Tyr(3-I)^2]oxytocin[a]	3c, 5a
102	[Tyr(3,5-I_2)^2]oxytocin	5a
103	[Tyr(3-I)^2]arginine vasopressin	5a
104	[Tyr(3,5-I_2)^2]arginine vasopressin	5a
105	[Tyr(3-I)^2]lysine vasopressin	5a
106	[Tyr(3,5-I_2)^2]lysine vasopressin	5a
107	[DL(α-$^2H_1$)Cys^1]oxytocin[b]	5b
108	[DL(β,β-$^2H_2$)Cys^1]oxytocin[b]	5b, 5c
109	[DL(α-2H_1)Cys^6]oxytocin[b]	5c
110	[α-$^2H_1$)Cys^1]arginine vasopressin	5d
111	[D(α-$^2H_1$)Cys^1]arginine vasopressin	5d
112	[(β,β-$^2H_2$)Cys^1]arginine vasopressin	5d
113	[α-$^2H_1$)Tyr_2]arginine vasopressin[b]	5d
114	[(α-$^2H_1$)Phe^3]arginine vasopressin	5d
115	[DL-(α,β,β-$^2H_3$)Cys^1]oxytocin[b]	5d
116	[(α,β,β-$^2H_3$)Tyr^2]arginine vasopressin	5d
117	[(α,α-$^2H_2$)Gly^9]arginine vasopressin	5d
118	[(^{3}H)Tyr^2]oxytocin	5a
119	[(^{3}H)Tyr^2]arginine vasopressin	5a
120	[(^{3}H)Tyr^2]lysine vasopressin	5a
121	[(^{13}C)Leu^3]oxytocin	5e
122	[(^{13}C)Pro^7]oxytocin	5f, 5g
123	[(^{13}C)Leu^8]oxytocin	5f, 5g
124	[(^{13}C)Ile,^{3}, Gly^9]oxytocin	5e
125	[(^{13}C)Gly^9]oxytocin	5e
126	[L(D)-(2-^{13}C)Cys^1]oxytocin	5h, 5i
127	[(2-^{13}C)Tyr^2]oxytocin	5j
128	[(2-^{13}C)Gly^9]oxytocin	5i—5k
129	[(2-^{13}C)Gly^9]arginine vasopressin	5k
130	[L(D)-(1-^{13}C)Cys^1]arginine vasopressin	5j

a Antagonist of oxytocin (see Table 3); *b* the resolution of diasteroisomers is also described.

5a G. Flouret, S. Terada, F. Yang, S. H. Nakagawa, T. Nakahara, and O. Hechter, *Biochemistry*, 1977, **16**, 2119.

5b V. J. Hruby, D. A. Upson, and N. S. Agarwal, *J. Org. Chem.* 1977, **42**, 3552.

5c D. A. Upson and V. J. Hruby, *J. Org. Chem.* 1976, **41**, 1353.

5d D. M. Yamamoto, D. A. Upson, D. K. Linn, and V. J. Hruby, *J. Amer. Chem. Soc.*, 1977, **99**, 1564.

5e J. H. Griffin, C. DiBello, R. Alazard, P. Nicolas, and P. Cohen, *Biochemistry*, 1977, **16**, 1579.

5f P. Cohen, O. Convert, J. H. Griffin, P. Nicolas, and C. DiBello, in ref. 3, p. 176.

5g O. Convert, J. H. Griffin, C. DiBello, P. Nicolas, and P. Cohen, *Biochemistry*, 1977, **16**, 5061

5h M. Blumenstein, V. J. Hruby, D. Yamamoto, and Y. Yang, *F.E.B.S. Letters*, 1977, **81**, 347.

5i M. Blumenstein and V. J. Hruby, *Biochemistry*, 1977, **16**, 5169.

5j V. J. Hruby, D M. Yamamoto Y. C. S. Yang, and M. Blumenstein, in ref. 3, p. 179.

5k M. Blumenstein and V. J. Hruby, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 1052.

titration of oxytocin and AVP receptors.[5a] The iodinated derivatives did not exhibit sufficient specificity to be useful for this purpose.[3c, 5a] These iodo-derivatives, however, were easily transformed into tritiated ligands for the successful characterization of neurohypophyseal receptor-sites in kidney and uterine membranes.[5a] Oxytocin and vasopressin analogues containing ^{13}C-enrichment in the amino-acid residue at some positions [5e—5g] [compounds (121)—(125)] or

even more specifically labelled [5h–5k] compounds [(126)—(130)] have been synthesized and used for studying conformational and microdynamical properties of the hormones bound to their physiological carrier-proteins, the neurophysins.[5e–5k]

The changes in ^{13}C-n.m.r. spectroscpic parameters (chemical shift, spin-lattice relaxation time and linewidth) before and after complex formation indicated that the first three residues, Cys-Tyr-Ile or Cys-Tyr-Phe, were rigidly bound to the neurophysins.[5e–5k] Convert *et al.* suggest that residues 7, 8, 9 of the tripeptide tail are free of any interactions with the proteins [5e–5g] while Blumenstein and Hruby [5i] conclude that the tail portion does interact with the neurophysin but that its interaction is weaker than that of the *N*-terminal part of the hormones.

Comments on Table 6.—New syntheses of oxytocin, AVP, and of their known analogues have been reported. These were carried out for a variety of reasons.

1. Oxytocin and vasopressin syntheses served as suitable models for investigation of new procedures in both classical [6a, 6b] and solid phase synthesis.[6c, 6i]
2. For spectroscopic investigations.[4g]
3. To clarify discrepancies in the literature regarding biological activities.[1a, 1b, 1f, 1h, 2f] In many instances higher activities and/or selectivities were subsequently reported. The most pronounced examples are: high A/P selectivity (3000) of

Table 6 *New syntheses of oxytocin, of arginine vasopressin, and of their analogues*

Compound number	*Peptide*	*Ref.*
25	Oxytocin	6*a*, 6*b*, 6*c*, 6*d*
131	Hydroxy-oxytocin	1*b*, 1*g*
132	[Penicillamine^1]oxytocin[a]	6*d*
133	[Trp^2]oxytocin	6*e*
134	[Phe^3]oxytocin	6*f*
135	[Phe^4]oxytocin	6*g*
136	[Gly^7]oxytocin	1*f*, 1*h*
137	[Lys^8]oxytocin	4*h*
138	[ε-Carbamoyl-Lys^8]oxytocin	4*h*
139	Oxytocinoic acid	6*h*
140	Tocinoic acid	6*h*
38	Arginine vasopressin	6*i*
141	[D-Arg^8]vasopressin	2*f*
142	Deamino-arginine vasopressin	2*f*
143	Deamino[D-Arg^8]vasopressin	2*f*
144	[D-Har^8]vasopressin	6*j*
145	Deamino[D-Har^8]vasopressin	6*j*

a penicillamine = $HS{-}C(CH_3)_2CH(NH_2)CO_2H$.

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6*b* S. Miksta, A. V. Rutkovska, O. S. Papsnevich, and G. Cipens, *Latv. PSR Zinat. Akad. Vestis. Kim. Ser.*, 1977, 503 (*Chem. Abs.*, 1977, **87**, 202 101r).
6*c* D. H. Live, W. C. Agosta, and D. Cowburn, *J. Org. Chem.*, 1977, **42**, 3556.
6*d* J. P. Meraldi and V. J. Hruby, *J. Amer. Chem. Soc.*, 1976, **98**, 6408.
6*e* B. M. Ferrier and L. A. Branda, *Canad. J. Biochem.*, 1976, **54**, 512.
6*f* R. Walter, C. W. Smyth, and J. Roy, *Proc. Nat. Acad. Sci. U.S.A.*, 1976, **73**, 3054.
6*g* C. W. Smith, C. R. Botos, G. Skala, and R. Walter, *J. Med. Chem.*, 1977, **20**, 1457.
6*h* C. A. Boicelli, A. F. Bradbury, and J. Feeney, *J.C.S. Perkin II*, 1977, 477.
6*i* L. E. Larsson, P. Melin, and U. Ragnarsson, *Internat. J. Peptide Protein Res.*, 1976, **8**, 39.
6*j* E. G. G. Lindeberg, P. Melin, and L. E. Larrson, *Internat. J. Peptide Protein Res.*, 1976, **8**, 193.

deamino[D-Arg^8]vasopressin [2f] and the high O/A selectivity (16 000) of [Gly^7]oxytocin.[1f]

4. [Trp^2]oxytocin [6e] may have been prepared because the authors did not know that this analogue had been prepared in 1972 (see this report, Vol. 5, p. 343, ref. 320).

Other Studies.—Interesting studies on the cross-reactivity of AVP and lysine vasopressin (LVP) and analogues with several antibodies have appeared recently.[185–188] Since these antisera have been produced in different species using different antigens coupled to different high molecular weight ligands it is hardly surprising that their binding characteristics differ. The use of selected structural analogues of AVP or oxytocin can be a helpful means by which to establish specificity of antisera, and equally important, to determine whether they discriminate against possible biologically inactive metabolites.[188]

Three very interesting reports showing that AVP analogues have other non-characteristic physiological and therapeutic effects may add an extra-dimension to structure–activity studies with synthetic analogues.

1. Cort *et al.*[189] have found that a series of AVP analogues can cause the release of plasminogen activator and that their effects follow a decreasing order of activity: deamino[6-carba, D-Arg^8]VP > deamino[D-Arg^8]VP > AVP > triglycyl-LVP > oxytocin.[189]
2. Legros *et al.*[190] have shown that LVP can improve memory in ageing subjects. This could be an important area for future research using synthetic analogues.
3. Mannuchi *et al.*[191] have reported that deamino[D-Arg^8]VP (dDAVP) can raise the plasma concentration of Factor VIII (the antihaemophilic factor) in patients with moderate or mild deficiencies due to haemophilia or von Willebrand's disease. dDAVP thus allows surgery in these patients without the use of antihaemophilic globulin that may carry hepatitis virus. However, the high antidiuretic activity of dDAVP is a serious side-effect. Thus the design of analogues with a selective F VIII response is highly desirable for extensive and safe therapeutic application of such an AVP-like drug.

3 Pancreatic Hormones

Contributed by D. Brandenburg and D. Saunders

Reviews have been published as 'Insulin today', the Banting Memorial Lecture 1976,[192] on preproinsulin,[193] proinsulin and C-peptide,[194] the molecular basis of

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[190] J. J. Legros, P. Gilot, X. Seron, J. Claessens, A. Adam, J. M. Moeglen, A. Audibert, and P. Berchier, *Lancet*, 1978, i, 41.
[191] P. M. Mannucci, F. I. Pareti, Z. M. Ruggeri, and A. Capitanio, *Lancet*, 1977, i, 869.
[192] D. F. Steiner, *Diabetes*, 1977, **26**, 322.
[193] S. J. Chan and D. F. Steiner, *Trends Biochem. Sci.*, 1977, **2**, 254.
[194] A. E. Kitabchi, *Metabolism*, 1977, **26**, 547.

insulin action,[195–197] on preparation and properties of cross-linked insulins,[198] and the aspects of physiological and biochemical basis of diabetes.[199] Further valuable information on insulin is contained in refs. 200, 201, and 202, particularly the sections on chemical and biological aspects of insulin and proinsulin,[203] and radioimmunological methods by R. Yalow.[204] In this context it is worth recalling that it was the fundamental work on insulin which led to the development of radioimmunoassays by S. Berson and R. Yalow, honoured by the Nobel Prize in 1977 (see ref. 205). Properties and regulation of the insulin receptor,[206] metal binding of insulin [207] (within a general review), as well as synthetic and semi-synthetic paths to insulin [208] have also been discussed.

The biosynthesis of proinsulin and proglucagon takes place in the microsomes.[209] While preproinsulin has so far not been found in whole cells, two proteins with molecular weights of 11 000 and 12 000 have now been observed in rat islets.[210] Bovine preproinsulin has been characterized chemically and immunologically.[211] It apparently folds and undergoes correct cystine formation less readily than reduced proinsulin. Extensive homology is found between the known positions of beef,[211] anglerfish,[212] sea raven,[212] and rat presequences. An even larger protein (mol. wt. 30 000) has been immunologically detected in virus-transformed rat B-cells.[213]

Insulin.—Ullrich *et al.*[214] (see also ref. 215) have isolated the coding region of the insulin gene by cloning in bacterial plasmids the complementary DNA (cDNA) synthesized *in vitro* from rat insulin mRNA, as well as cDNA fragments. Transformation of *E. coli* gave four combinant plasmids: one contained an inserted DNA fragment of about 410 nucleotides, the others smaller fragments. Sequence

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[199] J. Vallance-Owen, *Acta Diabetol. Latina*, 1977, **14**, 93.
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[201] 'Hormonal Receptors in Digestive Tract Physiology', INSERM Symposium 3, 1977, ed. S. Bonfils, P. Fromageot, and G. Rosselin, Elsevier, North Holland, Amsterdam.
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[206] B. H. Ginsberg, in 'Biochemical Action of Hormones', 1977, ed. G. Litwack, Academic Press, New York, Vol. 4.
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[214] A. Ullrich, J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rutter, and H. M. Goodman, *Science*, 1977, **196**, 1313.
[215] P. W. J. Rigby, *Nature*, 1977, **268**, 198.

analyses showed that (i) three plasmids cover the whole of the proinsulin coding sequence plus part of the prepeptide, (ii) the nucleotide sequence corresponds exactly to the amino-acid sequence of rat proinsulin I, proving additionally the linkages of the C-peptide, (iii) the missing presequence is ...Ala^{12}-*Leu-Leu*-Val-Leu-Trp-Glu-Pro-Lys-Pro-Ala-Gln-Ala^{0}-.... Two Leu-positions are confirmed, but Lys was placed 12 before.[193]

This work provides the basis for the isolation of the complete insulin gene and may be a first step towards the synthesis of the hormone in bacteria.

Chemical syntheses, semisyntheses, and modification have resulted in a variety of new analogues and derivatives which are listed in Table 7. Several known ones, on which new studies have been carried out, are included.

Table 7 *Analogues and derivatives of insulin*[a]

Compound number	*Structure*[b]		*Species*[c]	*Biological activity*[d]	*Test*[e]	*Ref.*
146a	Insulin		h			216
				++++	A, B, C, D, F	217
146b			p			218
146c			p		F	219
* 147	Gly^{A0}		b	+++	C	220
* 148	D-Phe^{A0}		b	+++	C, *f*	220
* 149	Trp^{A0}		b	+++	C	220
* 150	I_2-Tyr^{A0}		b	+++	C	220
151	Carbamoyl	A1	b	+++	C	220
* 152	Msc	A1	b	+++	C, *f*	220
* 153	Mtc	A1	b	+++	C	220
* 154	Palmitoyl	A1	b	++	C	220
155	Suc	A1	b	+++	C	220
156	Tfa	A1	b	+++	C	220
* 157	Arg^{A1}-Arg^{A0}		b	+++	C	220
* 158	Arg^{A2}-Arg^{A1}-Arg^{A0}		b	+++	C	220
159	Lys^{A1}-Arg^{A0}		b	+++	C	220
* 160	Boc-Gly^{A0}		b	+++	C	220
* 161	Boc-Trp^{A0}		b	++	C	220
* 162	[D-Lys^{A1}]		p		F	218
* 163	[Nle^{A2}]		o	++	A	221
* 164	[A_2Bu^{A2}, Glu^{A19}]		o	–	A, F	221
* 165	Biotinyl	B1	b	+++	C	222
166a	des-Phe^{B1}		b		*g*	223
166b			p			219

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Table 7 (*cont.*)

Compound number	*Structure*[b]		*Species*[c]	*Biological activity*[d]	*Test*[e]	*Ref.*
* 167	[*o*-F-Phe^{B2}]des-Phe^{B1}		p	+++	C	219
* 168	[Ala^{B1}]		p	+++	C	219
* 169	[*o*-F-Phe^{B1}]		p	+++	C	219
* 170	[*m*-F-Phe^{B1}]		p	+++	C	219
* 171	[Tyr^{B1}]		p	+++	C	219
* 172	[I_2-Tyr^{1B}]		p	++++	B, C, F, *h*	224
* 173	[Val^{B1}]		p	+++	C	219
* 174	[Arg^{B5}]					225
* 175	[Gly^{B5}]					225
* 176	[Gly^{B6}]					225
* 177	[D-Tyr^{B16}]		h			216
178a	des-(B26—B30) (despentapeptide)		p	+++	A, F, D	226
178b	des-(B26—B30)=R		b	++++	A, F, D	226
			b	+++	A	227
* 179	[Ala^{B22}]—R		b		A	227
* 180	[Cit^{B22}]—R		b		A	227
* 181	[Gly^{B22}]—R		b		A	227
* 182	[Lys^{B22}]—R		b		A	227
* 183	[Orn^{B22}]—R		b		A	227
184	des-(B23—B30) (desoctapeptide)			++	E	228
* 185	des-(B22—30) (desnonapeptide)		b		A	227
186	des-Ala^{B30}			++++	E	228
* 187	des-Met^{B31}		hg	++	C	229
				+++	E	
188	Dnp	B29	b		F	230
189	Ftc_3	A1, B1, B29	p		F	231
* 190	Trp_3	A1, B1, B29	b	+++	E	232
Tritiated and Iodinated Insulins						
191a	^{3}H-insulin		b			233
191b			b	++++	C, E, F	234
* 192	^{3}H-insulin, ^{35}S-Ptc_2	(A1, B1)	b			233
193a	^{125}I-insulin			++++	B, C, *h*	235

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[234] R. I. Misbin, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 690.
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Table 7 (*cont.*)

Compound number	*Structure*[b]		*Species*[c]	*Biological activity*[d]	*Test*[e]	*Ref.*
193b			c		E	236
* 193c			hg		*i*	229
193d						
193e	^{127}I-insulin			++++	B, C, *h*	235
				+++	C, E	237
193f			p	+++	C, E, *i*	237
193g	^{131}I-insulin				*h*	235
Intermediates						
194a	Boc	A1	b			220
194b		A1	p			218
* 195	Boc, (*Z*-Met)$_2$	A1, B1, B29	p			218
* 196	des-Gly^{A1} (*Z*-Met)$_2$	B1, B29	p			218
* 197	(*Z*-Met)$_2$	A1, B29	p			219
* 198	(*Z*-Met)$_2$	B1, B29	p			222
* 199	des-Phe^{B1} (*Z*-Met)$_2$	A1, B29	p			219
	A1, B29-(Eoc-Met)$_2$ = R′$_2$					
* 200	R′$_2$		p			219
* 201	des-Phe^{B1} R′$_2$		p			219
* 202	des-(B1-B2) R′$_2$		p			219
* 203	des-(B1-B3) R′$_2$		p			219
* 204	des-(B1-B4) R′$_2$		p			219
* 205	des-(B1-B5) R′$_2$		p			219
Derivatives of Incompletely Determined Structure, Monomeric						
206	Glu (^{3}H—NH—(CH$_2$)$_4$—NH$_2$) (A5, A15, B4)					238
207	Insulin, *N*-methylated		b		*g*	239
208	Insulin, sulphated		b		F, *l*	240
Heterolinked Insulins						
* 209	Glucose oxidase				F	241
210a	Dextran	(B29)	p	++	B, *j*, *k*	242
210b	Dextran	(B29)	p	–	B, *j*, *k*	242
211	Ferritin, monomeric		p		E	243
212a	Acrylic acid–acryloylpyrrolidone co-polymer B29					244
212b	Acrylic acid–styrene co-polymer B29					244
213	Polyvinylpyrrolidone			++	C, E	196, 245
214a	Sepharose 4B	(B1)	p			246
214b		(B29)	p			246

[236] J. Simon, P. Freychet, and G. Rosselin, *Diabetologia*, 1977, **13**, 219.
[237] S. Gammeltoft, J. Vinten, J. Gliemann, and S. Linde, *Horm. Metabol. Res.*, 1977, **9**, 186.
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[240] J. A. Little, R. Lee, M. Sebriakova, and A. Csima, *Diabetes*, 1977, **26**, 980.
[241] B. Mattiasson and H. Nilsson, *F.E.B.S. Letters*, 1976, **72**, 251.
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[243] L. Jarett and R. M. Smith, *J. Supramol. Struct.*, 1977, **6**, 45.
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[245] H. J. Kolb, B. U. v. Specht, R. Renner, and K. D. Hepp, *Diabetes*, 1976, **25**, Suppl. 2, 370.
[246] P. A. Jackson and K. Dixon, *J. Immunol. Methods*, 1977, **14**, 201.

Table 7 (*cont.*)

Compound number	*Structure*[b]	*Species*[c]	*Biological activity*[d]	*Test*[e]	*Ref.*
215	CH–sepharose 4B	p			246
216a	Succinyldiamino-dipropylamino-agarose				247
216b	(B1)				248
216c	(B29)				248
217	Biotinylinsulin + avidin-Sepharose B1	b	+++	C	222

[a] New compounds are marked by an asterisk. [b] Abbreviations: Msc = methylsulphoethyloxycarbonyl, Mtc = methylthioethyloxycarbonyl, A_2Bu = α,γ-diaminobutyryl, Dnp = 2,4-dinitrophenyl, Ftc = fluoresceinthiocarbamoyl, Ptc = phenylthiocarbamoyl, CH = carboxyhexyl. ( ): position not determined. [c] Human = h, Bovine = b, Porcine = p, Ovine = o, Chicken = c, Hagfish = hg. [d] Symbols for potency: 0—0.99% +, 1—9.99% ++, 10—99.9% +++, 100% ++++. [e] The following tests are indicated: A, mouse convulsion; B, *in vivo* blood-glucose assay; C, isolated fat-cell assay; D, glucose incorporation into diaphragm; E, receptor binding; F, immunological studies. [f] Preliminary *X*-ray studies. [g] Conformational studies. [h] Studies of metabolism. [i] Degradation studies. [j] ^{14}C-incorporation into adipose tissue *in vitro*. [k] Antilipolytic effect. [l] Diabetes therapy.

Sieber *et al.*[216] have now described in detail the final six steps of the total synthesis of human insulin 146a. It is remarkable that formation of the A7—B7 disulphide bridge occurs in over 70% yield not only in the 'H-peptide' A(1—21) B(1—30), but also its fully protected form, carrying 19 hydrophobic (3 Boc, 16 t-But) groups. Some racemization during the DCC–HOBt-coupling at B16/17 gave rise to the D-isomer (177). Compounds (163) and (164) were obtained from synthetic A-chains,[221] (174), (175), and (176) from synthetic B-chains [225] and the corresponding native chains. Cysteine protection with the benzyl group and fragment condensations in solution were used throughout.

Routes to the preparation of several A1-modified insulins are outlined.[220] Direct partial acylation of unprotected insulin gave (194a), (154), (155), and (156), as well as (160) and (161), which after deblocking gave (147) and (149). The derivatives (151) and (153) were obtained *via* B1-Msc-insulin. B1, B29-Boc_2-insulin, prepared from (156), served as the intermediate for the synthesis of (148), (150), (152), (157), (158), and (159).

A novel semisynthetic route is based on the *Z*-methionyl group.[218] The analogue (162) was obtained *via* the intermediates (194b), (195), (198), (196). The *method* was checked by re-synthesis of insulin (146b). Three different A1, B29-protected insulins were used for modifications at B1: the Boc-derivative for (165), the Msc-derivative for (172) (Vol. 9, p. 465, no. 32), and (197) for the removal of Phe^{B1} [compounds (199) and (166b)] and its replacement by a number of fluorinated and natural amino-acids [(168) (169) (170) (171) (173)].

