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Volume 14

A Specialist Periodical Report

Amino-acids, Peptides, and Proteins

Volume 14

A Review of the Literature Published during 1981

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(continued overleaf)

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Preface

This fourteenth Report reviews papers that are relevant to the chemistry of amino-acids, peptides, and proteins published during 1981, except in the contribution on Primary Structures, where the coverage is for 1980 and 1981 (making good a regretted omission from the thirteenth Report).

The format and style of the Report are very much as established by previous volumes, but in some sections the pressures of time and space have been met by cutting discussion even more drastically than before and presenting almost all the information in an easily scanned tabular form. Also, it is now the Society's policy to dispense with indexes in Specialist Periodical Reports except in special cases. This Report conforms with that policy by dropping the Author Index, which has been regular hitherto, as a contribution towards the minimization of production costs and delay. The coverage in Chapter 5, which deals with Structure and Biological Activity, is, as sometimes before, not quite comprehensive: the Gastrointestinal Hormones and Thyroid and Parathyroid Hormones are notable lacunae. It is hoped to remedy these gaps if this chapter remains a standard feature of the Reports.

The changeover in the Senior Reportership occasioned a little delay in making the necessary arrangements, and the contributors had to work under some pressure. I am grateful to them for accepting their tasks at rather short notice – in several cases in spite of other demanding commitments – and for their patience in the face of my harassment.

Bob Sheppard's run as Senior Reporter has been a long (Volumes 5—13) and distinguished one: I am very grateful to him for his efforts to make the changeover smooth and for much valuable advice. He was only the second Senior Reporter in the series: the first was Geoffrey Young (Volumes 1—4), who established the basic essentials of the structure to which the Reports still adhere. Geoffrey retires this year from Oxford, where he has been a Fellow of Jesus College since 1947 (Vice-Principal 1969—1972, Acting Principal 1973—1977), University Lecturer in Organic Chemistry 1952—70, and Aldrichian Praelector in Chemistry since 1970. He was trained in carbohydrate chemistry at Bristol under E. L. Hirst but, after wartime work in explosives research, he turned to amino-acids and peptides. These have retained his interest ever since. His principal numbered series of full papers, which has almost made its half century, records a succession of important contributions to the art of peptide synthesis – especially on the racemization problem, on protective groups, and on the application of basic 'handles' to facilitate isolation and purification. A correspondent of Josef Rudinger from about 1949, he was one of the pioneering handful who were invited by him to the first of the European Peptide Symposia (Prague, 1958) and has been very prominent in the regular series thus initiated. Nearer home, he was the leading figure in the foundation of the Chemical Society Peptide and Protein Group and was its first Secretary 1968—1969 and later Chairman 1972—1975. He has earned

the affection of his pupils and the respect of everyone in the field; he steps down as the doyen of British peptide chemistry.

Balliol College, Oxford JOHN JONES
September 1982

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Abbreviations

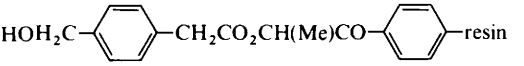
Abbreviations for amino-acids and their use in the formulations of derivatives follow, with some exceptions, the various Recommendations of the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature, which have been reprinted in Volumes 4, 5, and 8 in this series.

Other abbreviations that have been used are listed here or are defined in the text and table footnotes.

Ac	acetyl
Acm	acetamidomethyl
Ad	adamantyl
Adoc	adamantyloxycarbonyl
Adpoc	1-(1-adamantyl)-1-methylethoxycarbonyl
Aoc	t-amyloxycarbonyl
Aox	4,5-dianisoyl-4-oxazolin-2-one amino-acid derivative
Asu	α-aminosuberic acid
Asx	aspartic acid or asparagine (not yet determined)
ATP	adenosine 5′-triphosphate
Azoc	2-(4-phenylazophenyl)isopropyloxycarbonyl
Beoc	2-bromoethyloxycarbonyl
Boc	t-butoxycarbonyl
Bom	benzyloxymethyl
Bpoc	2-(4-biphenylyl)isopropoxycarbonyl
Br.Bom	4-Br-benzyloxymethyl
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-Cl$_2$)	2,6-dichlorobenzyl
Bzl(4-CN)	4-cyanobenzyl
Bzl(NO$_2$)	4-nitrobenzyl
Bzl(2-NO$_2$)	2-nitrobenzyl
Bzl(OMe)	4-methoxybenzyl
Cac	9-carbazolylcarbonyl
c.d.	circular dichroism
Cha	cyclohexylamine
CLIP	adrenocorticotropic hormone (18—39)
Cm	carboxymethyl
Cmc	*S*-carboxymethylcysteine