Shortening the B-chain by five amino-acids reduces the yield in chain combination with A-chain to about one-quarter, as observed with (178a) and (178b).[226]

[247] S. Jacobs, Y. Shechter, K. Bissell, and P. Cuatrecasas, *Biochem. Biophys. Res. Comm.*, 1977, 77, 981.

[248] Insulin Research Group, Division of Endocrinology, Peking Institute of Zoology, Academia Sinica, *Sci. Sin.* 1977, **20**, 794.

Carboxypeptidase B treatment of des-octapeptide (B23—B30) insulin pentamethylester gave the corresponding des-nonapeptide (B22—30) insulin ester,[227, 249] to which, after Boc-protection at A1 and B1, synthetic tetrapeptide-methyl esters were coupled with DCC–HONSu in pyridine. Saponification was carried out at an initial pH of 11.8 for 15 h at 4 °C. The possibility of imide formation from esterified Asn^{A21} under alkaline conditions has to be taken into account in these semisynthetic approaches [250, 251, 252] (see Vol. 9, p. 467).

Compound (187) was obtained from hagfish insulin by cleaving the uncommon Met^{B31} with carboxypeptidase B (*ca.* 80% yield).

^{3}H-insulin (191a) has been prepared with tritiated phosphoric acid–BF_3 complex (specific activity 6.5 mCi mmol^{-1}) and double-labelled with ^{35}S-phenylisothiocyanate (specific activity 40 μCi mmol^{-1}) to give (192). This was used for insulin determination to a limit of 0.017 μmol.[233] ^{3}H-labelling under microwave activation yielded intact (191b) (specific activity 240 mCi mmol^{-1}).[234] Iodine-labelled insulins (193) have been prepared following known procedures. It would be interesting to know whether the iodination pattern is affected by the altered primary structure of chicken and hagfish insulin.

For sequential Edman degradation of the B-chain, protection with ethoxycarbonyl-methionine (200) was superior to the Z-derivative (197) as the latter was slightly unstable.[219] Compounds (200)—(205) have been isolated and characterized. Glutamine residues can be effectively (2.6 of 3 residues) modified by means of transglutaminase.[238] Reductive methylation of insulin gave (207) in which 87% of the lysines are dimethylated and 70—80% of Gly and Phe modified.[239]

Insulin has been linked to several soluble and insoluble macromolecules.[196, 241–248] Such conjugates have attractive properties and potentials, but are problematic, particularly with respect to homogeneity and characterization. Thus, analytical data are rather incomplete. Coupling to glucose oxidase has been effected by periodate activation of the enzyme.[241] Compound (209) was used for insulin determination (down to 10^{-6} M) by means of an immunoelectrode. Unprotected insulin was linked to soluble, CNBr-activated dextrans [242] (mol. wt. 450 000 and 2 million) to give (210a + b), and to ferritin by means of glutaraldehyde.[243] Monomeric (211) was obtained by gel filtration or use of monomeric ferritin. The selectivity of insulin attachment was increased by reversible citraconylation of *N*-terminal amino groups [244, 245] or selection of pH.[246] Leakage could be completely [245] or largely [246] prevented by coupling *via* amide bonds, using activation of the polymer carboxy groups with DCC–HOBt,[244] water-soluble carbodi-imide [246] or their *N*-hydroxysuccinimide esters.[244, 246] Alternatively, copolymers containing acryloyl chloride were used.[244] The use of synthetic polymers containing metal chelating groups (212a) is a new approach for visualizing the hormone at target cells by electron microscopy. Compound (212a) is water-soluble, (212b) insoluble. The agarose-linked insulins, prepared according to earlier published procedures of Cuatrecasas, were used for the affinity chromatography of insulin receptors [247, 248] and antibodies.[246] Compound (217) represents

[249] H.-G. Gattner, E. W. Schmitt, and H. Zahn, in 'Peptides 1976', Proceedings 14th European Peptide Symposium, Wépion, 1976, p. 279.
[250] H.-G. Gattner and E. W. Schmitt, *Hoppe-Seyler's Z. Physiol. Chem.* 1977, **358**, 105.
[251] W. König and A. Volk, *Chem. Ber.*, 1977, **110**, 1.

a new type of carrier-bound insulin.[222] The bond between avidin and the support is effected by leak-proof linking with 2,4,6-trifluoro-5-chloropyrimidine. Compound (165) is specifically and non-covalently bound to the matrix due to the strong tendency to form the biotin–avidin complex ($K_D \sim 10^{-15}$).

Synthetic human insulin (146a) is equipotent in five systems to natural human and pork insulin.[217] These do not exhibit any species differences.[253] In sensitized individuals, (146a) can, like extracted insulins, elicit skin reactions.[254] *In vitro* assays of new A1-modified insulins substantiate earlier findings (*cf.* Vol. 9, p. 467) that substitutions at this amino group generally give rise to a marked decrease of activity.[220] The lowest values so far are found for (154) and (161) (about 5%). The potency of (149, 150) and (155) is about 15%, while the majority ranges between 22 and 35% (153), (160), (152), (159), (151), (156), (158), (148). The most active analogues are (157, 42%) and (147, 68%). Hydrophobic and acidic substituents cause the largest inactivation, a positive charge appears favourable (see also compounds 5 and 11, Vol. 9, p. 464). In contrast to partial inactivation is the finding that most derivatives crystallize in the 2-zinc rhombohedral form [except for (154), (157), (158), (159), and (161)]. *X*-ray diffraction patterns of (148) and (152) as well as those of Met-, Glu-, and Lys-insulin (Vol. 9) are similar to A1-Boc-insulin, indicating similar conformations. Several (unspecified) A1-substituted insulins have reduced potencies (23—49%) and metabolic clearance rates (MCR) in dogs, but similar distribution space.[256] An inverse relationship was found for MCR and $t_{0.5}$. The immuno-reactivity of (162) is somewhat reduced. The re-synthesized insulin (146b) crystallizes, which is a valid physical test in this case.[257] The contact between the side-chains of Ile^{A2} and Tyr^{A19} appear to be highly specific, as the potency of (163) is only 4%. The hydrophobic interaction cannot be replaced by a strongly polar one, as demonstrated by the inactivity of (164).[221] Compound (165) was prepared in order to obtain a new type of affinity support (see below). The high potency (94%) confirms this site to be optimal for linking. Compound (165) forms active complexes with avidin in solution. An activity range of 34—61% has been reported for the analogues (167)—(171).[219] Such a large decrease is surprising in comparison to (172) recent results [258] and also with respect to the mobility of the B1-side-chain.[223] New assays appear essential. The reported [219] immunological identity of (146c) and insulin is a proof of successful re-synthesis if the antiserum discriminates changes. Compounds (146c) (168), and (173) gave rhombohedral, (171) monoclinic crystals. Compound (172) was fully active *in vitro* and exhibited normal metabolic and urinary clearance, $t_{0.5}$, and apparent distribution space in dogs. It therefore fulfils, if labelled, all the necessary criteria for a tracer. For several (unspecified) B1-substituted insulins a remarkable, close correlation

[252] G. E. Means and R. E. Feeney, *Biochemistry*, 1968, 7, 2192.
[253] I. Balázsi, G. Cseh, I. Kurunczi, and L. Barta, *Diabetologia*, 1977, **13**, 380.
[254] A. Teuscher and P. Diem, *Diabetologia*, 1977, **13**, 435.
[255] R. A. Pullen, D. G. Lindsay, S. P. Wood, I. J. Tickle, T. L. Blundell, A. Wollmer, G. Krail, D. Brandenburg, H. Zahn, J. Gliemann, and S. Gameltoft, *Nature*, 1976, **259**, 369.
[256] R. H. Jones, K. H. Quine, M. A. Tatnell, D. I. Dron, D. Brandenburg, and D. Lindsay, *Diabetologia*, 1977, **13**, 405.
[257] D. Brandenburg, M. Biela, L. Herbertz, and H. Zahn, *Hoppe-Seyler's Z. Physiol. Chem.*, 1975, **356**, 961.
[258] H.-J. Weimann, Thesis, Technische Hochschule, Aachen, 1977.

between biopotency (93—102%, in dogs) and MCR was observed.[256] Data for compounds (174)—(177) have not yet become available.

Compounds (178a) and (178b) obtained by chain combination and restricted peptic cleavage were equipotent [(178b), 100%; (178a), 88% in system A and 67% in D].[226] In contrast, the activity of semisynthetic (178b)[227] is only 34%. These diverging findings raise again the question (*cf.* Vol. 9) of the actual intrinsic potency of des-pentapeptide insulin. Replacement of Arg caused a marked loss of activity [13% (182), 12% (183), 9% (179, 180), and 6% (181)]. This indicates a specific role of Arg, when incorporated in the peptide chain, which cannot even be played by Orn. The authors draw parallels to the insulin-like activity of Arg-peptides, but since neither receptor binding and *in vitro* biopotency data, nor structural information are known, interpretation at this stage appears difficult. Compounds (184) and (186) have been used to characterize receptors in human placenta[228] (100% and 3% binding, respectively).

Insulin from the atlantic hagfish (*Myxine glutinosa*), which is the phylogenetically oldest insulin known, has been subjected to a detailed study.[229] The biopotency *in vitro* is 4.6 ± 0.6%, while receptor binding is 23%, *i.e.* for a particular degree of lipogenesis receptor occupancy must be about five times higher than with mammalian insulin. This is the first case of discrepancy between these parameters. The reason is not the presence of the additional Met^{B31}, since (187) gave similar values (4% potency, 20% binding). Compound (190) also appears to possess a higher affinity for receptors in human- and rat-liver membranes[232] (*ca.* 50%) than biopotency *in vitro* (15—20%, J. Gliemann and D. Brandenburg, unpublished work).

Binding studies with the tritiated insulin (191b) to cultured human lymphocytes gave, like those with iodoinsulins, curvilinear Scatchard plots indicating that this effect is not the consequence of molecular distortions caused by iodine. The very low specific activity of (191b) (0.03% of normal mono-iodoinsulins) limits its applicability. The mono-iodoinsulins (193a) and (193e) (lactoperoxidase method,[259] iodine predominantly at Tyr^{A14},) were identical to insulin with respect to bioactivity *in vitro* and *in vivo* as well as MCR. Compound (193g) (chloramine T procedure, composition not specified) exhibited a markedly enhanced $t_{0.5}$ and reduced MCR. On the other hand, a comparative study[237] showed neither (193e) (different batch) nor (193f)[260] (30% iodine at A14, 70% at A19) to be fully active *in vitro* (78 ± 2 *versus* 65 ± 3%) and with respect to receptor binding (63 ± 4 *versus* 80 ± 6%). Interlaboratory assay variations may, at least in part, account for the differences found. For an insulin with Tyr^{A19} fully mono-iodinated, a potency of 50% can be deduced, which is a rather high value considering the size of iodine and the involvement of Tyr^{A19} in intra-monomeric and, possibly, in insulin–receptor interactions.[255]

The conjugates (210a), (210b), and (213) were reported to be stable, which is somewhat surprising for the former. Compounds (210a) and (213) are active *in vivo* and *in vitro* (about 10%), while (210b) is practically inactive. Compound (217) exhibited a potency of about 15% *in vitro*. Binding data of (213), which

[259] J. C. Sodoyez, F. Sodoyez-Goffaux, M. M. Goff, A. E. Zimmerman, and E. R. Arquilla, *J. Biol. Chem.*, 1975, **250**, 4268.

[260] S. Linde and B. Hansen, *Internat. J. Peptide Protein Res.*, 1974, **6**, 157.

cannot dimerize, give curvilinear Scatchard plots. This excludes self-association as a possible cause for non-linearity.[196]

These new results have further deepened our understanding of structure–function relations, but the molecular mechanism of insulin action, particularly the event(s) following formation of the hormone–receptor complex, remain unclear.[192, 195–197] Is insulin's role merely a physical interaction, or are subsequent chemical transformations of the molecule essential? The modifications reported affect primarily receptor binding, and in some cases also degradation. In hepatocytes, degradation is linked to binding,[192] and Steiner discusses a 'receptor–transducer–internalization model'.[192]

^{125}I-insulin (or the label?) has been observed to undergo a limited translocation in hepatocytes and to enter intact lymphocytes.[261] The possibility that insulin may regulate long-term effects (RNA and protein synthesis) through intracellular binding sites is considered.[197] These are immunologically different from plasma membrane sites.[262, 263] On the other hand, the generation of active fragments has been suggested,[192] *i.e.* 'built-in second messengers'. In this context the question of whether the insulin-like activity of Arg-peptides [227] is physiological or pharmacological is important. A new example is the sequence β-Ala-Arg-Gly-Phe-Phe-Phe-Tyr-NH_2, which exhibits activity *in vitro*, in the periphery, and potentiates insulin's action, but is not hypoglycaemic.[264]

Degradation of insulin has been studied under various conditions, but the physiological significance is mostly unclear. Degradation by concentrated suspensions of fat cells exhibits some structure-dependency under certain conditions.[229, 237] Enzymatic activity associated with liver cell membranes can easily be removed, but degrades receptor-bound insulin preferentially.[265] Purified liver membranes are devoid of degrading enzymes,[266] as are placental membranes to a large extent.[228] In isolated fat cells, antibodies against glutathione–insulin-transhydrogenase inhibit stimulation of cAMP phosphodiesterase (PDE) by insulin. The disulphide system of insulin or the resulting chains may be involved in PDE regulation.[267] Separated A- and B-chains exhibit small effects.[267] On the other hand, auto-antibodies against the insulin receptor, completely different molecules, exert stimulatory effects on glucose transport and metabolism in isolated muscle [268] and fat cells.[269] Cultured human lymphocytes appear to have receptors for A-chain-tetra-S-sulphonate, but not the S-carboxymethyl derivative or B-chain. The S-sulphonate competes for insulin binding sites (0.8%), inhibiting the weak insulin effect on glucose metabolism.[270]

[261] J.-L. Carpentier, P. Gorden, A. Le Cam, P. Freychet, and L. Orci, *Diabetologia*, 1977, **13**, 386.
[262] I. D. Goldfine, R. Vigneri, K. Y. Wong, G. J. Smith, N. B. Pliam, D. Cohen, and A. L. Jones, *Diabetologia*, 1977, **13**, 396.
[263] I. D. Goldfine, R. Vigneri, D. Cohen, N. B. Pliam, and C. R. Kahn, *Nature*, 1977, **269**, 698.
[264] M. Fujino, M. Wakimasu, S. Taketomi, and H. Iwatsuka, *Endocrinology*, 1977, **101**, 360.
[265] L. K. Dial, S. Miyamoto, and E. R. Arquilla, *Biochem. Biophys. Res. Comm.*, 1977, **74**, 545.
[266] M. H. Wisher, D. I. Dron, P. H. Sönksen, and J. H. Thomas, *Biochem. Soc. Trans.*, 1977, **5**, 313.
[267] B. H. Phelps and P. T. Varandani, *Biochem. Biophys. Res. Comm.*, 1977, **75**, 302.
[268] P. Freychet, Y. Le Marchand, P. Gorden, J. Flier, and C. R. Kahn, *Diabetologia*, 1977, **13**, 394.
[269] C. R Kahn, K. Baird, J. S. Flier, and D. B. Jarrett, *J. Clin. Invest.*, 1977, **60**, 1094.
[270] M. Kobayashi and J. C. Meek, *Diabetologia*, 1977, **13**, 251.

In fat cells and fat-cell plasma membranes, receptors are randomly distributed and occur in groups of up to six, as now shown with monomeric (211).[243]

The receptors of animals with insulins of different potencies have similar affinities for the hormone, but the binding capacity (number of receptors per unit cell surface) appears to be inversely related to the affinity of the homologous insulin to the receptor.[232, 236, 271] A first example of decreased affinity for insulin seems to be receptors of patients with generalized lipodystrophy.[272] Receptors from rat liver have been from purified to a binding activity of 0.4 pmol μg^{-1} [247] (seven times less than expected for a mol. wt. of 300 000), and from human placenta [228] 4000-fold.[273] Dissociation into active subunits of mol. wt. 50 000 with 6 M-guanidinium, HCl has been noted.[248]

A higher resolution of the insulin structure will be very helpful in our understanding of insulin–receptor interactions. Refinement of atomic positions with 1.3 Å data is in progress.[274] Models tend to suggest rigidity. However, insulin has to be regarded as a stable molecule with a limited flexibility, and its interaction with the receptor not as one of two solid body surfaces, but rather of two dynamic solution states.[275] Stability and some flexibility in solution are evident from recent physical studies. C.d.-spectra at pH 7.4 indicate that even in 3M-guanidinium, HCl the secondary structure is largely retained.[276] From the identity of the tyrosyl c.d. of insulin and (166a), thermal motion of the Phe^{B1} and Tyr^{A14} rings is deduced, and the predicted importance of this interaction in the hexamer ruled out.[223] The amino groups of (207) have been studied by means of ^1H- and ^{13}C-n.m.r. spectroscopy.[239] Besides yielding apparent p*K*-values of 6.7, 8.0, and 11.2 for Phe, Gly, and Lys (assignment tentative), small conformational changes are indicated around B29 on dimerization. Tetra-*S*-carboxymethyl-A-chain can be induced to form α-helix, with up to 43% in trifluoroethanol–water (83%).[277] The association of bovine insulin has been studied by sedimentation equilibrium at pH 7.4,[276] and at pH 7.0 in the presence of one atom Zn^{2+} per three monomers.[278] Finally, (184),[279] (188),[230] (189),[231] and several B1-aminoacylated insulins [280] have been used in studies on the mechanism of antibody formation. Experiments with eight species indicate that the formation of humoral antibodies depends essentially upon the species of origin of the hormone,[281] *i.e.* its chemical structure. The reduced immunogenicity of sulphated insulin (208) and the advantages of its application have clearly been demonstrated.[240]

In conclusion, although nature has so far provided the two most interesting insulins, that of chicken with elevated activity [236] and of hagfish with diverging binding and potency,[229] the chemically obtained insulins so far present a thesaurus which has been explored for biological potentials to a small extent only. It may

[271] W. Kemmler, A. Zynamon, R. Renner, and K. D. Herr, *Diabetologia*, 1977, **13**, 407.
[272] S. Oseid, H. Beck-Nielsen, O. Pedersen, and O. Søvik, *New England J. Med.*, 1977, **296**, 245.
[273] P. F. Williams and J. R. Turtle, *Proc. Austral. Biochem. Soc.*, 1977, **10**, 82.
[274] K. Sakabe and N. Sakabe, *Diabetes J. (Japan)*, 1977, **5**, 12.
[275] R. J. P. Williams, *Angew. Chem.*, 1977, **89**, 805.
[276] L. A. Holladay, M. Ascoli, and D. Puett, *Biochim. Biophys. Acta*, 1977, **494**, 245.
[277] L. A. Holladay, *Biophys. Chem.*, 1977, **7**, 41.
[278] B. K. Milthorpe, L. W. Nichol, and P. D. Jeffrey, *Biochim. Biophys. Acta*, 1977, **495**, 195.
[279] U. Kiesel and H.-G. Gattner, *Diabetologia*, 1977, **13**, 408.
[280] F. K. H. Jansen, U. Kiesel, and D. Brandenburg, *Ann. d'Immunologie*, 1977, **C128**, 313.
[281] H.-P. Neubauer, C. Baeder, and H.-H. Schöne, *Diabetologia*, 1977, **13**, 421.

even contain therapeutically valuable insulins – sulphated insulin is an encouraging example.

Glucagon.—Reviews of the role of glucagon in diabetes have appeared.[282–284] Increasing evidence favouring the concept of local feedback control of islet cell secretions has been discussed.[285–287] New cell types, *e.g.* PP-cells, will presumably be included in later refinements. The proceedings of a colloquium on the hormonal control of lipid metabolism have been published.[288] The reports of a glucagon symposium [289] appeared too late for last year's review. They provide a good survey of the whole field of glucagon research. A relation between the storage forms of glucagon in teleosts and crystals grown *in vitro* has been noted.[290, 291] The river lamprey possesses insulin- and glucagon-immunoreactive substances.[292] Preliminary investigations of the variations of the glucagon sequences of guinea-pig, shark, angler-fish, and dog-fish from that of pigs, cows, and humans have been made.[293] The guinea-pig sequence contains 11 residues more than porcine glucagon, but maintains histidine at the *N*-terminus. Porcine gut GLI has been compared to glucagon,[294] and considerable similarity noted.

Initial results from studies of glucagon biosynthesis in isolated angler-fish islets have been reported.[209, 294, 295] ^{3}H-Trp was used to label glucagon peptides, as this amino-acid is not present in proinsulin.[209, 294] No peptide of the size of somatostatin was observed, which may be due to the extraction technique. A precursor sequence for glucagon: molecular weight 12 000 (acidic) → 9000 (acidic) → 4900 (basic) → 3500 was postulated, with conversion occurring mainly in the secretory granule. Another group [296] found that the size of the observed glucagon species depended on the extraction technique, and on the degree of proteolytic activity in the homogenate. Extraction under neutral conditions produced mainly 50 000 and 9000 immunoreactivities, while extraction in acid gave predominantly a species with molecular weight 12 000.

Studies on glucagon receptors have continued apace. The mobile receptor theory [297] is gaining experimental evidence and has been briefly reviewed.[298] Both receptor and adenylate cyclase need to bind GTP before the full effect of glucagon

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[285] R. H. Unger and L. Orci, *Arch. Intern. Medicine*, 1977, **137**, 482.
[286] R. H. Unger and L. Orci, *Ann. Rev. Med.*, 1977, **28**, 119.
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[294] A. J. Moody, E. K. Frandsen, H. Jacobsen, F. Sundby, and L. Orci, *Metabolism*, 1976, **25**, Suppl 1, 1336.
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[298] M. F. Greaves, *Nature*, 1977, **265**, 681.

on cAMP accumulation is seen,[299, 300] despite GTP decreasing the affinity of the receptor. Probably both cyclase and receptor have distinct regulatory subunits.[300] GTP-sensitive glucagon–receptor and adenylate cyclase complexes have been partially purified after Lubrol PX solubilization of rat-liver plasma membranes.[301] Evidence has been obtained for exchange of partners within the receptor–cyclase complex.[302] A careful study[303] of the adipocyte glucagon receptor failed to detect homotropic co-operativity. Increased cyclase activity was evident within 20 s of binding. A K_d of 1.5 nM was measured, which is greater than the value of 0.24 nM measured in isolated adipocyte membranes. The difference was ascribed to the dissociative activity of GTP in the intact cells. Down-regulation of the glucagon receptor population is indicated by the reduced binding and sensitivity of liver cell membranes of chronically hypergluconaemic rats.[304] A report[305] of increased binding in patients with uraemic hypergluconaemia may be complicated by changes in the level of several hormones.

Glucagon, secretin, and VIP have obvious sequence similarities, and have been shown to bind weakly to the specific receptors of the others.[232] The cross-binding is, however, very weak and species-dependent. A theoretical approach[306] suggested that a possible two-fold symmetry within the glucagon sequence is maintained in secretin. It also exists in other hormones, and may reflect a corresponding symmetry at the level of the hormone–receptor complex.

A variety of techniques have been employed to look at the structure and structural transitions of glucagon. Analysis by the rules of Chou and Fasman indicates two possible conformers: one with residues 5—10 in a β-sheet, and 19—27 in a α-helix (31% α, 21% β); the other with both 5—10 and 19—27 in a β-sheet configuration (0% α, 52% β). A concentration-dependence of helix content has been shown by c.d. However, solutions left to stand for 10 days form fibrils,[307] indicating that it is the β-sheet that is most stable in solution, and not 55% α-helix as seen in crystals. The thermodynamics of the self-association of glucagon has been studied by c.d.[308] Negative enthalpy and entropy terms were measured. A positive entropy term is expected for a system interacting by hydrophobic forces. The authors suggest that the unawaited result indicates that substantial conformational changes are involved in aggregation. Another laboratory has used a combination of c.d., ^{1}H-n.m.r., difference u.v. spectroscopy,[309] fluorescence, and fluorescence polarization[310] to study the helix-promoting association of glucagon and dimyristoyl choline micelles. Binding of glucagon decreased lateral diffusion

[299] N. Kimura and N. Nagata, *J. Biol. Chem.*, 1977, **252**, 3829.
[300] P. M. Lad, A. F. Welton, and M. Rodbell, *J. Biol. Chem.*, 1977, **252**, 5942.
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[308] S. Formisano, M. L. Johnson, and H. Edelhoch, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 3340.
[309] R. M. Epand, A. J. S. Jones, and B. Sayer, *Biochemistry*, 1977, **16**, 4360.
[310] R. M. Epand, A. J. S. Jones, and S. Schreier, *Biochim. Biophys. Acta*, 1977, **491**, 296.

of lipid molecules, and some aromatic residues became buried in a more hydrophobic environment. Phosphorescence and optically detected magnetic resonance of tryptophan were used [311] to show that aggregation depends on concentration, and that the free-solution structure requires the presence of the three *C*-terminal residues. Chou and Fasman analysis predicts that these are vital in causing nucleation of α-helix. Similar results have been obtained by c.d.[312] However, des-(Met[27]-Asn[28]-Thr[29])-glucagon can form β-sheeted fibrils.

Chemical modification of glucagon has not yet reached the level of sophistication achieved with insulin. The literature has many contradictory results, and indicates how desirable it is that standardized analytical and assay techniques be applied in order properly to evaluate derivatives. Table 8 is a list of those derivatives which seem more trustworthy. An obvious difference between glucagon and

Table 8

Compound number	*Glucagon derivative*	*Biological test* A (%)	B (%)	C (%)	*Ref.*
218	des(His)[1]	2.4	2.0	–	312
		–	7—10	2	314
219	des(His[1]-Ser[2])	0	–	–	319
220	des(Met[27]-Asn[28]-Thr[29])	1.4	0.5	–	312
221	des(Asn[28]-Thr[29])	–	3	3	316
222	(CNBr-g)	–	2—3	2—3	314
223	(CNBr-g)—$NHNH_2$	–	3	3	314
224	(CNBr-g)—$NH(CH_2)_3CH_3$	–	3—4	3	314
225	(CNBr-g)—$NH(CH_2)_6NH$,Biotin	–	0.1	0.2	314
226	N^{ϵ}(Boc)[12]	–	–	very low	320
227	N^{ϵ}(TFA)[12]	–	–	*ca.* 30	320
228	Mono^{N}-(acetyl)	–	12	11	321
229	N^{α},N^{ϵ}-Di(acetyl)	–	0.5	0.7	321
230	*N*,*O*-Tetra(acetyl)	–	0.1	0.1	321
231	N^{α},N^{ϵ}-Bis(iodoacetyl)	–	0	0	314
232	N^{ϵ}-(NAP)	–	100	0	318
233	N^{α},N^{ϵ}-Di(TNP)	–	–	< 10	322
234	N^{α}(TNP),N^{ϵ}(guanidyl)	–	–	< 10	322
235	N^{α} (carbamoyl)-N^{ϵ}(TNP)	–	–	< 10	322
236	[3-Iodo-tyrosine[13]]	–	–	–	315
237	Mono(iodo)-	–	153	270	316
238	Di(iodo)-	–	178	380	316
239	Tri(iodo)-	–	195	–	316
240	Tetra(iodo)-	–	148	480	316

[311] J. B. A. Ross, K. W. Rousslang, D. A. Deranleau, and A. L. Kwiram, *Biochemistry*, 1977, **16**, 5398.
[312] W. W. Bromer, *Metabolism*, 1976, **25**, Suppl. 1, 1315.
[313] R. M. Epand, T. E. Cotz, D. H. B. Hoa, G. Rosselin, S. Schrier, *Metabolism*, 1976, **25**, Suppl. 1, 1317.
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[322] R. M. Epand and G. E. Wheeler, *Biochim. Biophys. Acta*, 1975, **393**, 236.

Table 8 (*cont.*)

Compound number	*Glucagon derivative*	*Biological test* A (%)	B (%)	C (%)	*Ref.*
241	Penta(iodo)-	–	51	160	316
242	[3-Nitro-tyrosine^{13}]	230	–	77	323
243	Di[3-nitro-tyrosine^{10,13}]	145	–	22	323
244	[3-Amino-tyrosine^{13}]	56	–	40	323
245	[3-Amino-tyrosine^{13}]	28	–	14	323
246	[*S*-Methyl-methionine^{27}]	–	–	0.2	317
247	[Hydroxynitrobenzyl-trypto-phan^{25}]	–	3	4	316
248	Cyclohexanedione derivative of arginine	–	2	–	316
249	Mono(des-amido) (mixture of 3, 20, 24 sites)	69	–	–	324
250	Glucagonyl tetra(glycinamide)	–	< 1	< 1	325
		–	–	0	326
251	Tetra(methyl)ester	–	< 1	< 1	325
252	Glucagonyl (taurine amide)^{29}	–	–	10	326
253	Glucagonyl tetra (taurine amide)	–	–	0	326
254	Amide with ethylene diamine ($2\frac{1}{2}$)	–	–	0	326

Biological test: A, *in vivo;* B, receptor binding; C, adenylate cyclase activation.

insulin derivatives is that the former can have differing receptor binding and adenylate cyclase potencies, whereas only one example of a difference between binding and *in vitro* activity has been shown for an insulin analogue.[229] Thus N^{α}-trinitrophenyl-glucagon binds to the receptor 100-fold better than it activates adenylate cyclase;[313] des-his^{1}-glucagon acts as a partial agonist by binding 3—4-fold better than it activates the cyclase.[314] The latter derivative has only 50% of the maximum cyclase-activating potential of glucagon,[303] albeit at much higher concentrations.

Controlled iodination with lactoperoxidase produces predominantly mono-iodotyrosine^{13}-glucagon.[315] Tyr^{10} reacts at only one-fifth of the rate of Tyr^{13} and very little di-iodination is observed. Increases in binding and cyclase activities [316] observed after iodination have been discussed.[314] Iodoglucagon has a different pH dependence for receptor binding and cyclase activation than the native hormone, possibly because the ionized iodophenoxide side-chains hinder binding. The resistance to proteolytic degradation, a common problem in normal assay procedures, is increased by iodination. The apparently stepwise increase in binding with level of iodination [316] indicates the need for a defined radioglucagon.

Treatment of glucagon with cyanogen bromide yields the weakly active des-(Asn^{28}, Thr^{29})-homoseryl lactone-glucagon.[314] The lactone moiety reacts with nucleophiles to produce secondary derivatives, but no recovery of activity was observed. The poor solubility of glucagon, and its tendency to form fibrils, make

[323] J. M. Patterson and W. W. Bromer, *J. Biol. Chem.*, 1973, **248**, 8337.
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[326] G. E. Wheeler, R. M. Epand, and D. Barrett, *Biochim. Biophys. Acta*, 1974, **372**, 440.

purification of derivatives by chromatography very difficult. A reversible modification of methionine increases the solubility of the hormone 500-fold.[317] The thioether side-chain reacts specifically with methyl iodide at pH 3.5 to form a *S*-methyl sulphonium compound, which is stable to acid hydrolysis, but reverts to the demethylated form on treatment with dithiothreitol at pH 10.5. *S*-methyl glucagon has only 0.2% potency in the cyclase assay, but, as with all derivatives with such low activities, this may be due to traces of contaminating glucagon.[312, 316]

A radioiodinated photoactivatable derivative, iodo-(ε-NAP)^{12}-glucagon, has been used to label components in liver plasma membranes.[318] Its binding was identical to ^{125}I-glucagon and was inhibited by native glucagon. After irradiation, a major specifically labelled component was found to have a molecular weight of 23 000—25 000 by SDS–polyacrylamide electrophoresis. The identity of this protein with the cyclase-activating glucagon receptor remains to be proven. The derivative was inactive in the cyclase assay.

Somatostatin.—Reviews of the role of somatostatin in diabetes mellitus,[327, 328] in carbohydrate homeostasis,[329] in gut physiology,[330, 331] and within the islet [332] have appeared. The use of somatostatin therapy in diabetes and pancreatitis continues to attract much attention, but is outside the scope of the review.

A patient with a pancreatic somatostinoma suffered from hypochlorhydria, steatorrhea, and a diabetic glucose tolerance.[333] Immunoreactive somatostatin has been detected in human, dog, and rat thyroid tissue.[334] Somatostatin release is not affected by insulin, but is increased by glucagon.[335] Hypophysectomy increases somatostatin secretion within the rat pancreas.[336] Pancreatic polypeptide levels are decreased by somatostatin [337] although this may be due to reductions in the level of the PP-secretagogue, secretin. Inhibition of insulin secretion is not a cAMP-dependent process, but requires Ca^{2+}-translocation within the B-cell.[338, 339] Incubation of isolated islets in the presence of antisomatostatin antibodies increases the insulin secretory response to sub-maximally effective glucose concentrations,[340] supporting the concept of paracrine control within the islet.[332] A somatostatin-binding protein has been detected in the cytosol of several

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tissues.[341] Binding may be followed by disulphide-bond cross-linking. Immunoreactive somatostatin was also detected in angler-fish islet homogenates, but as yet no precursors have been observed.[209]

The successful incorporation and expression of a fully synthetic DNA sequence coding for somatostatin has been reported.[342] The 'gene' was inserted into a β-galactosidase region of an *E. coli* plasmid using annealing techniques similar to those applied for the insertion of natural insulin genes into a plasmid.[214] The synthetic somatostatin nucleotide sequence began with the codon for methionine and finished with two nonsense codons. Thus CNBr-cleavage should release somatostatin from the translation product. This has been detected by radioimmunoassay. At present the yields are very low, and scaling-up experiments have not succeeded due to instability of the cultures, but the method obviously promises much for the future.

Selective alteration of the inhibitory activity of somatostatin analogues on the secretion of insulin, glucagon, and growth hormone has been achieved. Of especial interest for diabetes therapy are analogues which suppress glucagon and growth hormone but not insulin. [D-Cys^{14}]-,[343] [Ala^2, D-Cys^{14}]-, and [D-Trp^8, D-Cys^{14}]somatostatin may have these desirable properties. The effects of substitutions are independent in the latter derivatives. Thus [D-Trp^8]somatostatin was previously recognized as being more potent than the native hormone: [D-Trp^8, D-Cys^{14}]somatostatin is similarly more potent than the [D-Cys^{14}]-analogue, but retains the same selectivity of action. [D-Cys^{14}]- and [D-Trp^8]somatostatin are equipotent to somatostatin in their effects on gastric acid and pancreatic bicarbonate secretions.[344, 345]

A bicyclic analogue [346, 347] is more potent in suppressing growth hormone than glucagon or insulin, but insertion of D-Trp^8 returned the suppressive pattern to normal.[346] A carbacyclic [D-Trp^8]-analogue [348] has prolonged activity on all three hormones, possibly due to resistance to exopeptidases. Des(Ala^1, Gly^2)[desamino-Cys^3]somatostatin is very active for short periods.[348] Positions 1 and 2 and completion of the disulphide loop may be unimportant for activity.

Ultracentrifugation techniques and c.d. studies show some differences between normal somatostatin and eight synthetic analogues, and may eventually provide a structural basis on which to explain the differing activities.[349]

Pancreatic Polypeptide.—The role of this recently discovered 36-residue hormone is still very poorly understood. Originally found in avian pancreas, it has since

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[342] K. Italkura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heynekar, F. Bolivar, and H. W. Boyer, *Science*, 1977, **198**, 1056.
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[349] L. A. Holladay, J. Rivier, and D. Puett, *Biochemistry*, 1977, **16**, 4895.

been detected in many mammalian species, including man. It is stored in distinct cell types in dogs [350] (F-cells) and in man.[351] These cells are mainly peripheral to the islets, and some are even found in the acinar tissue. In alloxan-diabetic rats, the loss of B-cells is partly compensated in terms of mass by an increase in the number of PP-containing cells.[352] This will complicate the awaited hormonal and metabolic responses in such animals. Certain juvenile diabetics have been shown to have 'PP-cell' hyperplasia.[353] These cells occur as 'ribbon-like' islet formations independent of the usual 'atrophic' islets containing A and D cells. PP-cells may have been shown to have a function in mice. The hyperinsulinaemia, hyperglycaemia, abnormal glucose tolerance, and body-weight gain of New Zealand obese mice were returned to normal by the i.p. implantation of G albino rat islets encased in a Millipore sac.[354] A secreted factor seemed involved, and injection of bovine PP was found to produce similar effects. Similarly ob/ob mice treated with BPP have greatly diminished rates of body-weight increase and reduced food intake.[355] Such animals are deficient in PP- and somatostatin-containing cells. It was suggested that PP can act as a satiety factor, so that reduction in its level leads to hyperphagia.

BPP also has a trophic effect on pancreatic tissue.[356] Trophic effects previously ascribed to cholestokinin may in fact have been due to BPP, as cholestokinin-related peptides cause dramatic increases in plasma PP levels.[357] Caerulin and secretin also increase PP levels. Decreases in PP levels seen on infusion of somatostatin in gastronectomized patients may thus reflect the effect of this hormone in switching off secretin production.[336] Glucose, fat, or amino-acids increase PP levels if given orally but not intravenously. Insulin-induced hypoglycaemia provokes increased PP levels in normal but not in vagotomized patients. A complex entero-PP axis with nervous and hormonal control elements seems beyond dispute.

The released hormone may, like many others, be present in a heterogeneous form in plasma. Three immunoreactive species with molecular weights of *ca.* 100 000, 4000, and 1500 have been observed.[358] The globulin-sized material may be non-covalently linked PP and plasma protein, but no explanation is yet available for the mini-PP species. Other authors do not find such heterogeneity.[357]

Preliminary studies [359] on the structure of avian PP have been reported. The peptide was purified, and crystallized from neutral buffer by the 'hot-box' technique. The crystals were monoclinic, with space group C2. Isomorphous

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[351] B. H. Bergstrom, S. Loo, H. J. Hirsch, D. Schutzengel, and K. H. Gabbay, *J. Clin. Endocrinol. Metabol.*, 1977, **44**, 795.
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crystals with a high-occupany single site for $Hg(NO_3)_2$ were also obtained. From these, using anomalous scattering and Patterson function calculations, a low-resolution electron density map was constructed. This showed the molecule to be partly helical, and to form a compact dimer about the crystallographic two-fold axis. C.d. spectra also indicate considerable helix in solution. Use of the 'Schiffer–Edmunsen wheel' indicates a hydrophobic surface in the *C*-terminal half of the molecule which may provide the dimerization site for APP, much as similar sites provide the trimerization sites for glucagon.

Relaxin.—Although not a pancreatic hormone, the recent flurry of interest in relaxin has centred on its obvious relation to insulin. Differing sequences have been published for both chains of the molecule by two groups,[360, 361] and an insulin-like pattern of disulphide cross-links ascertained.[361] The differences may be due to intrinsic sequence variability at the *C*-terminus of the relaxin 'B'-chain.[362] An explanation of the known charge heterogeneity of relaxin preparations,[363] is not yet forthcoming. Although the sequence differences between insulin and relaxin demand 51 point mutations,[360] there has been nearly total conservation of residues known to contribute to the hydrophobic core of insulin.[361] Of the 20 positions that are invariant in the known insulin sequences, 15 are maintained in relaxin.

Possible relaxin structures have been produced by model-building[364] and computer-graphic techniques[365] by fitting the relaxin sequence to the insulin peptide backbone. New side-chains were accommodated without difficulty, and certain changes of amino-acid sequence between insulin and relaxin (*e.g.* $Ile^{A2} \rightarrow Leu$, $Leu^{A16} \rightarrow Ile$) were shown to be spatially compensatory. Tryptophan B15 can be easily inserted into the core of the molecule, although the alignment of the indole side-chain is disputed. The course of the *C*-terminus of the relaxin B-chain is less clear. Replacement of glycine B23 in insulin by L-serine in relaxin introduces a rotational restriction which may result in the structures of insulin and relaxin differing in this region. Provokingly, IGF (previously NSILA) may also have an insulin-like structure.[366] The relation of relaxin, insulin, and IGF has been discussed in terms of evolution from an early growth peptide.[367]

A recent report[368] describes the effect of chemical modification on the biological activity. It seems that oxidation of one tryptophan by NBS has no effect, and proceeds rapidly; oxidation of the second results in total inactivation. Reaction of the amino groups with TNBS or succinic anhydride leaves the activity intact; a water-insoluble 'tyrosylated' derivative is fully active. Similarly, reaction of the single methionine (A2) with iodoacetate is without effect, but cleavage of the *N*-terminal dipeptide with CNBr inactivates the hormone. At present, these results

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[367] H. D. Niall, ref. 220, p. 127.
[368] C. Schwabe and S. A. Braddon, *Biochem. Biophys. Res. Comm.*, 1976, **68**, 1126.

must be regarded as preliminary since none of the derivatives were fully characterized, and the assay procedures seem to indicate full or zero activity only. However, as the yield of relaxin from pregnant ovary tissue is high (*ca.* 120 mg kg^{-1} wet weight), higher than the yield of some mammalian insulins from pancreatic tissue, one can expect more such studies in the near future.

4 Gastrointestinal Hormones

Contributed by D. Gillessen, R. O. Studer, and U. Ludescher

The proceedings of the conference 'Chemistry and Biology of Gastrointestinal Hormones'[369] represent a comprehensive collection of reviews on all aspects of gastrointestinal hormones. Criteria for differentiating between physiological and pharmacological effects have been presented.[370] The aspects of gastrointestinal hormone–receptor interactions have been the subject of the 'First International Symposium on Hormonal Receptors in the Digestive Tract Physiology', published in the corresponding proceedings.[371]

Gastrins.—(Figure 1.) Several reviews [372–375] on gastrins have appeared. Two of them deal especially with the mechanism of action [373] and the regulation of gastric acid secretion,[374] another one with the inhibition of gastrin release and gastric acid secretion.[375]

Another synthesis of human 'little gastrin I' (G-17) [376] and a new total synthesis of human 'big gastrin I' (G-34) and its leucine-32 analogue have been reported.[377] G-34 proved to possess 50% higher biological activity than G-17.

G-14	Trp-Leu-
G-17	Pyr-Gly-Pro-Trp-Leu-
G-34	Pyr-Leu-Gly-Pro-Gln-Gly-His-Pro-Ser-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-Gln-Gly-Pro-Trp-Leu-
CCK	Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-
G-14	-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
G-17	-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
G-34	-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
CCK	-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-pancreozymin (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.

[369] *Acta Physiol. Acad. Sci. Hung.*, 1976, **47**, issue 4.
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The synthesis of new analogues of the *C*-terminal portion of gastrin has almost come to a standstill. In contrast to earlier findings that not only the aspartate residue in position 16 but also the terminal amide is essential for biological activity,[378] gastric secretory effects have been claimed for MBOC-Met-Asn-Phe-OH and MBOC-Met-Asp(OBzl)-Phe-OMe.[379] BOC-Trp-Met-Asp-D-Ala-NH_2 has been synthesized and reported to be a competitive inhibitor of pentagastrin and gastrin.[380]

The synthesis and purification of des-Trp[1]-[Leu[11]]-minigastrin I (G-13), an analogue which shows full gastrin activity, have been described.[381, 382] This G-13 fragment was considered as 'minigastrin'[383] before it became evident that in fact the G-14 fragment is the real 'minigastrin'.[384] The synthetic gastrin I fragments G-16, G-15, and G-13 were found to have about the same acid- and pepsin-stimulating activity as G-17, whereas G-10 and G4 had only activities of 26% and 2% respectively.[385] For equimolar rates of gastrin infusion, the increments in both the acid secretory rates and serum gastrin levels induced by G-34 were significantly greater than those obtained with [Leu[15]]-G-17 or [Leu[11]]-G-13. In both cases, however, the G-17 and G-13 analogues exhibited about the same activity.[386]

A number of radioimmunoassays of gastrin as well as their clinical use have been reported.[387–390] In a study on gastrin heterogeneity and its biological significance it has been found that the most abundant form of gastrin in gastric tissue extracts is G-17, accounting for 90—95% of extractable gastrin. In peripheral blood however G-34 is more abundant than G-17. One reason for this might be the slower disappearance of G-34 from the circulation.[391] Immunochemical studies on macromolecular gastrins have demonstrated that there are no detectable true macromolecular gastrins ('big big gastrins') in serum and non-neoplastic gastrointestinal mucosa. True gastrin macromolecules consisting of G-17 covalently coupled in its amino-terminus to proteins of varying length however are present in detectable amounts in some large gastrinomas.[392]

The distribution and molecular forms of gastrin in human foetuses have been reported and an important role of the hormone in the early development of the gastrointestinal tract suggested.[393]

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In contrast to human blood, cat blood seems to contain a converting enzyme capable of rapid conversion of G-17 into G-14.[394]

Gastrin in its molecular heterogeneity has also been identified in amphibians.[395]

Further evidence for separated stimulatory receptors for gastrin and histamine was presented.[396] On the other hand, however, results in favour of the hypothesis that gastrin stimulates acid secretion indirectly, *via* histamine secreting cells, were reported.[397]

A possible explanation for the 'fade' phenomenon observed in gastric acid secretion by continuous i.v. infusion of pentagastrin is that pentagastrin releases 5-hydroxytryptamine which at first potentiates and then inhibits the resulting stimulated secretion.[398]

The inactivation of gastrin has been studied *in vitro* by incubation with a fraction of rat small bowel mucosa and *in vivo* by perfusing gastrin through the small bowel vascular bed in dogs. In both cases there was a highly significant loss in the bioactivity of gastrin, but no significant change in its immunoreactivity indicating a subtle chemical change in the *C*-terminal tetrapeptide.[399]

The half-life of endogenous gastrin in dogs was calculated to be 8.62 min. This disappearance rate could be explained by two distinct half-lives: one of 2.8 min (which is similar to the half-time in dogs of both G-14 and G-17) and another of 15.4 min (which is similar to the half-time of G-34). As physiologically released gastrin is a mixture of three molecular forms the half-life of 8.62 min probably represents the disappearance half-time of this mixture.[400]

An autoradiographic light-microscopic study with ^{14}C-labelled pentagastrin has in principle confirmed earlier results on its metabolism.[401]