Cox	4,5-di-(4-chlorophenyl-4-oxazolin-2-one) amino-acid derivative
CPh_2Py	diphenyl-4-pyridylmethyl
DCCI	dicyclohexylcarbodi-imide
Dcha	dicyclohexylamine
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
Ddz	3,5-dimethoxy($\alpha\alpha$-dimethyl)benzyloxycarbonyl
DMA	dimethylacetamide
DMCBzl	dimethylcarbamoylbenzyl
DMCZ	dimethylcarbamoylbenzyloxycarbonyl
DMF	*NN*-dimethylformamide
DMSO	dimethyl sulphoxide
Dnp	2,4-dinitrophenyl
2,4-Dnps	2,4-dinitrosulphenyl
Dnpy	3,5-dinitro-1-(*p*-nitrophenyl)-4-pyridone
Dns	1-dimethylaminonaphthalene-5-sulphonyl (dansyl)
Dopa	3,4-dihydroxyphenylalanine
DP	degree of polymerization
DPA	diphenylacetyl
Dpp	diphenylphosphinoyl
Dppm	diphenyl-4-pyridylmethyl
DPtd	4,6-diphenylthieno[3,4-*d*][1,3]dioxal-2-one 5,5-dioxide
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
Dts	dithiosuccinoyl
Ec	ethylcarbamoyl
edta	ethylenediaminetetra-acetate
EEDQ	2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline
En	ethylenediamine
e.p.r.	electron paramagnetic resonance
e.s.r.	electron spin resonance
Et	ethyl
Fal	hexafluorovaline
Fmoc	9-fluorenylmethoxycarbonyl
Gal	galactose
g.c.–m.s.	gas chromatograph–mass spectrometer combination
g.l.c.	gas–liquid chromatography
Glc	glucose
Glp	2-pyrrolidone-5-carboxylic acid
Glx	glutamic acid or glutamine (not yet determined)
GTP	guanosine 5′-triphosphate
HMPA	hexamethylphosphoric triamide
HOBt	hydroxybenzotriazole
h.p.l.c.	high-performance liquid chromatography
Iboc	isobornyloxycarbonyl
i.r.	infrared
LDA	lithium di-isopropylamide
Mal=	maleoyl
Man	mannose

Mbh	4,4-dimethoxybenzhydryl
Mbs	4-methoxybenzenesulphonyl
Mds	4-methoxy-2,6-dimethylbenzenesulphonyl
Me	methyl
Mea	mercaptoethylamine
$MePh_2Peoc$	2-methyldiphenylphosphinioethyloxycarbonyl
Mhoc	1-methylcyclohexylcarbonyl
Moc	methoxycarbonyl
Msc	2-(methylsulphonyl)ethoxycarbonyl
Mtc	2-methylthioethyloxycarbonyl
MTM	methylthiomethyl
Mtr	2,3,6-trimethyl-4-methoxybenzenesulphonyl
Mts	mesitylenesulphonyl
NAD	nicotinamide-adenine dinucleotide (NAD^+ oxidized, NADH reduced)
NCA	*N*-carboxyanhydride
Nma	maleimido
Nmps	4-methyl-2-nitrosulphenyl
n.m.r.	nuclear magnetic resonance
Np	4-nitrophenyl
Nps	*o*-nitrophenylsulphenyl
Npys	3-nitropyridine-2-sulphenyl
Nsu	succinimido
OCp	2,4,5-trichlorophenoxy
OHFP	hexfluoroisopropyl ester
ONp	*p*-nitrophenoxy
ONp(*o*)	*o*-nitrophenoxy
ONp.Pic	2-nitro-4-(4-picolyloxycarbonyl)phenoxy
ONSu	succinimido-oxy
OPcp	pentachlorophenoxy
OPfp	pentafluorophenoxy
OPic	4-picolyloxy
Opop	phenacyl-2-hydroxyphenyl ester
o.r.d.	optical rotatory dispersion
OTAT	thiazoline-2-thione ester
OTce	2,2,2-trichloroethoxy
OTcp	2,4,5-trichlorophenoxy
Pac	phenacyl
PAC	phenylacetyl
Pam	4-(hydroxymethyl)phenylacetamidomethyl (resin)
Pcp	pentachlorophenyl
Peoc	2-triphenylphosphinioethyloxycarbonyl
Ph(SMe)	*p*-methylthiophenyl
Pic	4-picolyl
Picoc	4-picolyloxycarbonyl
Pipoc	piperidino-oxycarbonyl
Piv	pivaloyl