Cholecystokinin–Pancreozymin (CCK).—(Figure 1.) The previously reported synthesis of [Tyr^{28}]-CCK and [^{125}I-Tyr^{28}]-CCK [402] was reviewed in connection with structure–activity studies of other gastrointestinal hormones.[403] The central 9—20 CCK-dodecapeptide sequence was synthesized for immunological purposes,[404] and the protected heptapeptide hydrazide corresponding to the sequence 17—23 of CCK was prepared as fragment for the total synthesis of CCK.[405] The *C*-terminal octapeptide of CCK (OP-CCK) was labelled with tritium in the tryptophan residue.[406] An analogue of the *C*-terminal heptapeptide in which the

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[406] J. L. Morgat, J. P. Girma, and P. Fromageot, in ref. 371, p. 43.

tyrosine *O*-sulphate residue was replaced by serine *O*-sulphate showed only 0.4% of the biological potency of the parent compound when tested *in vitro* by measuring calcium outflux from dispersed acinar cells. This activity was less than that of the unsulphated parent heptapeptide reported to be about 1%.[407] Structure–activity relationship has been studied in a series of caerulein-like peptides closely related to the *C*-terminal sequence of CCK.[408]

A reliable method for generating antibodies against CCK has been reported [409] and antisera thus obtained were used for the development of a radioimmunoassay and for the immunohistochemical identification of CCK-producing cells in the human duodenum. Concentrations of immunoreactive CCK were determined in plasma samples of humans under various conditions using radioimmunoassay technique.[410]

The previously suggested presence of CCK peptides in brain [411] has been confirmed by the detection of two compounds resembling intact CCK and OP-CCK in extracts from pig brain [412] and of material with the immunoreactivity of OP-CCK in rabbit cerebral cortical neurons.[413] CCK-like immunoreactivity has also been observed in the brain and gastrointestinal tract of amphibians and in the gastrointestinal mucosa of teleost fish. The existence of a common evolutionary origin of gastrin and CCK has been postulated based on immunohistochemical evidence.[414] The presence of CCK-containing cells in mammalian gut has been verified by immuno-staining techniques using a highly specific CCK antiserum raised against the dodecapeptide sequence 9—20, which does not occur in any other known gastrointestinal hormone.[404] It has been demonstrated with the use of an *in vitro* assay that almost 80% of the CCK activity in the rat gut is found in the proximal third of the small intestine.[415] The effects of CCK, OP-CCK, and various analogues partially in combination with other gastrointestinal hormones on cAMP, cGMP, and on adenylate cyclase were studied in different *in vitro* [416–421] and *in vivo* [419, 422] systems in correlation to the different biological activities of these compounds. Binding of OP-CCK, its biologically fully active *N*-acetyl-derivative, and gastrin to cat gallbladder tissue was investigated *in vitro* and the

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physiological results obtained suggested that OP-CCK and gastrin act on a common receptor site.[423] Exocrine pancreatic tissue reacted under photolysis with the photoaffinity label 2-nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2, a mimetic of OP-CCK and caerulein. The resulting irreversible stimulation of protein discharge was indistinguishable from that observed with the most potent doses of caerulein.[424] Results from testing CCK, OP-CCK, gastrin II, and the corresponding desulphated derivatives indicated that the gallbladder of coho salmon contains relatively primitive CCK-receptors, which in contrast to mammalian ones cannot distinguish between agonists carrying the sulphated tyrosine residue in different positions to the *C*-terminus.[425] Studies on an isolated dog pancreas perfused *ex vivo* demonstrated that CCK is mainly responsible for the output of the enzymatic proteins while secretin stimulates predominantly the flow of the pancreatic juice.[426]

The role of CCK or OP-CCK with regard to satiety was studied in rats,[427–431] monkeys,[431] and man.[432] Since the results are somewhat controversial the question whether CCK serves as endogenous satiety signal still remains open. The effects of CCK or OP-CCK on the pressure of the lower oesophageal sphincter,[433] on intestine motility,[434–436] and on water absorption and motility of the gallbladder [437] were investigated. A comparison of the vascular effects of gastrointestinal hormones on various organs indicated that CCK may contribute to postprandial intestinal hyperemia.[438] Studies in humans revealed that CCK stimulates proportionally more the secretion of trypsinogen and lipase than that of amylase.[439] The release of immunoreactive somatostatin from the pancreas by CCK [440] as well as the inhibition of CCK release by somatostatin [441] has been demonstrated. It has been concluded from the inability of the unsulphated *C*-terminal hepta- and octapeptide of CCK either to stimulate hepatic bile flow in the dog or to antagonize sulphated OP-CCK in this system that the receptor in the biliary system is highly specific and requires sulphation of peptides for binding whereas the receptor in the stomach has little specificity and combines with both types of peptides.[442] The existence of a humoral factor with anti-CCK-activity

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with regard to pancreatic secretion has been postulated based on results obtained after intra-ileal infusion of oleic acid in parabiotic rats.[443]

Secretin.—(Figure 2, Table 9.) A new synthesis of porcine secretin has been published.[444] Several secretin peptide fragments [445, 446] and a number of secretin analogues have been synthesized in order to study structure–activity relationships [447, 448] immunological reactivities,[448–451] hormone–receptor interactions,[452] or to get compounds which can be iodinated in a much easier and more reproducible way.[448, 450, 451]

It seems that the secretin molecule is extremely sensitive to conformational changes in the amino-terminal region, since minor modifications are accompanied by a drastic loss of biological activity,[447, 448] immunological activity,[448] and receptor affinity.[452] In a comprehensive study on structure–activity relationships of the secretin family hormones, *i.e.* secretin (S), glucagon (GLU), vasoactive

	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)*

intestinal peptide (VIP), and gastric inhibitory peptide (GIP), a number of 'mixed hormones' have been synthesized by replacing the amino-terminal sequence 1—5 of secretin by the corresponding sequences 1—5 of GLU, VIP, and GIP thus yielding the secretin analogues [Gln^3]secretin (GLU-S), [Ala^4, Val^5]-secretin (VIP-S), and [Tyr^1, Ala^2, Glu^3]secretin (GIP-S).[453] The different biological potencies as measured in an *in vitro* adenylate cyclase stimulation assay were explained by variations in hydrophobic character and electric charge.

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[453] E. Wünsch, E. Jaeger, L. Moroder, and I. Schulz, ref. 371, p. 19.

Table 9 *Analogues of secretin*

Structure	*Test*	*Biological activity (%)*	*Ref.*
Natural porcine secretin	*a*	100	448
Synthetic porcine secretin	*a*	107.5	448
Tyr-secretin	*a*	25.6	448
Desamino-Tyr-secretin	*a*	4.1	448
[Tyr^1]secretin	*a*	4.3	448
[Asn^3]secretin	*a*	0.5	447
[Gln^3]secretin (GLU-S)	*a*	0.1	447
[Gln^3]secretin (GLU-S)	*b*	30	453
[Glu^3]secretin	*a*	2.2	447
[Ala^4]secretin	*a*	< 2.5	447
[D-Ala^4]secretin	*a*	8.1	447
[D-Ala^4]secretin	*a*	36.8	454
[Ala^4, Val^5]secretin (VIP-S)	*a*	9.8	454
[Ala^4, Val^5]secretin (VIP-S)	*b*	90	453
[Tyr^1, Ala^2, Glu^3]secretin (GIP-S)	*b*	50	453
Des-Tyr^1-secretin	*a*	inactive	454

[a] Biological activity assessed *in vivo* by measuring the exocrine pancreatic juice flow. [b] Biological activity assessed *in vitro* by measuring pancreatic adenylate cyclase stimulation potency.

The essential character of the carboxyl group of Asp^3 is confirmed by another study that found [Asn^3]- and [Gln^3]secretin to be almost inactive in an *in vivo* assay measuring pancreatic juice flow.[447]

The weak activity of [β-Asp^3]- and [Glu^3]secretin shows that not only the carboxy group but also its position is important.[447] For [Ala^4]secretin a biological activity of 2.1% was reported [447, 454] and for [D-Ala^4]secretin values of 8.1%[447] and 36.8%[454] were published. The His^1 residue seems to be of special importance for biological activity. From earlier investigations it is known that Des-His^1-secretin (S_{2-27}) is practically inactive.[455]

Amino-terminal elongation of secretin by the tyrosyl residue makes the activity drop to one-fourth.[448] Omission of the amino group of this tyrosyl residue in β-(4-hydroxyphenyl)-propionylsecretin leads to a further marked loss of activity.[448] Finally, the low but definite activity of [Tyr^1]secretin in contrast to the practically inactive S_{2-27} suggests that the aromatic property of the His residue in the *N*-terminus may in part contribute to the physiological activity of secretin.[448] Compared to secretin the Tyr^6 analogue shows a biological activity of only 1% and a considerably weakened immunoreactivity [448] indicating the importance of Phe^6. That is the reason why [^{125}I-Tyr^6]secretin is not suitable as a tracer for the immunoassay of secretin and therefore Tyr-secretin,[448] [Tyr^1]secretin,[448] β-(4-hydroxyphenyl)propionylsecretin,[448] and β-(4-hydroxyphenyl)propionyl-β-alanyl-secretin,[450] have been synthesized for the preparation of the corresponding radioactive tracers. The significant contribution of the Phe^6 residue to the interaction between secretin and its particular antiserum is also obvious when comparing the immunological reactivity of S_{4-27} and S_{5-27} on the one hand and S_{7-27} on the other hand.[448] Substitution of the acidic residues at positions 9 and 15 of

[454] N. Yanaihara, M. Sakagami, H. Sato, K. Yamamoto, T. Hashimoto, C. Yanaihara, Z. Ito, K. Yamaguchi, and K. Abe, *Gastroenterology*, 1977, **72**, 803.

[455] A. Bodanszky, M. A. Ondetti, V. Mutt, and M. Bodanszky, *J. Amer. Chem. Soc.*, 1969, **91**, 944.

S_{5-27} by the neutral amino-acids glutamine and asparagine resulted in a decrease in immunoreactivity which might correlate with conformational changes.[449]

Several methods for the radioimmunological determination of ^{125}I-secretin,[451, 456–458] [^{125}I-Tyr^6]secretin,[451] and ^{125}I-desaminotyrosyl-β-alanylsecretin (DATA-secretin)[451] have been elaborated. ^{125}I-DATA secretin showed, with several rabbit anti-secretin sera, superior immunoreactivity compared to [^{125}I-Tyr^6]secretin and to secretin iodinated at its *N*-terminal histidyl residue, and therefore appears to be most suitable as tracer for a sensitive secretin radioimmunoassay.[451] The radioiodination and distribution of [Tyr^6]secretin in normal rats have been studied.[459]

Plasma secretin immunoreactivity was related to administration of pentagastrin[460] as well as to pancreatic bicarbonate response to exogenous secretin in humans.[461]

In further studies the effect of secretin on plasma motilin in man[462] and on renal blood flow, interstitial pressure, and sodium excretion in dogs[463] was investigated.

The measurement of serum gastrin concentration after intravenous injections of secretin proved to be useful in the diagnosis of the Zollinger–Ellison syndrome.[464]

Secretin plasma levels were found to be elevated in patients with diabetes mellitus.[465]

Vasoactive Intestinal Peptide (VIP).—(Figure 2.) The isolation, purification, amino-acid sequence, and biological activity of VIP from fowl,[466] the synthesis of porcine VIP and of the corresponding derivative labelled with radioactive iodine have been described in patent reports.[467] The methyl ester of the *N*-terminal decapeptide of VIP was synthesized in solution by the stepwise strategy. This fragment showed about half of the potency of the *C*-terminal fragment VIP_{18-28} in the smooth muscle assay. However, its effect on blood flow was stronger than that of fragments VIP_{18-28} or VIP_{15-28}.[468] The usefulness of diphenyl phosphorazidate as coupling reagent was demonstrated in syntheses of the *N*-terminal hexapeptide sequence by fragment condensation and stepwise approach.[469]

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A review on structure, distribution, receptor-binding, as well as pharmacological and physiological activities of VIP, has been published.[470] The biological and immunological activities of various VIP-fragments were studied.[454] From the cross-reactivities of fragments VIP_{7-28}, VIP_{11-28}, VIP_{18-28}, VIP_{1-18}-amide and VIP_{1-22}-amide in a radioimmunoassay system using antiserum raised against synthetic VIP and ^{125}I-VIP as tracer it has been concluded that VIP carries antigenic sites in the *N*- and *C*-terminal region. The concentration of material with VIP-like immunoreactivity was determined in extracts from various parts of the intestine including pancreas and from pituitaries.[454] The concentration of VIP was seven-fold higher in cerebrospinal fluid [471] than in plasma of humans [472] when measured by radioimmunoanalysis. A radioreceptorassay for VIP was developed and has been used to determine the concentration of VIP in the intestine of developing rats.[473] The same technique and radioimmunoassay were used for measuring VIP in various parts of the intestine of neonates and premature infants [474] and in rats.[475]

Evidence for the possible role of VIP as neurotransmitter or modulator is steadily increasing by the results of immunocytochemical and radioimmunological studies. In the reporting period VIP was localized in nerves of the female genito–urinary tract,[476] in various parts of the brain and here especially in the synaptosomal fraction,[477, 478] in granules of the terminals of p-type neurons,[479] and in nerve fibres of the earthworm.[480] A neuroendocrine function of VIP has also been postulated from the presence of VIP-positive terminals in respective brain areas.[481] Binding of VIP, and in some investigations also ofVIP-fragments, to receptors, activation of the adenylate cyclase system, and the effects of other gastrointestinal hormones on these processes were studied in different cell and membrane preparations,[420, 452, 482–486] The effects of VIP on colonic transport [487, 488] and on secretion of hydrolase from pancreatic fragments [489] in view of

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[483] M. Bodanszky, ref. 371, p. 13.
[484] D. Bataille, J. Besson, C. Bastard, M. Laburthe, and G. Rosselin, ref. 371, p. 113.
[485] J. D. Gardner, J. Christophe, P. Robberecht, and T. P. Conlon, ref. 371, p. 227.
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its action on adenylate cyclase have been reported. A VIP-sensitive adenylate cyclase isolated from guinea-pig brain has been characterized.[490]

It has been found that VIP, like secretin, inhibits fluid absorption and induces secretion in the isolated gallbladder of the guinea-pig.[491] Since, in an *in vitro* study, insulin and glucagon were released from rat pancreas by the addition of VIP in very low concentrations, the possibility of a physiological function of VIP on the endocrine pancreas has been suggested.[492] Similar conclusions have been drawn from *in vivo* experiments using intrapancreatical infusions of VIP.[493, 494] A local control of pancreatic endocrine function by VIP has been suggested, based on results of experiments investigating the influence of glucose concentration on VIP-induced release of glucagon and insulin.[495] The effect of VIP on pancreatic juice flow and bicarbonate output has been studied in comparison to secretin in man [496] and cats.[497] It was observed that VIP acts in man only as a partial secretin-like agonist, while it is a full agonist in the cat, although at a ten times higher dose than secretin. Since VIP decreases basal gallbladder pressure and antagonizes the pressure response of an infusion of CCK in the opossum, it was suggested that the regulation of gallbladder motor function depends on the interaction of the gastrointestinal hormones.[498] The role of VIP as mediator of increased vascular permeability has been reviewed.[499]

Increases of the plasma levels of immunoreactive VIP were observed after electrical stimulation of vagal nerves,[500] intraduodenal infusion of hypertonic saline,[501] of hydrochloric acid, fat, and ethanol,[502] and after i.v. application of calcium [503] indicating that VIP is released under these conditions.

Since no significant difference with regard to serum levels of VIP and inhibition of pentagastrin-stimulated acid secretion was observed with dogs, whether VIP was given by portal or systemic infusions, it has been concluded that the liver does not play a major role in the inactivation of VIP.[504] However, similar studies in cats, measuring the effect of VIP on pancreatic secretion, showed a dose-dependent decrease of response, when VIP was administered by the portal route.[497]

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Gastric Inhibitory Peptide (GIP).—(Figure 2.) A new synthesis of GIP by means of fragment condensation has been reported [505] and the *N*-terminal decapeptide was prepared by two alternative routes using diphenyl phosphorazidate and diethyl phosphorocyanide as coupling reagents.[506] The previously reported synthesis and biological activities of GIP [507] and of some of its partial sequences were discussed.[403] The effects of synthetic GIP and of its unprotected fragments GIP_{1-28}, GIP_{22-43}, and GIP_{15-43} on insulin and glucagon secretion were studied *in vitro* and *in vivo* and the results suggested that GIP stimulated insulin and glucagon release but that the fragments were inactive.[508]

The site of GIP release in man has been studied by perfusing different parts of the intestine with glucose and measuring the GIP concentration in the perfusate by radioimmunoassay technique. The results indicated that the proximal small intestine was the primary site of GIP release but that small quantities were also released by the distal small bowel.[509] Quantitative immunocytochemical studies revealed that perfusion of the duodenum loops with 0.1N-HCl had no influence on the number of GIP, motilin, and gastrin cells, but that after acidification of the duodenum the number of secreting cells decreased significantly.[510]

The influence of GIP on glucose or otherwise induced insulin release [511, 512] and on adenylate cyclase [482] has been studied in isolated pancreatic islet preparations.

It has been found the CCK, gastrin, and pentagastrin augment glucose-stimulated GIP secretion [513] and that glucagon inhibits GIP release.[514] The glucose-induced secretion of GIP was studied in diabetics in relation to its action on insulin and glucagon release.[515] The effects of GIP on human jejunal water and electrolyte transport were investigated.[516] It has been suggested that the kidney may be an important site for the removal of GIP from the circulation.[517]

Motilin.—(Figure 3.) Syntheses of motilin by the solid-phase technique [518] and the fragment condensation approach [519] and of [Leu^{13}, Glu^{14}]motilin [520] by the latter procedure have been described in patent reports. Various approaches to

[505] M. Kubota, *Japan. Kokai*, 77/10 273 (*Chem. Abs.*, 1977, **87**, 53 598).
[506] Y. Hamada, S. Rischi, T. Shioiri, and S. Yamada, *Chem. and Pharm. Bull. (Japan)*, 1977, **25**, 224.
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[515] S. A. Ross, J. C. Brown, and J. Dupré, *Diabetes*, 1977, **26**, 525.
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[518] S. Yamada, *Japan. Kokai* 77/39 670 (*Chem. Abs.*, 1977, **87**, 85 248).
[519] S. Mihara and F. Shimizu, *Japan. Kokai* 77/73 863 (*Chem. Abs.*, 1977, **87**, 202 131).
[520] E. Wünsch, G. Wendlberger, and E. Jaeger, *Ger. Offen.*, 2 544 348.

the synthesis of motilin based on the example of two known and one new synthesis were discussed.[403] The *N*-terminal hexadecapeptide was prepared in conventional manner and exhibited a biological activity of 3% of that of synthetic motilin when tested *in vitro* on rabbit duodenal muscle.[521] That the *N*-terminal region is also

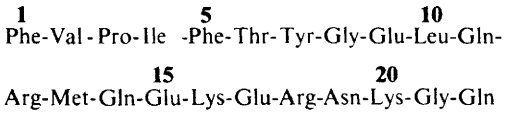

Figure 3 *The sequence of porcine motiline*

important for the exhibition of biological activity can be seen from the results of motilin fragments which lack residues in this part. Fragment M_{6-22}, for example, showed only 2% of the activity of that of motilin and fragment M_{9-22} was inactive.[403]

Motilin has been localized at the ultrastructural level in enterochromaffin cells of the rabbit bile tract with the use of an immunoelectron cytochemical technique.[522] The pharmacokinetic behaviour of motilin has been studied in man and disappearance half-lives of 4.36 min and 4.56 min were measured for exogenous [Nle^{13}, Glu^{14}]motilin and endogenous motilin, respectively.[523]

Two reviews dealing with the biological role of motilin have been published.[524,525] The effects of motilin or its equally potent [Nle^{13}, Glu^{14}]motilin analogue [526] on gastric motor-activity,[527] on gastric emptying,[528] on gastrointestinal myoelectric activity,[529] on gastric secretion, serum gastrin levels, and mucosal blood flow,[530] and on the lower oesophageal sphincter [531] have been studied. It has been found that infusions of secretin [462] and insulin [532] and oral administration of glucose [533] result in decreased plasma levels of motilin.

Urogastrone.—(Figure 4.) The primary structure of urogastrone, a potent inhibitor of gastric acid secretion present in human urine, has been elucidated. Two separate polypeptides, β- and γ-urogastrone, have been isolated. β-Urogastrone is a 53-amino-acid residue polypeptide containing three disulphide bonds, and γ-urogastrone has an identical sequence but lacks the *C*-terminal

[521] K. Ueda, K. Kitagawa, T. Akita, S. Honma, T. Segawa, Y. Kai, Y. Mori, and H. Yajima, *Chem. and Pharm. Bull. (Japan)*, 1977, **25**, 2123.
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[524] W. Domschke, *Amer. J. Dig. Dis.*, 1977, **22**, 454.
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[529] D. L. Wingate, H. Ruppin, H. H. Thompson, W. E. R. Green, W. Domschke, E. Wünsch, L. Demling, and H. D. Ritchie, *Acta Hepato-Gastroenterol.*, 1977, **24**, 278.
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[532] Y. Seino, K. Mori, T. Denko, T. Chiba, S. Matsukura, H. Imura, N. Yanaihara, *Igaku No Ayumi*, 1977, **102**, 757 (*Chem. Abs.*, 1978, **88**, 651).
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arginine residue. Urogastrone is structurally related to mouse epidermal growth factor in that 37 of the 53 residues are commonly located in each polypeptide. As both peptides have similar effects upon gastric acid secretion and epidermal growth, urogastrone is also a human epidermal growth factor. The smallest biologically active unit has not yet been defined but at least six residues can be removed from the *C*-terminus without causing a reduction in potency.[534] A method to purify urogastrone has been published [535] and the absorption of urogastrone from rat intestine was investigated.[536]

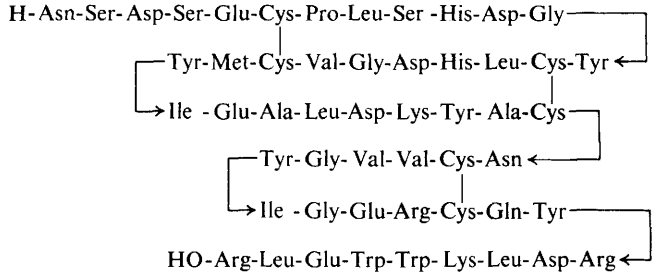


Figure 4 *The amino-acid sequence of β-urogastrone*

5 Vasoactive Peptides

Contributed by P. D. Roy

Bradykinin.—Reviews have covered bradykinin inhibitors and potentiators,[537] inhibitors of kinin-forming enzymes,[538] the inactivation of bradykinin in the pulmonary circulation,[539] and kallikreins and kinins in hypertension.[540]

Table 10 lists bradykinin analogues with available biological data which have appeared in publications during 1976 and 1977. Structure–activity relationship studies have examined the effect of variations in the side-chains of amino-acids in the 1-, 5-, 8-, and 9-positions, and of reduction in chain length. In the main these modifications have diminished bradykinin activity, the only exception being *para*-halogenated phenylalanine analogues. There are no analogues reported to antagonize bradykinin activity.

The importance of the side-chains of the arginine residues in positions 1 and 9 has been further demonstrated. Separate or simultaneous replacement of the terminal residues by 4-guanidinophenyl-L-alanine gives analogues (255), (258), or (286) with only 1—3% activity of the parent peptide on isolated guinea-pig ileum. Activity of the monosubstituted analogues (255) and (286) on the isolated rat uterus was 2 and 0.1% respectively, whilst the bis-substituted analogues (258) was

[534] H. Gregory and B. M. Preston, *Internat. J. Peptide and Protein Res.*, 1977, **9**, 107.
[535] H. Yajima, *Japan. Kokai* 76/110 013 (*Chem. Abs.*, 1977, **86**, 8617).
[536] R. Hori, K. Okumura, K.-I. Inui, N. Nakamura, A. Miyoshi, and T. Suyama, *Chem. and Pharm. Bull. (Japan)*, 1977, **25**, 1974.
[537] 'Fogarty International Center Proceedings, No. 27', ed J. J. Pisons and K. F. Austen, U.S. Government Printing Office, Washington, 1976, pp. 287—294.
[538] S. Fujii, *Adv. Exp. Med. Biol.*, 1976, **70**, 75.
[539] J. M. Stewart, *Agents Actions*, 1976, **6**, 494.
[540] H. R. Croxatto, R. Albertine, J. Corthorn, and R. Rosas, in 'Hypertension: Physiopathology and Treatment', ed. J. Genest, E. Koiw, and O. Kuchel, McGraw-Hill, New York, 1977, pp. 364—73.

Table 10 *Analogues of bradykinin*

1 5 9
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Bradykinin

Compound number	*Structure*[a]	*Biological activity*[b]	*Test*[c]	*Ref.*
255	[Phe(4-Gu)^1]	++	A	541
		++	B	541
256	[Nar^1]	+	A	543
		+	B	543
		+	C	543
257	[Nar ^{1,9}]	−	A	543
		−	B	543
		−	C	543
258	[Phe(4-Gu)^{1,9}]	−	A	541
		++	B	541
259	[Phe(4-F)^5]	++++	D	546
260	[Phe(4-Cl)^5]	++/+++	D	546
261	[Phe(4-Br)^5]	++/+++	D	546
262	[Phe(4-I)^5]	++/+++	D	546
263	[Trp^5, Gly^6]	+	B	542
264	[Trp^5, Tyr^8]	−	B	542
265	[Trp^5, Gly^6]-(2-9)	−	B	542
266	[Trp^5, Gly^6]-(5-9)	−	B	542
267	[Trp^5, Tyr^8]-(2-9)	−	B	542
268	[Trp^5, Tyr^8]-(5-9)	−	B	542
269	[Tyr^5, Gly^6]	+	A	548
		−	B	548
270	[Gly^6, Tyr^8]	+	A	548
		+	B	548
271	[Tyr^{5,8}, Gly^6]	−	A	548
		−	B	548
272	[Gly^6, MePhe^8]			549
273	[Gly^6, Chl^8]			549
274	Lysyl[Gly^6, Phl^8]			549
275	Phe(4-F)^8	++++	D	546
276	Phe(4-Cl)^8	++++	D	546
277	Phe(4-Br)^8	++++	D	546
278	Phe(4-I)^8	+++	D	546
279	[MePhe^8]			
280	[HPh^8]	+	A	545
		−	B	545
		+	C	545
281	[Gly(Phe)^8]	−	A	544
		−	B	544
		−	C	544

[541] S. Moore, H. D. Law, D. E. Brundish, D. F. Elliott, and R. Wade, *J.C.S. Perkin I*, 1977, 2025.
[542] S. Reissmann, H. Arold, M. P. Filatova, and H. Feist, *J. Prakt. Chem.* 1977, **319**, 987.
[543] H. Arold and D. Gersch, *J. Prakt. Chem.*, 1976, **318**, 336.
[544] H. Arold, R. Reissmann, and S. Reissmann, *J. Prakt. Chem.*, 1976, **318**, 441.
[545] H. Arold, S. Reissmann, and D. Orzschig, *J. Prakt. Chem.*, 1976, **318**, 420.
[546] G. H. Fisher, A. Chung, and J. W. Ryan, *Circulation*, 1977, **56**, 241.
[547] S. Reissman, I. Paegélow, and H. Arold, *Acta Biol. Med. Germanica*, 1974, **63**, 117.
[548] S. Reissmann, M. P. Filatova, T. O. Reutova, H. Arold, and V. T. Ivanov, *J. Prakt. Chem.*, 1976, **318**, 429.
[549] N. A. Krit, G. A. Ravdel, and V. T. Ivanov, *Bio-org. Khim.*, 1976, **2**, 1455.

Table 10 (*cont.*)

Compound number	*Structure*[a]	*Biological activity*[b]	*Test*[c]	*Ref.*
282	[Gly(Phe)^8, Nar^9]	−	A	544
		−	B	544
		−	C	544
283	[Gly(Phe)^8, Har^9]	−	A	544
		+	B	544
		−	C	544
284	[HPh^8, Nar^9]	+	A	545
		−	B	545
		−	C	545
285	[HPh^8, Har^9]	+	A	545
		−	B	545
		−	C	545
286	[Phe(4-Gu)^9]	+	A	541
		++	B	541
287	[Nar^9]	++	A	543
		++	B	543
		++	C	543
288	carbohydrate carbohydrate | | Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-bradykinin	++++	D	551
289	carbohydrate carbohydrate | | Thr Thr Arg Arg-ArgGly-bradykinin			551

[a] Chl = β-cyclohexyl-L-lactic acid, Phl = phenyl-lactic acid. [b] Symbols for potency are used as in Table 7. [c] A, Rat uterus; B, guinea-pig ileum; C, rabbit blood pressure; D, rat blood pressure.

essentially inactive in this sensitive system. Loss in activity is believed to be associated with the rigidity of the benzenoid side-chains in positions 1 and/or 9 which prevents the folding required for the presentation of the guanidino-group to the receptor. Interesting arginine side-chain modifications of intermediate flexibility have been proposed in order to test this hypothesis. The three analogues (255), (258), and (286) were inactive when tested as antagonists.[541]

Synthesis of 1- and/or 9-norarginine bradykinin analogues (256), (257), and (287) has been described[543] following the previous publication of biological activities.[547] Shortening of the arginine side-chain by one CH_2 group results in substantial reduction of activity presumably due to difficulty in simultaneous binding of all essential groups to the receptor.

Similarly, the synthesis has been described of six bradykinin analogues (280)—(285) in which either the 8-phenyl group alone or both the 8-phenyl and 9-guanidino groups are varied in length by one CH_2 from the peptide backbone.[544, 545]

The generally poor bradykinin activity of these derivatives has been reported earlier.[547] It is of some interest that the homophenylalanine analogues (280) and

[550] G. A. Popkova, M. V. Astapova, I. Yu. Lisunkin, G. A. Ravdel, and N. A. Krit, *Bio-org. Khim.*, 1976, **2**, 1606.

[551] H. Yoshida, R. G. Geller, and J. J. Pisano, *Biochemistry*, 1976, **15**, 61.

(285) do not retain activity which differs from the case of the 1- and/or 9-homoarginine analogues where side-chain extension is consistent with activity.

The effect of substituting phenylalanine in position 5 of bradykinin by an alternative hydrophobic residue, tryptophan, has been studied. The [Trp^5, Gly^6]-(263) and [Trp^5, Tyr^6]-(264) analogues of bradykinin retain only 0.15% and 0.05% activity, respectively, on guinea-pig ileum. Analogues (264) with modifications in the 5- and 8-positions showed no inhibitory activity. The omission of *N*-terminal arginine or *N*-terminal tetrapeptide sequence from either (263) or (264) gave partial sequences (265) to (268) with no bradykinin activity.[542]

In contrast replacement of phenylalanine in position 8 by *para*-halogenated phenylalanines (F-, Cl-, Br, or I-Phe) affords analogues (275)—(278) which retain high bradykinin activity (50—360% on rat blood pressure). In addition [F-Phe^5]-bradykinin (259) is also highly active (143%), but the other halogeno-Phe^5 analogues (260)—(262) show relatively decreased blood pressure effects (2—45%). It is suggested therefore that Phe^5 is more critical than Phe^8 for hormone–receptor interaction.[546]

Apart from compound (263) a number of other derivatives of 6-glycine bradykinin have been described. The high order of activity of 6-glycine bradykinin is again largely lost on further substitution of phenylalanine by tyrosine in positions 5 and/or 8, as in the analogues (269), (270), and (271).[548] Other 6-glycine bradykinin analogues modified in position 8 by *N*-methylphenylalanine (272), β-cyclohexyl-lactic acid (273), or phenyl-lactic acid (274) (with the *N*-terminus also substituted by lysine) have also been reported.[549, 550]

Structurally related bradykinin-like peptides (288) and (289), termed Vespulakinins 1 and 2 respectively, have been isolated from the venom of wasps of the genera *Vespula*. They are the first reported naturally occurring vasoactive glycopeptide derivatives of bradykinin. Both are extremely basic and (288) is at least twice as potent as bradykinin in lowering rat blood pressure.[551] It would be interesting to know whether the hypotensive action of these peptides is enhanced by other pharmacological effects in particular histamine release from mast cells. Bradykinin and other basic peptides from the venom of wasps and bees are known to be potent releasers of histamine from rat mast cells *in vitro*. The extent to which the carbohydrate prosthetic groups contribute to activity is not known.

The sequence of residues preceding the bradykinin moeity in high molecular weight bovine kininogen is reported to be Ser-Leu-Met-Lys. This differs from the tetrapeptide sequences Ser(and Gly)-Arg-Met-Lys identified by other investigators as being contiguous with the kinin moeity. The possibility remains that different kininogens are involved in these studies.[552]

Angiotensin.—Reviews have covered structure–activity studies of antagonists of the renin–angiotensin system,[553] newer angiotensin II agonists and antagonists,[554] angiotensin II antagonists as diagnostic and pharmacological tools,[555] the clinical

[552] Y. N. Han, H. Kato, and S. Iwanaga, *F.E.B.S. Letters*, 1976, **71**, 45.
[553] G. R. Marshall, *Fed. Proc.*, 1976, **35**, 2494.
[554] F. M. Bumpus, *Fed. Proc.*, 1977, **36**, 2128.
[555] W. A. Pettinger and H. C. Mitchell, *Prog. Biochem. Pharmacol.*, 1976, **12**, 203.

pharmacology of 'Saralasin',[556] the renin–angiotensin system,[557] and angiotensin III (des-Asp'-angiotensin II),[558] among other related topics.[559, 560, 561, 562]

Table 11 lists new angiotensin* analogues within their biological activities reported during 1976 and 1977. Structure–activity relationship studies have continued with the objective of finding potent antagonists of the pressor and myotropic activity of the parent hormone showing no or low residual pressor activity, and with an increased *in vivo* half-life. For purposes of longer term administration, clinically used angiotensin antagonists should be either devoid of these limitations or sufficiently potent to be used at dose levels that do not induce other responses.

Table 11 *Analogues of angiotensin*

1 5 8
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Angiotensin II

Compound number	*Structure*[a]	*Biological activity*[b] *Pressor*	*Other test*[c]	*Ref.*
290	[Sar^{1,2}, Thr^{8}]	−	C, D	567
291	[Asn^{1}, aza-α'-homo-L-Val^{3}, Val^{5}]	+++	D	573
292	[Asn^{1}, aza-α'-homo-Tyr^{4}, Val^{5}]	+++		573
293	[Asn^{1}, aza-α'-homo-D-Tyr^{4}, Val^{5}]	+++		573
294	[Sar^{1}, MeTyr^{4}, Ile^{8}]	+ *	C, D	566
295	[Sar^{1}, MeIle^{5}, Ile^{8}]	+ *		566
296	[Sar^{1}, MeIle^{5,8}]	−*		566
297	[Sar^{1}, Thr(Me)^{5}, Ile^{8}]	++ *		567
298	[Sar^{1}, Thr(Me)^{5,8}]	+ *		567
299	[Sar^{1}, Val^{5}, Val(α-Me)^{8}]	− *	A	568
		− *	D	568
300	[Suc^{1}, Val^{5}, Ala^{7}]			572
301	1-DL-BzlMal-[retroenantio-Suc^{1}, Val^{5}, Ala^{7}]	++	A, B	572
302	[Suc^{1}, Val^{5}, β-Ala^{7}]	+		572
303	1-DL-BzlMal-[retroenantio-Suc^{1}, Val^{5}, β-Ala^{7}]	+++		572
304	1-BzlMal-[retroenantio-Val^{5}][d]	− −	A, B	572
305	1-BzlMal-[retroenantio-Suc^{1}, Val^{5}][d]	− −		572
306	[Ac-Asn^{1}, Val^{5}, Mel^{8}]	*	A	570
307	[Ac-Asn^{1}, Val^{5}, Mel-OMe^{8}]	*		570
308	[Ac-Asn^{1}, Val^{5}, Mel-OBzl^{8}]	*		570
309	[Ac-Asn^{1}, Val^{5}, Hfv^{8}]	*		570
310	[Ac-Asn^{1}, Val^{5}, Hfv OEt^{8}]	*		570
311	[Ac-Asn^{1}, Val^{5}, Hfv OBzl^{8}]	*		570
312	[Ac-Asn^{1}, Val^{5}, Phe-ol^{8}]	− −		571
313	[Ac-Asn^{1}, Val^{5}, Pmk^{8}]	− −		571
314	[Ac-Asn^{1}, Val^{5}, Phe-OMe^{8}]	++		571
315	[Ac-Asn^{1}, Val^{5}, Phe(α-Me)OMe^{8}]	− −		571
316	des-Asp^{1}-[Thr^{8}]	+ *	C, D	565

[556] W. A. Pettinger and H. C. Mitchell, *Fed. Proc.*, 1976, **35**, 2521.
[557] L. T. Skeggs, F. E. Dorer, J. R. Kahn, K. E. Lentz, and M. Levine, *Amer. J. Med.*, 1976, **60**, 737.
[558] R. H. Freeman, J. O. Davis, T. E. Lohmeier, and W. S. Spielman, *Fed. Proc.*, 1977, **36**, 1766.
[559] J. P. Buckley and B. S. Jandhyala, *Life Sci.*, 1977, **20**, 1485.
[560] W. F. Ganong, *Fed. Proc.*, 1977, **36**, 1771.
[561] L. T. Skeggs, M. Levine, K. E. Lentz, J. R. Kahn, and F. E. Dorer, *Fed. Proc.*, 1977, **36**, 1755.
[562] F. M. Bumpus and M. C. Khosla, ref. 540, pp. 183—201.

* The 'term 'angiotensin' is used to refer specifically to [Asp^{1},Ile^{5}]angiotensin II; other derivatives are named with reference to this.

Table 11 (*cont.*)

Compound number	*Structure*[a]	*Biological activity*[b] *Pressor*	*Other test*[c]		*Ref.*
	NH ‖		*	E	565
317	[H_2N-C-Gly[1], Thr[8]]	+	*		565
318	[Me_2Gly[1], Thr[8]]	+	*		565
319	[MeIle[1], Thr[8]]	+	*		565
320	[Sar[1], Ser(Me)[8]]	+	*		565
321	[Sar[1], Thr(Me)[8]]	+	*		566
322	[Sar[1], Ser[8]]	+	*		566
323	[Sar[1], Met[8]]	++	*	C, D	566
324	[Sar[1], Ala(α-Me)[8]]	+	*		567
325	[Ala(α-Me)[1, 8]]	+	*		567
326	[MeAsp-NH_2[1], Ile[8]]	+	*		566
327	[Sar[1], MeIle[8]]	++	**		566
328	[Phe(α-Me)[4], Val[5]]	+++		A	568
		++		D	568
329	[Val[5], Phe(α-Me)[8]]	++++		A	568
		+++		D	568
330	N^{α}-Oct-[Leu[8]]	+	*	D	569
331	angiotensinyl-Ser-Leu	+++			574

[a] Mel = melphalan, Phe [*p*-N($CH_2CH_2Cl)_2$], Oct = n-octanoyl, Pmk = methyl ketone of phenylalanine, Hfv = hexafluorovaline, BzlMal = Benzylmalonyl $HO_2CCH(CH_2Ph)CO$—. [b] Symbols for potency are used as in Table 7; * and ** indicate inhibition (of increasing potency). [c] A, rat uterus; B, guinea-pig ileum; C, rabbit aorta; D, rat blood pressure; E, aldosterone release. [d] Activity refers to diastereoisomers with either L- or D-benzylmalonyl residue.

The antagonistic potency of 'Saralasin' ([Sar[1], Ala[8]]angiotensin) has been compared with [Sar[1], Ile[8]]angiotensin in normal subjects on various Na balances. The [Ile[8]]-antagonist was equipotent to or better than 'Saralasin' in subjects on low Na diet, but its pressor effects were greater in all Na states.[563] Comparative studies of 'Saralasin', [Sar[1], Ile[8]]- and [Sar[1], Thr[8]]angiotensin in dog suggest the latter antagonist would be best suited for clinical use.[564]

New inhibitory analogues of angiotensin with variations to both positions 1 and 8 have been described. Analogues of the antagonist [Sar[1], Thr[8]]angiotensin with replacement of position 1 by hydrogen atom (316), guanidineacetic acid (317), dimethyl glycine (318), or *N*-methylisoleucine (319) all show reduced initial pressor activity (bolus injection in rat) but are less potent as antagonists than the parent structure. Moreover, infusion studies in rat indicate that dimethylglycine in position 1 (318), contrary to other substituents, induces an increase in initial pressor activity greater than [Sar[1], Thr[8]]angiotensin.[565]

The inhibitory activities of a series of [Sar[1]]angiotensin analogues with variations in position 8 have been compared with [Sar[1], Thr[8]]angiotensin.[566] Substitution of the threonine hydroxy group by the *O*-methyl group gave an analogue (321)

[563] T. Ogihara, T. Hata, H. Mikami, T. Mandai, and Y. Kumahara, *Life Sci.*, 1977, **20**, 1855.
[564] E. L. Bravo, M. C. Khosla, and F. M. Bumpus, *Prog. Biochem., Pharmacol.*, 1976, **12**, 33.
[565] M. C. Khosla, M. M. Hall, H. Munoz-Ramirez, P. A. Khairallah, and F. M. Bumpus, *J. Medicin. Chem.*, 1977, **20**, 253.
[566] M. C. Khosla, H. Muñoz-Ramirez, M. M. Hall, R. R. Smeby, P. A. Khairallah, and F. M. Bumpus, *J. Medicin. Chem.*, 1976, **19**, 244.

with increased potency and *in vivo* half-life. Substitution of position 8 with serine (322), methionine (323), or with *O*-methylserine (320),[565] reduces antagonistic activity suggesting that branching in the side-chain of position 8, as in [Sar^1, Thr(Me)^8]angiotensin, is an important way of optimizing antagonistic activity in this series. The *in vitro* antagonistic activity of [Sar^1, Ser(Me)^8]angiotensin (320) (rabbit aortic strips) is higher than any other competitive antagonist of angiotensin. Preliminary data indicate the heptapeptide analogue (316) is almost equipotent to des-Asp^1-[Ile^8]angiotensin in inhibiting steroidogenesis induction by angiotensin or angiotensin III.[565]

The effect of isosteric replacement of Ile by Thr(Me) in position 5 of [Sar^1, Ile^8]- and [Sar^1, Thr(Me)^8]angiotensin is reported to increase significantly *in vivo* and *in vitro* antagonistic potencies of these peptides. Comparative infusion studies in rat indicate that the isosteric analogues (297) and (298) have slightly increased initial pressor activity. The analogue [Sar^1, Thr(Me)^{5,8}]angiotensin is currently the most potent antagonist of the pressor action of angiotensin in rat.[567]

Modifications employing non-mammalian amino-acids have been introduced into antagonist structures in attempts to increase the *in vivo* half-life. Earlier findings on the pattern of degradation of angiotensin in human plasma have prompted synthesis of analogues of the inhibitor [Sar^1, Ile^8]angiotensin containing *N*-methylated amino-acids at positions, 1, 4, 5, and 8.[566] Replacement of position 1 with *N*-methylisoasparagine gave an analogue (326) equipotent to the [Sar^1, Ile^8] derivative when tested in rat. Substitution of position 4 (Tyr) with MeTyr (294), or position 5 and/or 8 (Ile) with MeIle [compounds (296), (295), and (327), respectively] reduced the antagonistic properties of [Sar^1, Ile^8]angiotensin. Moreover, apart from compound (294) none of these analogues showed any enhanced duration of action. This indicates *N*-methylation at position 5 or 8 did not afford protection against proteolytic enzymes, whilst in the case of the position 4 derivative (294) *in vivo* antagonistic activity was too low for the analogue to be of sufficient interest. Compounds (294) and (296) exhibited reduced initial pressure activity compared to [Sar^1, Ile^8]angiotensin, but again were less potent antagonists. Substitution of analine in [Sar^1, Ala^8]angiotensin by α-methylalanine has given an analogue (324) with increased *in vivo* antagonistic activity. Simultaneous replacement of position 1 with α-methylalanine to give compound (325) reduced activity.[567] A similar approach has led to the antagonist [Sar^1, Val^5, Val(α-Me)^8]angiotensin (299) which was found to be more potent *in vivo* than the corresponding [Sar^1, Val^{5,8}] derivative.[568] In general however the above α-methyl-substituted analogues are of lower potency than [Sar^1, Ile^8]- or [Sar^1, Thr^8]angiotensin.

Substitution of arginine by sarcosine in position 2 of [Sar^1, Thr^8]angiotensin gave an equipotent analogue (290) with prolonged duration of action (*ca.* 3 h) *in vitro*. Compound (290) showed poorer activity *in vivo*, presumably not as a result of increased degradative action.[567]

An alternative attempt to obtain longer acting angiotensin antagonists has been

[567] M. C. Khosla, H. Muñoz-Ramirez, M. M. Hall, P. A. Khairallah, and F. M. Bumpus, *J. Medicin. Chem.*, 1977, **20**, 1051.

[568] J. Turk, P. Needleman, and G. R. Marshall, *Mol. Pharmacol.*, 1976, **12**, 217.

to increase the duration of peptide in the biophase by introducing lipophilic moieties into the structure.[569]

Contrary to expectations, the antagonistic activity of [Leu^8]angiotensin was not prolonged on addition of the n-octanoyl residue to give analogue (330) indicating that this parameter was not rate-limiting at the receptor level. Lipophilic antagonists like compound (330) may be useful for depot administration. In this regard the release of angiotensin antagonists is reported to occur over a 6—8 h period when administered in a lipophilic vehicle such as linseed oil.[567]

Replacement of Phe^8 in [Ac-Asn^1, Val^5]angiotensin by the potential alkylating moiety melaphan (Mel) or its methyl or benzyl esters gave analogues (306), (307), and (308) respectively, which showed potent antagonistic activity *in vitro*. Interestingly, the antagonism of the melaphan analogues was completely reversible. The corresponding series of analogues (309), (310), and (311) containing derivatives of the hydrophobic residue hexafluorovaline (Hfv) in position 8 are likewise good antagonists. The latter series are potentially useful as ^{19}F n.m.r. probes in receptor-binding studies.[570]

The role of the *C*-terminal carboxylate in angiotensin activity has been studied. The alcohol (312), ketone (313), and methyl ester analogues (314) and (315) of [AcAsn^1, Val^5]angiotensin have been synthesized but apart from (314), none showed either agonist or antagonist activity. Weak agonist activity associated with (314) is probably due to its partial conversion back to the *C*-terminal free-acid form and it seems likely that the activity of other angiotension analogues with modified *C*-terminal carboxyl groups is due to a similar conversion.[571]

Retroenantiomers of angiotensin peptides have been reported to show appreciable activity.[572] In the examples studied the *N*-terminal Phe residue was replaced by a benzylmalonyl residue to maintain the *C*-terminus of angiotensin. Whilst the retroenantiomers (304) and (305) of [Val^5]- and [Suc^1, Val^5]angiotensin respectively were inactive as agonists or antagonists, retroenantiomers (301) and (303) had activity in the range 8—24% when compared to the respective parent compounds [Suc^1, Val^5, Ala^7]- (300) and [Suc^1, Val^5, β-Ala^7]angiotensin (302). The direction of the amide bonds appears not to be essential for activity in examples where Pro^7 in angiotensin has been substituted to avoid the 'proline problem'.