Pme	pentamethylbenzenesulphonyl
Pms	*p*-tolylmethylsulphonyl
Pop	
Ppoc	phenylisopropoxycarbonyl
Ppt	diphenylphosphinothioyl
Pth-Gly	the phenylthiohydantoin derived from glycine, *etc.*
Pz	*p*-phenylazobenzyloxycarbonyl
SBu^t	t-butylthio
SCB	t-butyloxycarbonylsulphenyl
Scm	carboxymethylsulphenyl
SDS	sodium dodecyl sulphate
Spr^i	isopropylthio
Sub	5-dibenzosuberyl
SZ	benzyloxycarbonylsulphenyl
Tac	toluene-*p*-sulphonylaminocarbonyl
tBA	t-butylammonium
TCBoc	2,2,2-trichloro-1,1,-dimethylethoxycarbonyl
Tcp	2,4,5-trichlorophenyl
Tcroc	2-CF_3-chromonylmethylenecarbonyl
Tcrom	2-CF_3-chromonylmethyl
Tfa	trifluoroacetyl
Thp	tetrahydropyranyl
t.l.c.	thin-layer chromatography
TMB	2,4,6-trimethylbenzyl
Tmeda	*NNN'N'*-tetramethylethylenediamine
TMG	tetramethylguanidine
TMSE	2-trimethylsilylethoxycarbonyl
Tnps	2,4,6-trinitrosulphenyl
Tos	toluene-*p*-sulphonyl
Troc	2,2,2-trichloroethyloxycarbonyl
Trt	triphenylmethyl
Tse	2-(toluene-*p*-sulphonyl)ethyl
u.v.	ultraviolet
Xan	9-xanthydryl
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

As in the preceding volumes of this Specialist Periodical Report, the coverage of the chemical and biochemical literature on the amino-acids is intended to be thorough, but the biological aspects, such as metabolism, biosynthesis, and distribution in the bio- and geo-spheres of the common amino-acids, are only covered through representative citations.

Textbooks and Reviews.—A textbook [1] and symposium proceedings,[2] covering general aspects and analysis, respectively, have been published in 1981. The analytical theme also features as a component of a review of the analysis of amino-acids and other constituents of single cells.[3] Physical and chemical properties of amino-acids [4] and theoretical analysis of hydration of amino-acids and peptides [5] have also been reviewed; other reviews appearing in the recent literature are cited in the appropriate sections of this chapter.

2 Naturally Occurring Amino-acids

Occurrence of Known Amino-acids.—The biosynthesis, occurrence, and properties of γ-carboxyglutamic acid have been reviewed,[6] and, on an extra-terrestrial level, the results for amino-acid content of moon soil and meteorites have been brought together.[7]

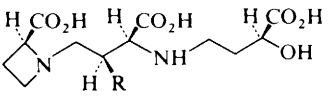
In selecting papers from the 1981 literature for inclusion in this section, the broad routine literature on the distribution of the common amino-acids is largely excluded. As in earlier volumes, however, items of exceptional interest, usually relating to geological or extra-terrestrial sources, are cited. The distribution of common and uncommon amino-acids in the Isua rocks of south-western Greenland of age *ca.* 3800 My has been determined [8] to counter a claim, based on laser-Raman spectroscopic analysis, that amino-acids in microfossil crystal regions (and therefore insulated from leaching and infusion processes) may be of ancient derivation. Since amino-acids that are unstable to geological processes were found

[1] 'Aminosäuren', J. Breuer and S. Kowalewsi, Thieme Verlag, Stuttgart, 1981.
[2] 'Proceedings of a Symposium on Amino-acid Analysis', ed. J. M. Rattenbury, Horwood, Chichester, 1981.
[3] N. N. Osborne in 'Methods in Neurobiology', ed. R. LaRue, Plenum Press, New York, 1981, Vol. 1, p. 135.
[4] P. O. Larsen in 'Biochemistry of Plants', ed. B. J. Miflin, 1980, Vol. 5, p. 225.
[5] Y. Paterson, G. Nemethy, and H. A. Scheraga, *Ann. N.Y. Acad. Sci.*, 1981, **367**, 132.
[6] J. P. Bumier, M. Borowski, B. C. Furie, and B. Furie, *Mol. Cell. Biochem.*, 1981, **39**, 191.
[7] S. W. Fox, K. Harada, and P. E. Hare, *Subcell. Biochem.*, 1981, **8**, 357.
[8] B. Nagy, M. H. Engel, J. E. Zumberge, H. Ogino, and S. Y. Chang, *Nature (London)*, 1981, **289**, 53.