Other modifications of the angiotensin backbone have been reported. The Aza-α-homoamino acid analogues of [Asn^1, Val^5]angiotensin containing an 'extra' NH-group inserted between the α-CH and CO of Tyr^4 (292), D-Tyr^4 (293), and Val^3 (291) retain relatively high activity, indicating recognition by the receptor system. These 'aza-analogues' and constituent fragments have been useful as model compounds in studies on the mode of hormone–receptor interaction.[573]

[569] A. C. M. Paiva, V. L. A. Nouailhetas, and T. B. Paiva, *J. Medicin. Chem.*, 1977, **20**, 898.

[570] K. Hsieh, W. H. Vine, P. Needleman, and G. R. Marshall, Abstracts, American Chemical Society Meeting, 1976 (172), MEDI 15.

[571] K. Hsieh, P. Needleman, and G. R. Marshall, Abstracts, American Chemical Society Meeting, 1976 (172), MEDI 16.

[572] G. Goissis, V. L. A. Nouailhetas, and A. C. M. Paiva, *J. Medicin. Chem.*, 1976, **19**, 1287.

[573] G. Chipens, J. Ancan, G. Afanasyeva, J. Balodis, J. Indulen, V. Klusha, V. Kudryashova, E. Liepinsh, N. Makarova, and N. Mishlyakova, 'Peptides 1976', Proceedings of the 14th European Peptide Symposium, ed., A. Loffet, Editions de l'Université de Bruxelles, Brussels, 1976, pp. 353—360.

The majority of angiotensin analogues described have been synthesized by the solid-phase procedure. Coupling of sterically hindered amino-acids has presented difficulties in certain cases. Attempts to introduce Boc-Val(α-Me) into position 3 or 5 of angiotensin were unsuccessful,[568] but Boc-Ala(α-Me) activated with Woodward's reagent K is reported to be an effective acylating agent in solid-phase synthesis.[567]

The sequence of angiotensin I from fowl differs in position 9 (Ser in place of His) from that of known angiotensins I.[574] The [Val^5, Ser^9]decapeptide (331) showed a higher pressor activity in rat than either [Val^5]- or [Ile^5]angiotensin I, presumably indicating that it is more readily converted to the corresponding active angiotensin II form. If the latter is the case it indicates that the converting enzyme (dipeptidyl carboxypeptidase) is not specific for the Phe-His bond.

Evidence for an alternative or additional pathway for the formation of angiotensin III, namely from des-Asp^1-angiotensin I, has been provided.[575, 576] However, the analogue [Sar^1]angiotensin, which resists enzyme conversion to angiotensin III, is equipotent to the heptapeptide form in stimulating aldosterone secretion. This result indicates that angiotensin at the octapeptide level could also be a stimulus for aldosterone secretion.[576]

Studies in man with the des-Asp^1-derivative of the [Ile^8]angiotensin antagonist indicate that it exhibits a selective inhibitory effect on steroidogenic but not pressor action of angiotensin.[577]

The conformational properties of angiotensin have been summarized, and the results of further c.d. and n.m.r. studies reported. A well-defined orientation of the side-chain of Tyr^4 and His^6 is important for activity.[578] Other studies carried out on the effect of pH on the n.m.r. spectrum of [Asn^1, Val^5]angiotensin analogues show the existence of a major and minor conformation.[579] The effect of primary sequence, solvent, and pH on the c.d. spectra of angiotensin and analogues has been reported.[580]

Conformationally restricted analogues of [Val^5]angiotensin with a methyl group in the α-carbon of positions 4 (328) and 8 (329) retain significant agonist activity. The relatively high activity of (328) has, in conjunction with previous data, been used to derive torsional angles at position 4 for the receptor-bound conformation of angiotensin. As this differs from the calculated values for angiotensin in solution, it is suggested that a conformational change occurs on binding to the receptor.[568]

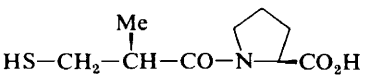
Studies on the mechanism of angiotensin tachyphylaxis in smooth muscle indicate that contrary to earlier reports there is no correlation between tachyphylaxis and peptide affinity for a receptor. A closer relationship was observed between the degree of amino group protonation and tachyphylaxis in the case of

[574] M. C. Khosla and F. M. Bumpus, *J. Medicin. Chem.*, 1977, **20**, 315.
[575] W. B. Campbell, J. M. Schmitz, and H. D. Itskovitz, *Endocrinology*, 1977, **100**, 46.
[576] M. C. Khosla, E. L. Bravo, R. R. Smeby, and F. M. Bumpus, ref. 573, pp. 371—377.
[577] T. Kono, F. Oseko, F. Ikeda, M. Nanno, and J. Endo, *J. Clin. Endocrinol., Metabol.*, 1976, **43**, 940.
[578] S. Fermandjian, K. Lintner, W. Haar, P. Fromageot, M. C. Khosla, R. R. Smeby, and F. M. Bumpus, ref. 573, pp. 339—352.
[579] R. E. Galardy, H. E. Bleich, P. Ziegler, and L. C. Craig, *Biochemistry*, 1976, **15**, 2303.
[580] K. Lintner, S. Fermandjian, P. Fromageot, M. C. Khosla, R. R. Smeby, and F. M. Bumpus, *Biochemistry*, 1977, **16**, 806.

angiotensin and seven analogue peptides. Events subsequent to the agonist–receptor interaction may also be involved in angiotensin tachyphylaxis.[581]

Potentiating Peptides and Enzyme Inhibitors.—Reviews have dealt with inhibition of converting enzyme and the regulation of blood pressure.[582, 583] The nonapeptide angiotensin I-converting enzyme inhibitor, Gly-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro (Squibb 20 881), is reported to show certain advantages over the angiotensin II competitive antagonist 'Saralasin'. In particular there were no pressor effects associated with SQ 20 881 which is a distinct advantage over 'Saralasin'.[584] However, a limitation of SQ 20 881 as a potential antihypertensive drug is its lack of oral activity.

In this connection an important new class of orally active, potent, and specific inhibitors of angiotensin converting enzyme has been developed, based on a hypothetical model of the active site of the enzyme. The most active compound from the series of synthetic L-proline derivatives tested was 2-D-methyl-3-mercaptopropanoyl-L-proline (332) (SQ 14 225). It was orally active in rat models and



(332)

exhibited potent *in vitro* inhibitory active of the order of SQ 20 881. The corresponding L-methyl derivative was approximately a hundred-fold less active.[585]

SQ 14 225 is reported to be a powerful inhibitor of the pressor effect of angiotensin I and a potentiator of the depressor effect of bradykinin in several animal models of experimental hypertension.[586] Clinical studies have now demonstrated that SQ 14 225 is an orally active inhibitor of angiotensin-converting enzyme in man and is potentially useful for the diagnosis and treatment of renovascular hypertension.[587]

The stepwise solution synthesis of bradykinin-potentiating pentapeptide, Glp-Lys-Trp-Ala-Pro (BPP5a), has been reported to give good yields of pure product, and to be more suitable for commercial preparation purposes than the original solid-phase synthesis procedure.[588]

The activity of BPP5a has been compared with that of another potentiating peptide Val-Glu-Ser-Ser-Lys (A-V15), in a number of isolated smooth-muscle preparations. No essential qualitative difference was observed suggesting that the two structurally unrelated peptides may share a similar mechanism of action.[589]

[581] T. B. Paiva, M. E. Miyamoto, L. Juliano, and A. C. M. Paiva, *J. Pharmacol. Exp. Ther.*, 1977, **202**, 294.
[582] A. C. Barger, *Agents Actions*, 1976, **6**, 538.
[583] E. Haber and A. C. Barger, *Prog. Biochem. Pharmacol.*, 1976, **12**, 16.
[584] D. B. Case, J. M. Wallace, H. J. Keim, M. A. Weber, J. I. Drayer, R. P. White, J. E. Sealey, and J. H. Laragh, *Amer. J. Med.*, 1976, **61**, 790.
[585] M. A. Ondetti, B. Rubin, and D. W. Cushman, *Science*, 1977, **196**, 441.
[586] R. J. Laffan, M. E. Goldberg, J. P. High, T. Schaeffer, M. H. Waugh, and B. Rubin, *Fed. Proc.*, 1977, **36**, 1049.
[587] R. K. Ferguson, G. A. Turini, H. R. Brunner, H. Gavras, and D. N. McKinstry, *Lancet*, 1977, **1**, 775.
[588] A. Ali, M. A. Guidicci, and D. Stevenson, *Experientia*, 1976, **32**, 1503.
[589] J. G. Ufkes, P. N. Aarsen, and C. Van Der Meer, *European J. Pharmacol.*, 1976, **40**, 137.

Tachykinins.—Naturally occurring peptide kinins have been classified into groups based on sequence homology and pharmacological properties. Structurally well-defined groups are the bradykinins, tachykinins (including substance P), bombesins (including litorin), and caerulein-like peptides, with additional families awaiting more precise characterization.[590]

Reviews have appeared on the pharmacology of substance P[591] and on caerulein.[592] Structure–activity relationships in ceruletide-like peptides have been reported.[593]

The activity of substance P and its *C*-terminal penta- to decapeptides has been expressed in terms of affinity and intrinsic activity contributions. In guinea-pig ileum intrinsic activity reached a maximum at the nonapeptide level where biological activity was greater than that of substance P. There was a marked increase in affinity and biological activity on extension in chain length from penta- to hexapeptide. Similarly when the *C*-terminal pentapeptides of substance P, physalaemin, or eledoisin were acylated at the *N*-terminus, biological activity

Table 12 *Analogues of substance P and other tachykinins*

Compound number	*Structure*[a]	*Biological activity* g.p.i.[b]	g.p.c.[c]	*Ref.*
	1 5 10			
	Arg-Pro-Lys-Pro-Glu-Glu-Phe-Phe-Gly-Leu-Met-NH_2 substance P			
	Glp-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 physalaemin			
	Glp-Pro-Ser-Lys-Asp-Ala-Phe-Ile Gly-Leu-Met-NH_2 eledoisin			
333	Lys-Phe-Ile-Gly-Leu-Met-NH_2	100	100	594
334	Phe-Phe-Gly-Leu-Met-NH_2	5	n.t.	
335	HO-Phac-Phe-Phe-Gly-Leu-Met-NH_2	600	145	
336	*Ala*-Phe-Gly-Leu-Met-NH_2	0.6	<0.1	
337	HO-Phac-*Ala*-Phe-Gly-Leu-Met-NH_2	3.8	<0.1	
338	*Gln*-Phe-Gly-Leu-Met-NH_2	0.3	n.t.	
339	Phe-Phe-Gly-*Gly*-Met-NH_2	0.7	n.t.	
340	Ho-Phac-Phe-Phe-Gly-*Gly*-Met-NH_2	3.6	2.4	
341	Phe-Phe-Gly-Leu-*Nle*-NH_2	0.6	25	
342	Lys-Phe-Phe-Gly-Leu-*Nle*-NH_2	5.1	17	

[a] HO-Phac = 4-hydroxy-phenylacetyl-. [b] Guinea-pig ileum *in vitro*. [c] Guinea-pig colon *in vitro*.

increased up to 100-fold or more, as seen for compounds (334) and (335) in Table 12. The greatest increase in activity was produced by the *p*-hydroxyphenylacetic acid residue.[594]

A series of analogues of *C*-terminal pentapeptide of substance P has been described. Substitution of the Phe, Leu, or Met residues, and *N*-terminal acylation with the *p*-hydroxyphenylacetyl residue gave analogues (336)—(341) with weak

[590] V. Ersparmer, G. F. Ersparmer, and L. Negri, ref. 537, pp. 153—164.
[591] R. W. Bury, *Austral. J. Exp. Biol. and Med. Sci.*, 1977, **55**, 671.
[592] F. Ganzina and A. Santamaria, *Acta Gastro-Enterol. Belg.*, 1976, **39**, 169.
[593] R. de Castiglione, in 'Hormonal, Receptors in Digestive Tract Physiology', ed. S. Bonfils, North-Holland, Amsterdam, 1977, pp. 33–42.
[594] H. Niedrich, M. Bienert, J. Bergmann, R. Kuehne, R. Franke, and P. Oehme, ref. 573, p. 407.

biological activity in guinea-pig ileum. These modifications produced partial agonists but not antagonists.[594]

The order of potency of tachykinins on the longitudinal muscle of guinea-pig ileum is eledoisin > uperolein > substance P > phyllomedusin > physalaemin and is thought to be a function of the *N*-terminal part of the respective peptides.[595]

The tachykinins phyllomedusin and uperolein have been synthesized in solution employing a fragment strategy.[596] The effect of bombesin and structurally related peptides on thermoregulation in the rat has been studied.[597] Synthesis of a nonapeptide corresponding to the proposed sequence of natural Glu(OMe)^2-litorin (343) has been described. Biological and physico-chemical properties of synthetic and naturally occurring peptides are reported

Glp-Glu(OMe)-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
(343)

to be identical.[598]

6 Enkephalins and Endorphins: A Review of Structure–Activity Relationships

Contributed by B. A. Morgan

Introduction.—Two years have now elapsed since the publication of the structures of Met^5- and Leu^5-enkephalin. During this period research activity has been intense, and approximately five hundred publications relating to the chemistry and biology of the opioid peptides have appeared. In this review attention is focused on the relationship between structure and activity of enkephalin and endorphin analogues. This volume covers work published or disclosed at scientific meetings during 1977; information disclosed in the patent literature has not been included. The initial sources of information have been 'Current Contents – Life Sciences' (Institute for Scientific Information, U.S.A.) and 'Ringdoc' (Derwent Publications Ltd., England).

Much of the work published during 1977 has sought to examine physiological processes involving the opioid peptides. A detailed review of these investigations is beyond the scope of this volume; however, the following 'summary' of reviews is intended to assist the interested reader to obtain an insight into current research on opiate pharmacology, biochemistry, and physiology. Many general reviews on opiates and analgesia have been published, including those by Snyder.[599, 600] The reviews by Kosterlitz and Hughes[601] and Snyder and Simantov[602] concentrate more on the opioid peptides. The role of peptides in neurotransmission has been reviewed by Otsuka and Takahashi[603] and by Hökfelt.[604] A detailed examination

[595] J. Széli, E. Molina, L. Zappia, and G. Bertaccini, *European J. Pharmacol.*, 1977, **43**, 285.
[596] R. de Castiglione and F. Angelucci, ref. 573, p. 529.
[597] M. Brown, J. Rivier, and W. Vale, *Science*, 1977, **196**, 998.
[598] M. Mazzoli and R. de Castiglione, *Experientia*, 1977, **33**, 990.
[599] S. H. Snyder, *Chem. Eng. News*, 1977, **55**, 26.
[600] S. H. Snyder, *Scientific American*, 1977, **236**, (3), 44.
[601] H. W. Kosterlitz and J. Hughes, *Brit. J. Psychiat.*, 1977, **130**, 304.
[602] S. H. Snyder and R. Simantov, *J. Neurochem.* 1977, **28**, 13.
[603] M. Otsuka and T. Takahashi, *Ann. Rev. Pharmacol. Toxicol.*, 1977, **17**, 425.
[604] T. Hökfelt, *Acta Pharmacol. Toxicol.*, 1977, **41** (Suppl. 4), 25.

of the current evidence for the physiological role of the enkephalins in neurotransmission has been published by Frederickson.[605] The relationship between peptides common to brain and intestine and the 'APUD' concept has been reviewed,[606] as has the effects of peptides on behaviour.[607] The structural relationships between the enkephalins and other opiate drugs have also been reviewed.[608]

Most spheres of research related to peptide opiates were discussed in several symposia held during 1977. The proceedings of the London Symposium on 'Centrally Acting Peptides'[609] and the Brescia meeting on 'The Endorphins' have been published.[610] A considerable amount of research relevant to the opioid peptides was presented at the 5th American peptide symposium.[611]

Structure–Activity Relationships.—The activities of enkephalin and endorphin analogues published or disclosed during the review period are tabulated in Tables 13—16. These contain mainly novel analogues; analogues mentioned in previous volumes of this series are included only if an important novel activity is discussed or a previous result amended.

Opiate-like activity may be detected using isolated tissue preparations, receptor binding techniques, biochemical reactions, and behavioural effects in animals. Given this wide range of procedures, and the variety of test conditions which can be applied, it is noteworthy that a high degree of agreement is observed on comparison of results from individual laboratories.

One of the objectives of this review is to gather together results and conclusions from individual laboratories and to attempt to collate these in a cohesive manner. For this reason the data presented in Tables 13—16 have been transformed, where possible, to give potencies relative to a standard opiate (Met^5-enkephalin in the case of *in vitro* tests, morphine in the case of *in vivo* results). It is suggested that the practice of including a standard compound in an investigation will enable valid correlations to be made between results from different laboratories.

For the purpose of this review information relating to different test methods will be considered separately. A concluding section will consider the relative merits of each type of assay and the correlation between assay methods.

Isolated Tissue Assays.—The activities of enkephalin analogues tested on isolated tissues are listed in Table 13. Investigators have used either the electrically stimulated mouse vas deferens preparation (MVD) or the electrically stimulated guinea-pig ileum preparation (GPI).

Analogues bearing single substitution have substantiated the trends outlined in the last volume of this series. These may be summarized:

(a) An *N*-terminal nitrogen function is vital for activity, although in some cases this may be alkylated or acylated.

[605] R. C. A. Frederickson, *Life Sci.*, 1977, **21**, 23.
[606] A. G. E. Pearse, *Med. Biol.*, 1977, **55**, 115.
[607] D. de Wied, *Life Sci.*, 1977, **20**, 145.
[608] A. S. Horn and J. R. Rodgers, *J. Pharm. Pharmacol.*, 1977, **29**, 257.
[609] 'Centrally Acting Peptides', ed. J. Hughes, Macmillan, London, 1978.
[610] 'The Endorphins', Advances in Biochemical Psychopharmacology, Vol. 18, ed. E. Costa and N. Trabucchi, Raven Press, New York, 1978.
[611] 'Peptides', Proceedings of the 5th American Peptide Symposium, ed. M. Goodman and J. Meienhofer, John Wiley and Sons, New York, 1977.

Table 13 *Evaluation of analogues by isolated tissue methods*

(*a*) *Enkephalins*

Compound number	*Structure**	*MVD potency*		*Test Ref.*	*GPI potency*		*Ref.*
344	Ile^5	25	*a*	612			
345	Nle^5	5.1	*a*	612			
346	Val^5	1.3	*a*	612			
347	Ala^5	0.1	*a*	612	2.9	*b*	615
348	Gly^5	0.1	*a*	612			
349	Met (O)^5	11.3	*a*	612			
350	Tyr^4, Leu^5	0.5	*a*	612			
351	Ala^3, Leu^5	0.8	*a*	612			
352	Pro^3, Leu^5	0.01	*a*	612			
353	Sar^3, Leu^5	0.1	*a*	612			
354	Ala^2, Leu^5	0.3	*a*	612			
355	Pro^2, Leu^5	<0.01	*a*	612			
356	Sar^2, Leu^5	0.12	*a*	612			
357	Tyr (OMe)^1, Leu^5	0.75	*a*	612			
358	β-Homo Tyr^1, Leu^5	1.2	*a*	612			
359	D-Leu^5	45	*a*	612	15	*b*	615
360	D-Phe^4, Leu^5	0.01	*a*	612			
361	D-Ala^3, Leu^5	0.1	*a*	612			
362	D-Ala^3	0.05	*a*	612	4	*b*	615
363	D-Pro^3, Leu^5	0.17	*a*	612			
364	D-Ala^2, Leu^5	267	*a*	612	230	*b*	615
365	D-Ala^2	500	*b*	616	625	*b*	616
					316	*b*	615
					0.41	*a*	614
366	D-Leu^2, Leu^5	6.4	*a*	612			
367	D-Trp^2, Leu^5	1.6	*a*	612			
368	D-Tyr^1, Leu^5	0.16	*a*	612			
369	D-Tyr^1, D-Ala^2, Leu^5	0.7	*a*	612			
370	D-Ala^2, D-Ala^3, Leu^5	0.35	*a*	612			
371	D-Ala^2, D-Pro^3, Leu^5	0.02	*a*	612			
372	D-Ala^2, D-Phe^4, Leu^5	0.20	*a*	612			
373	D-Ala^2, D-Leu^5	1227	*a*	612	1.95	*a*	612
					684	*b*	615
374	D-Ala^2, D-Met^5	228	*a*	612	0.92	*a*	612
					419	*b*	615
375	D-Tyr^1, D-Ala^2, D-Leu^5	0.3	*a*	612			
376	D-Ala^2, D-β-homoLeu^5	44	*a*	612			
377	Phe^1, D-Ala^2, Leu^5	2.5	*a*	612			
378	3,5-I_2 Tyr^1, D-Ala^2, Leu^5	0.4	*a*	612			
379	D-Ala^2, Ala^3, Leu^5	12	*a*	612			
380	D-Ala^2, Asn^3, Leu^5	0.9	*a*	612			
		1.28	*a*	613			
381	D-Ala^2, Sar^3, Leu^5	18	*a*	612			
382	D-Ala^2, Leu^4, Leu^5	0.01	*a*	612			
383	D-Ala^2, His^3, Leu^5	0.08	*a*	612			
384	D-Ala^2, C-PhGly^4, Leu^5	0.04	*a*	612			
385	D-Ala^2, Nle^5	209	*a*	612			
386	D-Ala^2, Thr^5	9	*a*	612			
387	D-Ala^2, Pro^5	4	*a*	612			
388	Tyr (OAc)^1, D-Ala^2, Leu^5	28	*a*	612			
389	Phe^1, Tyr^4, Leu^5	0.01	*a*	612			
390	Tyr(OMe)^1, Tyr(OMe)^4, Leu^5	0.03	*a*	612			

Table 13 (*cont.*)

Compound number	*Structure**	*MVD potency*		*Test Ref.*	*GPI potency*		*Ref.*
391	Ala^2, Ala^3, Leu^5	0.04	*a*	612			
392	Ala^2, D-Ala^3, Leu^5	0.04	*a*	612			
393	Ala^2, D-Ala^3	0.01	*a*	612			
394	α-MeAla^2, D-Leu^5	1.5	*a*	612			
395	α-MeAla^2, D-Leu^{5}OMe	0.3	*a*	612			
396	D-Ala^2, Leu^5, Thr^6	461	*a*	612	0.87	*a*	613
397	D-Ala^2, Thr^6	229	*a*	612	0.63	*a*	613
398	Thr^6	22	*a*	612			
399	D-Ala^2, D-Leu^5, Thr^6	19	*a*	612			
400	D-Ala^2, D-Met^5, Thr^6	4	*a*	612			
401	Arg^0, Leu^5, Thr^6	10	*a*	612			
402	Leu^5, Tyr^6, Gly^7	9	*a*	612			
403	D-Ala^2, D-Leu^5, Phe^6, Gly^7	6.3	*a*	612			
404	Arg^0, Leu^5	2.5	*a*	612			
405	D-Ala^2, D-Leu^5, Lys^6, Lys^7	1.6	*a*	612			
406	D-Ala^2, D-Leu^5, D-Thr^6	11.1	*a*	612			
407	Met^5 NHEt	10	*a*	612			
408	Met^{5}OMe	5	*a*	612			
409	Leu^{5}OMe	8.9	*a*	612	100	*c*	620*a*
410	Leu-ol^5	4	*a*	612			
411	BocTyr^1, Leu^5	0.01	*a*	612			
412	*N*-Me Tyr^1, Leu^5	24	*a*	612	0.93	*a*	613
413	*N*-Me Tyr^1, Leu^{5}OMe	6.7	*a*	612	0.75	*a*	613
414	*N*-DiMe Tyr^1, Leu^5	0.4	*a*	612			
415	*N*-DiMeTyr^1, Leu^{5}OMe	0.7	*a*	612			
416	D-Ala^2, Met^{5}OMe	94	*a*	612	1.38	*a*	613
417	D-Ala^2, Leu^{5}OMe	95	*a*	612	2800	*c*	620*a*
418	*N*-MeTyr^1, D-Ala^2, Leu^5	66	*a*	612			
419	*N*-MeTyr^1, D-Ala^2, Leu^{5}OMe	21.5	*a*	612			
420	*N*-DiMeTyr^1, D-Ala^2, Leu^5	12	*a*	612			
421	*N*-Di MeTyr^1, D-Ala^2, Leu^{5}OMe	9.7	*a*	612	0.65	*a*	613
422	D-Ala^2, D-Met^{5}OMe	148	*a*	612	1.76	*a*	613
423	D-Ala^2, D-Leu^{5}OMe	74	*a*	612	6.4	*a*	613
424	*N*-MeTyr^1, D-Ala^2, D-Leu^5	229	*a*	612	1.0	*a*	614
425	*N*-MeTyr^1, D-Ala^2, D-Leu^{5}OMe	18.3	*a*	612	3.3	*a*	614
426	*N*-DiMeTyr^1, D-Ala^2, D-Leu^5	33.6	*a*	612			
427	*N*-DiMeTyr^1, D-Ala^2, D-Leu^{5}OMe	3	*a*	612			
428	D-Ala^2, *p*-ClPhe^4, D-Leu^5	1695	*a*	613	1.0	*a*	613
429	D-Ala^2, *p*-ClPhe^4, D-Leu^{5}OMe	226	*a*	613			
430	D-Ala^2, D-Leu^5 O (*p*-ClPh)	177	*a*	613			
431	Dopa^1, Leu^5	2.5	*a*	614			
432	*N*-MeTyr^1, D-Ala^2, D-Met^{5}NH$_2$	30	*a*	614	0.69	*a*	614
433	D-Ala^2, D-Met^{5}NH$_2$	24	*a*	614	0.66	*a*	614
					246	*b*	615
434	*N*-MeTyr^1, D-Ala^2, D-Met^{5}OMe	39	*a*	614	2.4	*a*	614
435	*N*-MeTyr^1, D-Ala^2, D-Met^5	150	*a*	614	0.40	*a*	614
448	Ala^1				0	*b*	615
449	Ala^2				6.4	*b*	615
450	Ala^3				< 1	*b*	615
451	Ala^4				0	*b*	615
452	D-Ala^1				0	*b*	615
454	D-Ala^4				< 1	*b*	615

Table 13 (*cont.*)

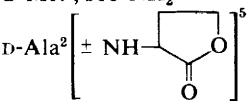
Compound number	*Structure**	*MVD potency*		*Test Ref.*	*GPI potency*		*Ref.*
455	D-Ala^5				6.5	*b*	615
456	Gly^1				0	*b*	615
457	D-Tyr^1, Thr^4				< 1	*b*	615
458	D-Met^5				10.5	*b*	615
459	D-Ala^{2}NH$_2$				528	*b*	615
460	D-Ala^2, Leu^{5}NH$_2$	160	*b*	616	528	*b*	615
461	D-Ala^2, D-Leu^{5}NH$_2$				406	*b*	615
462	D-Ala^2, D-Ala^5				205	*b*	615
463	D-Ala^2, D-Lys^5				328	*b*	615
464	D-Ala^2, D-Phe^5				522	*b*	615
465	D-Ala^2, D-Pro^5				32	*b*	615
466	D-Ala^2, *N*-MeLeu^5				387	*b*	615
467	D-Ala^2, *N*-MeLeu^{5}NH$_2$				711	*b*	615
468	*N*-MeTyr^1	20	*b*	616	100	*b*	616
469	*N*-CpmTyr^1	0.8	*b*	616	1.5	*b*	616
470	*N*-MeTyr^{1}NH$_2$	10	*b*	616	525	*b*	616
471	*N*-MeTyr^{1}NHPr	25	*b*	616	470	*b*	616
472	D-Ala^{2}NH$_2$	190	*b*	616	800	*b*	616
473	D-Ala^{2}NHPr	520	*b*	616	480	*b*	616
474	D-Ala^2, DesMet^{5}NHCH$_2$CH$_2$-CHMe$_2$	44	*b*	616	940	*b*	616
475	Tyr-D-Ala-Gly-Phe NH$_2$	6	*b*	616	515	*b*	616
476	Tyr-D-Ala-GlyNHCH$_2$CH$_2$Ph	2.3	*b*	616	440	*b*	616
477	Tyr-D-AlaGlyN (Me)CH$_2$-CH$_2$Ph	7.5	*b*	616	530	*b*	616
478	D-Ala^2, Des Met^{5}NH-n-pentyl				390	*b*	650
479	*N*-MeTyr^1, Des Met^{5}NH-n-pentyl				120	*b*	650
482	D-Ala^2, Pro^{5}NHEt				450	*c*	620*a*
484	D-Met^2, Pro^{5}NH$_2$	8.0	*d*	628	7.9	*d*	628
485					1600	*c*	620*a*
486	D-Ala^2, Leu^{5}NH(CH$_2$)$_2$NHMe				2350	*c*	620*a*
487	D-Ala^2, Leu^{5}NH(CH$_2$)$_2$NMe$_2$				1300	*c*	620*a*
488	D-Ala^2, Leu^5 O(CH$_2$)$_2$OH				1350	*c*	620*a*
489	D-Ala^2, Leu^{5}NH(CH$_2$)$_2$OH				850	*c*	620*a*
490	D-Ala^2, AzLeu^{5}NH$_2$				750	*c*	620*a*
491	D-Ala^2, Leu^5, D-Thr^6				500	*c*	620*a*
492	[(Gly)$_3$]^0, D-Ala^2, Leu^{5}OMe				1000	*c*	620*a*
494	D-Ala^2, AzGly^3, AzLeu^{5}NH$_2$				550	*c*	620*a*
495	Lys^0, D-Ala^2, AzLeu^{5}NH$_2$				500	*c*	620*a*
496	Lys^0, D-Ala^2, OMe				1350	*c*	620*a*
497	D-Ser^2, Pro^5 NHEt				350	*c*	620*a*
498	*N*-MeTyr^1, D-Ser^2, Pro^{5}NHEt				450	*c*	620*a*
499	Lys^0, D-Ser^2, OMe				7000	*c*	620*a*
500	Lys^0, D-Ser^2, AzLeu^{5}NH$_2$				750	*c*	620*a*
501	D-Ser^2, Leu^{5}OCH(CH$_2$ OAc)$_2$				500	*c*	620*a*
502	D-Ala^2, [Phe (6H)]^4, Leu^5				150	*c*	620*a*
503	D-Ser^2, Leu^{5}OMe				5500	*c*	620*a*
504	D-Met^2, Leu^{5}OMe				4000	*c*	620*a*
505	AzAla^2, Leu^{5}OMe				2850	*c*	620*a*

Table 13 (*cont.*)

Compound number	*Structure**	*MVD potency*		*Test Ref.*	*GPI potency*		*Ref.*
506	AzPhe[4], Leu[5]				25	*c*	620*a*
507	*N*-Me Phe[4], Leu[5]				25	*c*	620*a*
508	AzGly[3], Leu[5]OMe				400	*c*	620*a*
509	AzLeu[5] NH_2				300	*c*	620*b*
510	D-Ser[2], OMe				2000	*c*	620*b*
519	*S*-Benzylsulphonium Met[5]				10	*b*	651
521	*N*-Allyl Tyr[1]				0.08	*d*	617
522	*N*-Allyl Tyr[1], Leu[5]				0.36	*d*	617
523	Arg^0	72	*b*	618	90	*b*	618
(*b*) *Endorphin analogues*							
524	β-LPH 61—68				67	*b*	615
525	β-LPH 61—69				60	*b*	615
526	β-LPH 61—76 (α-End.)	22	*b*	621	32	*b*	615
527	β-LPH 61—77 (γ-End.)	73	*b*	621	23	*b*	615
528	β-LPH 61—79				37	*b*	615
529	β-LPH 61—87 (δ-End.)				48	*b*	615
530	β-LPH 61—91 (β-End. porcine)				450	*b*	615
531	β-LPH 61—91 (β-End. ovine)				450	*b*	615
532	β-LPH 61—91 (β-End. human)	29	*b*	621			
533	α-End. NH_2				72	*b*	615
534	Leu[5] α-End.				10	*b*	615
535	D-Ala[2] α-End.	610	*b*	621	37	*b*	615
536	D-Ala[2] D-Leu[5] α-End.				1.7	*b*	615
537	γ-End. NH_2				41	*b*	615
538	Leu[5] γ-End.				8.2	*b*	615
539	D-Ala[2] γ-End.	647	*b*	621	37	*b*	615
540	D-Ala[2], D-Leu[5] γ-End.				2.8	*b*	615
541	Leu[5] β-End.				75	*b*	615
542	β-LPH 66—91				0	*b*	615
543	β-LPH 62—91				0	*b*	615
544	Leu[5] β-End. human	61	*b*	621			
545	D-Ala[2] β-End. human	262	*b*	621			

Potency: [a] $IC_{50}M$ (morphine)/$IC_{50}M$ (peptide). [b] % Potency relative to Met[5]-enkephalin. [c] % Potency relative to Leu[5]-enkephalin. [d] Potency relative to normorphine.

* The number denoting the position of individual amino-acids refer to the Met[5] enkephalin sequence (Tyr[1]-Gly[2]-Gly[3]-Phe[4]-Met[5]) unless the full structure is given or a β-LPH sequence is specified.

(b) An intact L-Tyrosine[1] moiety is vital for activity.

(c) Substitution of D-amino-acids at position 2, in particular D-alanine, leads to increased activity. Substitution of L-amino-acids or sarcosine at this position leads to a decrease in activity.

(d) *N*- or *C*-substitution at position 3 generally leads to a dramatic loss of activity.

(e) The Phe side-chain at position 4 is vital for activity, though the amino group of this residue may be methylated, *e.g.* (477).

(f) A wide variety of manipulations are possible at the *C*-terminus without destroying activity. Truncation (*e.g.* removal of carboxy group), extension (by either an alkyl group or an amino-acid or peptide residue), oxidation

(to methionine sulphoxide), reduction [to the corresponding alcohol, *e.g.* (444)] or incorporation of D-amino-acids all lead to active analogues, though of widely varying potency.

The effects of multiple substitution on potency are more difficult to categorize. Usually the strategy of combining D-amino-acid substitution at position 2 with a variety of changes at the *C*-terminus has been employed.

The Burroughs–Wellcome group have published detailed accounts of combining D-amino-acid substitution at positions 2 and 5.[612, 613, 614] The D-Ala^2, D-Leu^5 series has provided some interesting results. D-Ala^2,D-Leu^5-E (373) is particularly potent, and it is interesting to note that this compound is one of the few analogues which possesses high potency in the MVD assay relative to the GPI assay. The corresponding *p*-chloro-Phe analogue (428) is even more potent. *N*-methylation (418), substitution of D-Met for D-Leu (374), or derivatization of the carboxy group (423, 429) all lead to a reduction in potency relative to (373).

Combination of D-Ala^2 with other substitution has also been investigated by Ling *et al.*[615] D-Ala^2,*N*-MeLeu^{5}NH$_2$ (467) was particularly interesting, being seven times more potent than Met^5-E.

Morgan *et al.* have presented results on a series of analogues combining D-Ala^2 substitution with increasing degrees of truncation at the *C*-terminus.[616] All the truncated analogues showed increased potency in the GPI assay; potency in the MVD assay was reduced. The smallest fragment with good potency (4.4 × Met^5-E) was the tripeptide (476). The related tripeptide (477), *N*-methylated at the position corresponding to Phe^4, retained good potency in the GPI assay. *N*-cyclopropyl and *N*-allyl groups have been widely used to induce antagonist character in morphine-based analgesic agents. It has been reported that the *N*-cyclopropylmethyl analogue (469) (*N*-CpmTyr^1, Met^5-E) possesses only weak activity as an agonist and showed no ability to antagonize the effects of the potent pure agonist, etorphine, on the MVD preparation.[616]

It is interesting to note that Hahn *et al.* have reported that *N*-allylTyr^1,Leu^5-E (522) possesses antagonist character against normorphine on the guinea-pig ileum. The corresponding Met^5 analogue (521) was considerably less potent.[617]

Law *et al.* have reported that β-LPH$_{60-65}$ (523) has agonist-like activity with a potency similar to Met^5-E in both the MVD and GPI assays.[618] However, their results *in vivo* are not consistent with an earlier report by Ungar who postulated that (523) may be an endogenous antagonist of opiate effects.[619]

[612] C. R. Beddell, R. B. Clark, G. W. Hardy, L. A. Lowe, F. B. Ubatuba, J. R. Vane, S. Wilkinson, K. J. Chang, P. Cuatrecasas, and R. J. Miller, *Proc. Roy. Soc. B*, 1977, **198**, 249.

[613] R. J. Miller, K. J. Chang, P. Cuatrecasas, S. Wilkinson, L. A. Lowe, C. Beddell, and R. Follenfant, see ref. 609, p. 195.

[614] C. R. Beddell, R. B. Clark, R. L. Follenfant, L. A. Lowe, F. B. Ubatuba, S. Wilkinson, and R. J. Miller, in 'Biological Activity and Chemical Structure', ed. J. H. Keverling Buisman, Elsevier Scientific Publishing Co., Amsterdam, 1977, p. 177.

[615] N. Ling, S. Minick, L. Lazarus, J. Rivier, and R. Guillemin, ref. 611, p. 96.

[616] B. A. Morgan, J. D. Bower, K. P. Guest, B. K. Handa, G. Metcalf, and C. F. C. Smith, ref. 611, p. 111.

[617] E. F. Hahn, F. Fishman, Y. Shiwaka, F. F. Foldes, H. Nagashima, and D. Duncalf, *Res. Comm. in Chem. Path. and Pharmacol.*, 1977, **18**, 1.

[618] P. Y. Law, E. T. Wei, L. F. Tseng, H. H. Loh, and E. L. Way, *Life Sci.*, 1977, **20**, 251.

[619] G. Ungar, A. L. Ungar, and D. H. Malin in 'Opiates and Endogenous Opioid Peptides', ed. H. W. Kosterlitz, Elsevier–North Holland Biomedical Press, Amsterdam, 1976, p. 121.

Dutta and co-workers have reported structure–activity relations on a wide range of analogues.[620] The incorporation of aza-amino-acids is of particular interest. The AzGly^3 compound (508) is a rare example of an analogue with substitution at position 3 with a greater potency than the Gly^3 counterpart. The AzAla^2 (505) and AzLeu^5 analogues (509) are also active. A series of *N*-terminally extended analogues were also investigated. The Lys series was particularly interesting, Lys^0,D-Ser^2,Met^5-EOMe (499) having a potency of 70 × Leu^5-E in the GPI assay.

A series of endorphin analogues has also been investigated (Table 13b). Ling *et al.* have reported the incorporation of D-Ala^2 or Leu^5 into α-endorphin (535, 534), γ-endorphin (539, 538) and Leu^5 into β-endorphin (541).[615] In the GPI assay these analogues are at least ten times less potent than the natural β-LPH sequence. In contrast, Coy *et al.* have reported that D-Ala^2 incorporation in α-, β-, and γ-endorphin (535, 545, 539) results in higher potency in the MVD assay.[621]

Opiate Receptor Binding Assay.—Interaction of the enkephalins with opiate receptors may also be investigated by performing binding assays utilizing radiolabelled alkaloid and peptide opiates. Assays of this type can be performed in a wide variety of ways. Variables include tissue source, preparation of tissue fractions, temperature and duration of incubation, composition of incubation medium, and choice of radiolabelled ligand.

In general, structure–activity relationships derived using this technique correspond well with the concepts evolved using isolated tissue methods.

The data reported using this technique are set out in Table 14. Miller *et al.* comment that high correlation may be seen between the potency observed in the mouse vas deferens preparation and the opiate receptor affinity.[613]

Table 14 *Enkephalin analogues. Binding data*

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
345	Nle^5	0.044	*a*	612
		60	*f*	625
346	Val^5	0.044	*a*	612
347	Ala^5	0.006	*a*	612
348	Gly^5	0.0004	*a*	612
350	Tyr^4, Leu^5	0.0002	*a*	612
351	Ala^3, Leu^5	0.0004	*a*	612
352	Pro^3, Leu^5	0.0001	*a*	612
353	Sar^3, Leu^5	0.0039	*a*	612
354	Ala^2, Leu^5	0.0007	*a*	612
		14	*f*	625
355	Pro^2, Leu^5	< 0.0004	*a*	612
356	Sar^2, Leu^5	0.0004	*a*	612
357	Tyr(OMe)^1, Leu^5	0.0008	*a*	612

[620a] A. S. Dutta, J. J. Gormley, C. F. Hayward, J. S. Morley, J. S. Shaw, G. J. Stacey, and M. T. Turnbull, *Life Sci.*, 1977, **21**, 559.

[620b] A. S. Dutta, J. J. Gormley, C. F. Hayward, J. S. Morley, J. S. Shaw, G. J. Stacey, and M. T. Turnbull, *Brit. J. Pharmacol.*, 1977, **61**, 481P.

[621] D. H. Coy, P. Gill, A. J. Kastin, A. Dupont, L. Cusan, F. Labrie, D. Britton, and R. Fertel, ref. 611, p. 107.

Table 14 (*cont.*)

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
358	β-homoTyr[1], Leu[5]	< 0.0004	*a*	612
359	D-Leu[5]	0.14	*a*	612
360	D-Phe[4], Leu[5]	< 0.0004	*a*	612
361	D-Ala[3], Leu[5]	0.00007	*a*	612
362	D-Ala[3]	< 0.0004	*a*	612
363	D-Pro[3], Leu[5]	< 0.0004	*a*	612
364	D-Ala[2], Leu[5]	1.094	*a*	612
		44	*b*	613
		70	*c*	613
365	D-Ala[2]	0.921	*a*	613
367	D-Trp[2], Leu[5]	0.438	*a*	612
369	D-Tyr[1], DAla[2], Leu[5]	0.0018	*a*	612
370	D-Ala[2], D-Ala[3], Leu[5]	0.0009	*a*	612
371	D-Ala[2], D-Pro[3], Leu[5]	0.0004	*a*	612
372	D-Ala[2], D-Phe[4], Leu[5]	0.0004	*a*	612
373	D-Ala[2], D-Leu[5]	1.346	*a*	612
		40	*b*	613
		100	*c*	613
374	D-Ala[2], D-Met[5]	0.438	*a*	612
375	D-Tyr[1], D-Ala[2], D-Leu[5]	< 0.0004	*a*	612
377	Phe[1], D-Ala[2], Leu[5]	0.005	*a*	612
379	D-Ala[2], Ala[3], Leu[5]	0.044	*a*	612
380	D-Ala[2], Asn[3], Leu[5]	0.005	*a*	612
381	D-Ala[2], Sar[3], Leu[5]	< 0.0004	*a*	612
382	D-Ala[2], Leu[4], Leu[5]	< 0.0004	*a*	612
383	D-Ala[2], His[4], Leu[5]	0.0008	*a*	612
384	D-Ala[2], C-PhGly[4], Leu[5]	0.0008	*a*	612
385	D-Ala[2], Nle[5]	0.389	*a*	612
386	D-Ala[2], Thr[5]	0.039	*a*	612
387	D-Ala[2], Pro[5]	0.0006	*a*	612
388	Tyr(OAc)[1]D-Ala[2], Leu[5]	0.0044	*a*	612
389	Phe[1], Tyr[4], Leu[5]	0.0004	*a*	612
390	Tyr(OMe)[1], Tyr(OMe)[4], Leu[5]	0.004	*a*	612
391	Ala[2], Ala[3], Leu[5]	0.0004	*a*	612
392	Ala[2], D-Ala[3], Leu[5]	0.0004	*a*	612
393	Ala[2], D-Ala[3]	0.0004	*a*	612
394	α-MeAla[2], D-Leu[5]	< 0.0004	*a*	612
395	α-MeAla[3], D-Leu[5]OMe	< 0.0004	*a*	612
396	D-Ala[2], Leu[5], Thr[6]	0.438	*a*	612
397	D-Ala[2], Thr[6]	0.625	*a*	612
399	D-Ala[2], Leu[5], Thr[6]	0.117	*a*	612
400	D-Ala[2], D-Met[5], Thr[6]	0.050	*a*	612
401	Arg[0], Leu[5], Thr[6]	0.023	*a*	612
402	Leu[5], Tyr[6], Gly[7]	0.0035	*a*	612
403	D-Ala[2], D-Leu[5], Phe[6], Gly[7]	0.0044	*a*	612
404	Arg[0], Leu[5]	0.007	*a*	612
		17	*f*	625
405	D-Ala[2], Leu[5], Lys[6], Lys[7]	0.0039	*a*	612
409	Leu[5]OMe	0.058	*a*	612
412	*N*-MeTyr[1], Leu[5]	0.005	*a*	612
413	*N*-MeTyr[1], Leu[5]OMe	0.007	*a*	612
414	*N*-DiMeTyr[1], Leu[5]	0.001	*a*	612
415	*N*-DiMeTyr[1], Leu[5]OMe	0.0004	*a*	612

Table 14 (*cont.*)

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
416	D-Ala^2, Met^{5}OMe	0.700	*a*	612
417	D-Ala^2, Leu^{5}OMe	0.389	*a*	612
418	*N*-MeTyr^1, D-Ala^2, Leu^5	0.018	*a*	612
419	*N*-MeTyr^1, D-Ala^2, Leu^{5}OMe	0.005	*a*	612
420	*N*-DiMeTyr^1, D-Ala^2, Leu^5	0.0018	*a*	612
421	*N*-DiMeTyr^1, D-Ala^2, Leu^{5}OMe	0.0007	*a*	612
422	D-Ala^2, D-Met^{5}OMe	0.234	*a*	612
423	D-Ala^2, D-Leu^{5}OMe	0.389	*a*	612
426	*N*-DiMeTyr^1, D-Ala^2, D-Leu^5	0.0012	*a*	612
427	*N*-DiMeTyr^3, D-Ala^2, D-Leu^{5}OMe	0.0005	*a*	612
428	D-Ala^2, *p*-ClPhe^4, D-Leu^5	3.5	*a*	612
429	D-Ala^2, *p*-ClPhe^4, D-Leu^{5}OMe	1.75	*a*	612
431	Dopa^1, Leu^5	1	*d*	614
432	*N*-MeTyr^1, D-Ala^2, D-Met^{5}NH$_2$	0.01	*a*	614
433	D-Ala^2, D-Met^{5}NH$_2$	0.39	*a*	614
435	*N*-MeTyr^1, D-Ala^2, D-Met^5	0.01	*a*	614
444	Met^5-ol	1.50	*e*	622
445	D-Ala^2, Met^5-ol	3.6	*e*	622
446	D-Ala^2, Met(O)^5-ol	1.2	*e*	622
447	D-Ala^2, MePhe^4, Met(O)^{5}ol	6.1	*e*	622
453	Carboranylalanine^4, Leu^5	118	*d*	652
518	Phe^1	0.017	*f*	625
520	*N*-AllylTyr^1, D-Ala^2	0.02	*a*	623

Conditions: [a] IC_{50}M (morphine)/IC_{50}M (peptide). Na^+ absent. Whole rat brain pellet [^{3}H]naloxone. [b] IC_{50}M (morphine)/IC_{50}M (peptide). Whole rat brain. ^{125}I-D-Ala^2, Leu^5-enkephalin. [c] IC_{50}M (morphine)/IC_{50}M (peptide) 4T G1 cells. ^{125}I-D-Ala^2, D-Leu^5-enkephalin. [d] Activity relative to Met^5-E = 100; rat brain, [^{3}H]naloxone. [e] IC_{50}M (morphine)/IC_{50}M (peptide); rat brain, [^{3}H]naloxone. [f] K_D (Met^5-E)/K_D (peptide); rat brain membranes.

Roemer *et al.* have reported receptor binding data on a series of analogues; the results are in good agreement with their studies *in vivo*.[622]

Several workers have investigated the 'sodium shift' ratio as a means of determining agonist–antagonist character. The Sandoz group report that D-Ala^2, MePhe^4,Met-(O)^5-ol (447) has a sodium shift ratio of 8.2.[622] This is calculated from the ID_{50} values obtained in the presence and absence of NaCl. A value of 8.2 would suggest that the analogue possesses 'partial antagonist' character similar to the benzomorphan pentazocine. As there is no evidence *in vivo* to suggest antagonist character for (447), it is concluded that the value of the sodium shift ratio as a predictor of the degree of antagonist character of opioid peptides is uncertain. In contrast, Pert *et al.* conclude that *N*-allyl-Tyr^1,D-Ala^2-E (520) 'seems to be a weak agonist with mild antagonist character *in vivo* with a small sodium shift *in vitro* like that of opiate partial antagonists.'[623]

Biochemical Assays.—Several workers have reported that opiates inhibit transmitter release and the activity of certain 'secondary messenger' systems. Soon after their discovery the enkephalins were shown to inhibit both basal and prostaglandin-stimulated adenylate cyclase activity using a neuroblastoma X

[622] D. Romer, H. H. Buescher, R. C. Hill, J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser and R. Huguenin, *Nature*, 1977, **268**, 547.
[623] C. B. Pert, D. L. Bowie, A. Pert, J. L. Morell, and E. Gross, *Nature*, 1977, **269**, 73.

glioma hybrid cell, and several groups have used this method to investigate structure–activity relationships (Table 15). Agarwal and co-workers have examined several analogues using this method.[624] In general, results are in agreement with other *in vitro* assay methods. A similar study has been reported by Wahlstrom *et al.*[625] These workers suggest the hypothesis that the hybrid cell lining has only enkephalin 'sites' with high affinity for the peptide opiates but only low affinity for the alkaloid opiates.

Table 15 *Evaluation of enkephalin analogues by inhibition of adenylate cyclase activity in neuroblastoma X glioma hybrid cells*

Compound number	*Structure*	*Activity*	*Method*	*Ref.*
345	Nle^5	300	*a*	625
354	Ala^2, Leu^5	2	*b*	624
		6	*a*	625
404	Arg^0, Leu^5	10	*a*	625
409	Leu^{5}OMe	30	*b*	624
514	Ser^3, Leu^5	1	*b*	624
515	Aba^2, Leu^5	0.5	*b*	624
516	Tyr-Gly-Phe-Leu	0.4	*b*	624
517	Ser^2, Leu^5	0.1	*b*	624
518	Phe^1	0.003	*a*	625

[a] Inhibition of PGE_1, effect on cyclic AMP levels; potency expressed relative to Leu^5-enkephalin = 100%. [b] Inhibition of opiate-sensitive adenylate cyclase activity; potency expressed relative to Leu^5-enkephalin = 100%.

***In vivo* Tests.**—Opiate drugs produce a wide spectrum of pharmacological effects in animal tests. A large number of enkephalin analogues have been tested in a variety of assay methods during the review period. The results of antinociceptive testing are shown in Table 16. As in the *in vitro* sections, the variety of species, route of administration, nociceptive stimuli, and method of assessment complicate the task of comparing the results of individual laboratories. Wherever possible, results are tabulated as 'potency relative to morphine'.

Miller *et al.* have evaluated a selection of analogues in several tests.[613] In the mouse hot-plate assay D-Ala^2,D-Leu^5 and D-Ala^2,D-Met^5 acids, amides, and esters were all active at <0.1 μg per mouse i.c.v. *N*-MeTyr^1,D-Ala^2,D-Met^5NH_2 (432) was the most potent with an ED_{50} of 0.005 μg per mouse i.c.v. The same series of analogues were among the most potent in the anti-diarrhoeal test. In this case, *N*-MeTyr^1,D-Ala^2,D-Leu^5NH_2 (437) was the most potent with an ED_{50} of 0.3 mg kg^{-1} (s.c. administration).

Wei and co-workers have reported that D-Ala^2,D-Leu^5 enkephalin (373) is approximately 32 times as potent as morphine in the mouse tail flick (i.c.v.).[626]

A series of Pro^5 analogues has been described by Bajusz *et al.*[627] The most potent compound, D-Met^2,Pro^5NH_2 (484), is 55 times as potent as morphine in

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[626] E. T. Wei, L. F. Tseng, H. H. Loh, and C. H. Li, *Life Sci.*, 1977, **21**, 321.
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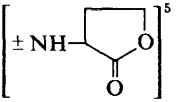
Table 16 *Enkephalin analogues:* in vivo *data*

Compound number	*Structure*	*Activity*	*Method*	*Ref.*
373	D-Ala^2, D-Leu^5	0.5	*a*	613
		3.0	*b*	613
		31.7	*k*	626
374	D-Ala^2, D-Met^5	0.5	*a*	613
		2.0	*b*	613
380	D-Ala^2, Asn^3, Leu^5	3.0	*b*	613
386	D-Ala^2, Thr^5	0.5	*a*	600
		3.0	*b*	613
397	D-Ala^2, Met^5, Thr^6	0.24	*b*	613
399	D-Ala^2, D-Leu^5, Thr^6	0.1	*b*	613
403	D-Ala^2, D-Leu^5, Phe^6, Gly^7	0.1	*a*	613
406	D-Ala^2, D-Leu^5, D-Thr^6	0.1	*a*	613
417	D-Ala^2, D-Leu^{5}OMe	0.07	*a*	613
418	*N*-MeTyr^1, D-Ala^2, D-Leu	0.07	*a*	613
419	*N*-MeTyr^1, D-Ala^2, D-LeuOMe	0.05	*a*	613
423	D-Ala^2, D-Leu^{5}OMe	0.5	*a*	613
424	*N*-MeTyr^1, D-Ala^2, D-Leu^5	0.07	*a*	613
428	D-Ala^2, *p*-ClPhe^4, D-Leu^5	0.07	*a*	613
430	D-Ala^2, D-Leu^5, O(*p*-ClPh)	0.05	*a*	613
432	*N*-MeTyr^1, D-Ala^2, D-Met^{5}NH$_2$	0.005	*a*	613
		0.8	*b*	613
		0.7	*c*	614
434	*N*-MeTyr^1, D-Ala^2, D-Met^{5}OMe	0.02	*a*	613
		2.0	*b*	613
435	*N*-MeTyr^1, D-Ala^2, D-Met^5	0.07	*a*	613
		2.0	*b*	613
436	D-Ala^2, D-Leu^{5}NHEt	0.01	*a*	613
		0.8	*b*	614
		2.0	*c*	614
437	*N*-MeTyr^1, D-Ala^2, D-Leu^{5}NH$_2$	0.05	*a*	613
		0.3	*b*	613
		1.0	*c*	614
438	*N*-MeTyr^1, D-Ala^1, D-Met^{5}NHEt	0.07	*a*	613
		0.7	*b*	613
440	Tyr-D-Ala-Gly-PheOMe	0.2	*a*	613
		0.7	*b*	613
		1.4	*c*	614
441	Tyr-D-Ala-Gly-Phe	0.7	*a*	613
442	D-Ala^2, *p*-ClPhe^4, D-Leu^{5}NHEt	1.0	*b*	613
443	Tyr-Ile-Asn-Met-Leu	8.0	*b*	613
444	Met^5-ol	43	*d*	622
		52	*e*	622
445	D-Ala^2, Met^5-ol	0.04	*d*	622
		12	*e*	622
446	D-Ala^2, Met (O)^5-ol	0.01	*d*	622
		12	*e*	622
447	D-Ala^2, *N*-MePhe^4, Met (O)^5-ol	0.002	*d*	622
		0.7	*e*	622
470	*N*-MeTyr^{1}NH$_2$	200 μg, 100%, 3 h	*f*	616
471	*N*-MeTyr^{1}NHPr	100 μg, 100%, 3 h	*f*	616
365	D-Ala^2	250 μg, 72%, 2 h	*f*	616
472	D-Ala^{2}NH$_2$	100 μg, 88%, 2 h	*f*	616
473	D-Ala^{2}NHPr	100 μg, 76%, 3 h	*f*	616

Table 16 (*cont.*)

Compound number	*Structure*	*Affinity*	*Method*	*Ref.*
474	D-Ala^2, Leu^{5}NH$_2$	100 μg, 100%, 2 h	*f*	616
475	D-Ala^2, DesMet$_5$NH(CH$_2$)$_2$CHMe$_2$	100 μg, 20%	*f*	616
476	Tyr-D-Ala-GlyNH(CH$_2$)$_2$Ph	100 μg, 83%, 4 h	*f*	616
477	Tyr-D-Ala-GlyN(Me)(CH$_2$)$_2$Ph	100 μg, 35%, 3 h	*f*	616
478	D-Ala^2, DesMet^{5}NHn-pentyl	2.2 μg	*d*	650
479	*N*-MeTyr^1, DesMet^{5}NHn-pentyl	4.4 μg	*d*	650
480	D-Ala^2, Pro^{5}NHamyl	0.08	*g*	627
		0.1	*h*	627
481	D-Ala^2, Pro^{5}NHEt	16.9	*g*	627
		0.19	*h*	627
		5 mg kg^{-1}, 5 s	*i*	620*a*
482	D-Ala^2, Pro^{5}NH$_2$	3.9	*g*	627
		0.22	*h*	627
483	D-Met^2, Pro^{5}NHEt	16.9	*g*	627
		0.55	*h*	627
484	D-Met^2, Pro^{5}NH$_2$	49.8	*g*	627
		5.5	*h*	627
485	D-Ala^2 	5 mg kg^{-1}, 5 s	*i*	620*a*
486	D-Ala^2, Leu^{5}NH (CH$_2$)$_2$NHMe	5 mg kg^{-1}, 3 s	*i*	620*a*
487	D-Ala^2, Leu^{5}NH (CH$_2$)$_2$NMe$_2$	10 mg kg^{-1} 10 s	*i*	620*a*
488	D-Ala^2, Leu^{5}O(CH$_2$)$_2$OH	25 mg kg^{-1} 5 s	*i*	620*a*
489	D-Ala^2, Leu^{5}NH (CH$_2$)$_2$OH	25 mg kg^{-1} 6 s	*i*	620*a*
490	D-Ala^2, AzLeu^{5}NH$_2$	25 mg kg^{-1}, 9 s	*i*	620*a*
491	D-Ala^2, Leu^5, D-Thr^6	25 mg kg^{-1}, 11 s	*i*	620*a*
492	[(Gly)$_3$]^0, D-Ala^2, Leu^{5}OMe	25 mg kg^{-1}, 5 s	*i*	620*a*
493	[(Leu)$_3$]^0, D-Ala^2, Leu^{5}OMe	10 mg kg^{-1}, 4 s	*i*	620*a*
494	D-Ala^2, AzGly^3, AzLeu^{3}NH$_2$	25 mg kg^{-1}, 11 s	*i*	620*a*
495	Lys^0, D-Ala^2, AzLeu^{5}NH$_2$	25 mg kg^{-1}, 10 s	*i*	620*a*
496	Lys^0, D-Ala^2, Met^{5}OMe	25 mg kg^{-1}, 4 s	*i*	620*a*
497	D-Ser^2, Pro^{5}NHEt	10 mg kg^{-1}, 5 s	*i*	620*a*
498	*N*-MeTyr^1, D-Ser^2, Pro^{5}NHEt	25 mg kg^{-1}, 10 s	*i*	620*a*
499	Lys^0, D-Ser^2, Met^{5}OMe	25 mg kg^{-1}, 4 s	*i*	620*a*
500	Lys^0, D-Ser^2, AzLeu^{5}NH$_2$	25 mg kg^{-1}, 7 s	*i*	620*a*
501	D-Ser^2, Leu^{5}OCH(CH$_2$OAc)$_2$	25 mg kg^{-1}, 7 s	*i*	620*a*
502	D-Ala^2, [Phe (6H)]^4, Leu^5	50 mg kg^{-1}, 10 s	*i*	620*a*
509	AzLeu^{5}NH$_2$	100 mg kg^{-1}, 2 s	*i*	620*b*
510	D-Ser^2, Met^{5}OMe	25 mg kg^{-1}, 4 s	*i*	620*b*
511	D-Met^2, Thz^{5}NH$_2$	4.2	*j*	629
		7.7	*k*	629
512	D-Thr^2, Thz^{5}NH$_2$	4.8	*j*	629
		27.1	*k*	629

[a] ED_{50} μg per mouse; mouse hot-plate i.c.v. administration. [b] Rat anti-diarrhoeal activity ED_{50} mg kg^{-1}, s.c. [c] Guinea-pig anti-tussive test ED_{50} mg kg^{-1}, s.c. [d] ED_{50} μg per mouse, 15 min mouse tail flick i.c.v. [e] ED_{50} mg kg^{-1}, 15 s mouse tail flick i.v. [f] Rat tail flick i.c.v., dose μg per rat, 'extent' of analgesia, duration of analgesia. [g] Rat tail flick central administration, *potency* relative to morphine. [h] Rat tail flick, i.v. potency relative to morphine. [i] Mouse hot-plate, i.v. minimum dose (mg kg^{-1}) causing increase (sec) in reaction time. [j] Mouse tail flick, i.v., potency relative to morphine. [k] Mouse tail flick, i.c.v., potency relative to morphine.

the rat tail flick test (i.c.v.). A more detailed account of the pharmacology of (484) has also been published.[628]

Dutta *et al.*[620a] have reported results on a series of analogues in the mouse hot-plate test (i.v.). These workers observed poor correlation between *in vitro* screens and *in vivo* findings. The most potent compounds included D-Ala^2,Pro^{5}NHEt (481), and the *C*-terminal homoserine lactone (485).

The Pro^5 series, initially described by Bajusz,[627] has been extended by Yamashiro *et al.* These workers have substituted thiazolidine-4-carboxylic acid (Thz) at position 5 and obtained analogues (511) and (512).[629] Although direct comparison is not possible, the D-Met^2,Thz^5 analogue (511) appears to be approximately equipotent to the corresponding Pro^5 analogue (484) on intravenous administration.

Morgan *et al.* have described a series of analogues with varying degrees of truncation at the *C*-terminus.[616] Again there is little correlation between *in vivo* and *in vitro* findings. It is interesting to note, however, that the tripeptide derivatives (476) and (477) give rise to good analgesia after i.c.v. administration.

A fascinating series of analogues has been reported by the Sandoz group.[622] Four structural modifications led these workers to D-Ala^2,MePhe^4,Met(O)^{5}ol (447) which was found to be 1000 times as potent as morphine on i.c.v. administration and up to five times as potent i.v. in the mouse tail flick test. Results of several other tests, including self-administration and withdrawal studies in monkeys, are also reported.

Conclusions: Structure–Activity Relationships.—A brief examination of the data collected in Tables 13—16 of this section reveals many apparent inconsistencies between the results obtained with various assay procedures. Dutta *et al.* [620a] report that although Lys,^{0}D-Ser^2,Met^{5}OMe (499) is 70 times as potent as Leu^5-enkephalin in the guinea-pig ileum assay, its activity *in vivo* (mouse hot-plate test) is considerably less than analogues such as D-Ala^2,Pro^{5}NHEt (482) which displays only four times the potency of Met^5-E on the ileum. Similarly Miller *et al.*[613] showed that while D-Ala^2,D-Leu^5-E (373) is approximately six times as potent as its *N*-MeTyr^1 analogue (424) on the mouse vas deferens preparation, the situation *in vivo* is reversed with the *N*-Me analogue (424) being ten times more potent than (373) in the mouse hot-plate test after intracereboventricular administration. These examples support many previous findings that *in vitro* assays of peptides for opiate-like action do not necessarily predict antinociceptive activity *in vivo*. In many cases such anomalies may of course be explained by differences in biotransformation or pharmacokinetic behaviour.

It is more difficult to explain the variations between the results of *in vitro* assays by such phenomena. D-Ala^2,D-Leu^5-enkephalin (373), for example, is 1200 times more potent than morphine on the MVD assay but only twice as potent on the GPI assay.[612] This result is contrasted by analogues such as Tyr-D-Ala-GlyNH-$(CH_2)_2$Ph (476) which is less potent than morphine on the MVD and five times more potent on the GPI assay.[616] It is unlikely that such dramatic variations

[628] J. I. Székely, A. Z. Rónai, Z. Dunai-Kovács, E. Miglécz, I. Bérzetei, S. Bajusz, and L. Gráf, *European J. Pharmacol.*, 1977, **43**, 293.

[629] D. Yamashiro, L. F. Tseng, and C. H. Li, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 1124.

between *in vitro* assays are due to biotransformation and the alternative explanation of receptor multiplicity has been suggested by many groups. Although this phenomenon has been accepted in the cholinergic and adrenergic systems, and more recently has been demonstrated in the histaminergic system, its demonstration in the opiate field has been more elusive. The existence of pharmacologically differentiable populations of opioid receptors was first postulated by Martin in 1967 [630] and more recently his group extended the concept and postulated the existence of three distinct receptor types producing different pharmacological syndromes.[631] These receptors were named μ, for which morphine was the prototype agonist, κ (ketocyclazocine as agonist), and σ (*N*-allylnormetazocine as agonist). The discovery of the opioid peptides and the development of opiate receptor binding techniques initiated further work in this area. Kosterlitz and co-workers have used the isolated guinea-pig ileum and mouse vas deferens preparations, and receptor binding studies using [^{3}H]Leu^5-enkephalin and [^{3}H]-naloxone to investigate receptor heterogeneity with respect to a range of opioid peptides.[632, 633] One of the most interesting findings of these studies was the fact that β-endorphin was equally effective in inhibiting Leu^5-enkephalin and naloxone binding, and was equipotent on both the GPI and MVD. This observation may be interpreted as indicating that β-endorphin interacts equally well with several (perhaps all) opiate receptors. Met^5-enkephalin however differs from its putative precursor in several aspects and Lord *et al.* speculate therefore that Met^5-enkephalin 'may be designed for a more specialized function than β-endorphin'.[632] Further evidence has been published by other groups of workers. Ronai *et al.* have found that the antagonist naltrexone can differentiate between the Pro^5 series and other enkephalin analogues in the GPI assay.[634] Terenius has investigated the binding properties of opiate receptors in rat cerebellum using a variety of radio-labelled ligands.[635] He concludes that at least three kinds of binding sites may be observed.

To summarize, there are now a wealth of data which can best be explained by postulating multiplicity of opiate receptors. It remains to be seen whether this heterogeneity can be exploited therapeutically to produce agents with an improved pharmacological profile. In any event, the following statement may now be considered sound advice for researchers in this area: 'a single assay-system can no longer be considered a reliable method for prediction of the pharmacological activity of new opioid compounds, particularly of those with peptide structure. For such compounds multiple parallel assays . . . may be useful'.[632]

Conformation Studies.—Many workers have investigated possible conformations of the enkephalins; n.m.r. techniques have been widely used. Khaled *et al.*, have suggested two conformational states; a monomeric form containing a β-turn at Gly^3-Phe^4 or an associated form with an antiparallel cross β-structure.[636]

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[634] A. Z. Rónai, I. Bérzetei, S. Bajusz, *European J. Pharmacol.*, 1977, **45**, 393.
[635] L. Terenius, *Psychoneuroendocrinology*, 1977, 53.

β-Turnstructures have previously been suggested by several groups (see last year's review). Jones *et al.* have reported that only the zwitterionic form of Met[5]-E yields ^{1}H-n.m.r. parameters indicative of a Gly-Phe β-I turn.[637] This conclusion is substantiated by the results of Fournie Zaluski.[638, 639] A ^{1}H-n.m.r. study of Met[5]-enkephalin conformation as a function of pH has been reported by Anteunis *et al.*[640] Other reports include a ^{1}H and ^{13}C study of the conformational behaviour of Leu[5]-enkephalin [641] and a ^{1}H study of several enkephalin analogues.[642]

Several empirical analyses of enkephalin conformation have been reported. Beddell *et al.* have compared results obtained with analogues on the MVD preparation *in vitro* with predictions derived from a series of possible conformations and conclude that only a small sub-set are compatible with experimental data.[643] Isogai *et al.* suggest a Gly[3]-Phe[4] β-II′ conformation on the basis of empirical energy calculations.[644] A β-I turn was suggested by De Coen *et al.*, after theoretical conformation analysis.[645] Conformational energy calculations on Met[5]-E and several analogues have been carried out by Momany.[646] In a different approach Gorin and Marshall fitted the tetrapeptide HTyr-Gly-Gly-PheOH to an opiate receptor model based on *X*-ray crystallographic data of morphine and oripavine derivatives.[647]

Conclusions.—The review period has witnessed a dramatic increase in our knowledge of opioid peptide structure–activity relationships, including the disclosure of several highly active analogues. Whether or not these analogues will provide a therapeutic advance is still to be determined. A second area of particular interest to those engaged on peptide research is that of the possible interactions between the enkephalins and other centrally active peptides. Evidence has already been presented to suggest that enkephalin can inhibit the release of substance P in the substantia gelatinosa.[648] Evidence of a physiological interplay between TRH and endorphins has also been suggested.[649]

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