in the crushed rock samples,[8] it was contended that these were laid down since the last of the Isua metamorphic episodes; the extent of racemization is by no means complete, suggesting a date of a few ten thousand years and also implying that the amino-acid content is the result of continuous seepage into the rocks from lichen since the end of the last ice age.[8] The structures of a group of amino-acids found in the Murchison meteorite have also been discussed in relation to inferences that might be drawn – are they synthesized by the condensation of simple precursors under the influence of energy input and catalysts, or are they the remnants of living organisms, racemized and degraded or rearranged through the trauma undergone by the meteorite preceding its arrival on Earth? The presence of six of the seven acyclic six-carbon amino-acids in the Murchison meteorite has been confirmed,[9] although their relative proportions differ from those for amino-acid mixtures formed through electric discharge and Fischer–Tropsch-type syntheses. The major structural type represented in the amino-acid content of this meteorite is the α-branched α-amino-acid; the positive identification of 2-amino-2-ethylbutyric acid, 2-amino-2,3-dimethylbutyric acid, pseudo-leucine (*C*-t-butylglycine), and 2-methylnorvaline represents the first report of the 'natural' occurrence of these amino-acids.[9] Leucine and isoleucine (also *allo*-isoleucine), reported earlier, were also confirmed to be present, and the presence of norleucine is described as 'probable'.[9]

Thirteen common amino-acids have been identified in the 6M hydrochloric acid hydrolysate of humic acids extracted from Spanish lignite.[10]

Some other 'first occurrences' of amino-acids in plant sources have been reported. N^{δ}-Methylornithine has been found in *Atropa belladonna* and shown to be the biosynthetic precursor of atropine.[11] *N*-Carboxymethyl-L-serine is present



(1) R = OH, mugineic acid
(2) R = H, 2′-deoxymugineic acid

in asparagus shoots.[12] Mugineic acid[13] (1) (see Vol. 12, p. 3) and 2′-deoxymugineic acid[14] (2) are iron(II)-chelating constituents of wheat and barley; the 2′-deoxy compound was isolated from root washings of water-cultured wheat (*Triticum aestivum*) under iron-deficient media.[14] *N*-Malonyl-1-aminocyclopropanecarboxylic acid has been recognized as a metabolite of 1-aminocyclopropanecarboxylic acid in buckwheat,[15] while α-(methylenecyclopropyl)glycine, formed from threonine and a one-carbon unit from methionine, is an intermediate in the biosynthesis of β-(methylenecyclopropyl)alanine (the toxic amino-acid,

[9] J. R. Cronin, W. E. Gandy, and S. Pizzarella, *J. Mol. Evol.*, 1981, **17**, 265.
[10] R. Moliner and J. M. Gavilan, *Fuel*, 1981, 64 (*Chem. Abstr.*, 1981, **94**, 124358).
[11] S. H. Hedges and R. B. Herbert, *Phytochemistry*, 1981, **20**, 2064.
[12] T. Kasai and S. Sakamura, *Agric. Biol. Chem.*, 1981, **45**, 1483.
[13] Y. Sugiura, H. Tanaka, Y. Mino, T. Ishida, N. Ota, M. Inoue, K. Nomoto, H. Yoshioka, and T. Takemoto, *J. Am. Chem. Soc.*, 1981, **103**, 6979.
[14] K. Nomoto, H. Yoshioka, M. Arima, S. Fushiya, S. Takagi, and T. Takemoto, *Chimia*, 1981, **35**, 249.
[15] N. Amrhein, D. Schneebeck, H. Skorupka, S. Tophof, and J. Stoeckigt, *Naturwissenschaften*, 1981, **68**, 619.

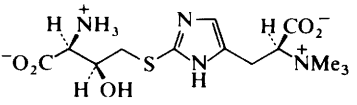
hypoglycin, present in *Blighia sapida*).[16] Briefer mention can be made of other amino-acids in new locations: L-4-oxalysine in *Streptomyces roseoviridofuscus*,[17] 2,4-*trans*-4,5-dihydroxypipecolic acid and *cis*-5-hydroxypipecolic acid from leaves of *Calliandra angustifolia* and the sap of *Calliandra confusa*,[18] 4-methyleneglutamine and 4-methyleneglutamic acid in newly germinated peanut (*Arachis hypogaea*),[19] γ-methyl-L-glutamic acid (*erythro*/*threo* mixture) in *Mycena pura* and *Wynnea gigantea* fruiting bodies,[20] (2*S*,4*S*)-4-hydroxyglutamic acid as the predominant isomer of this amino-acid in *Phlox* species, the (2*S*,4*S*) isomer also found there being the first finding for this isomer in this species,[21] of which *Ledenbergia rose-aenea* and *Pandanus veitcheii* also contain 4-hydroxy-4-methylglutamic acid.[21]

Although non-tolerant seedling roots of *Armeria maritima* contain 1.5—3.0% L-proline, copper-tolerant strains contain extraordinarily high levels (11.0%) of this imino-acid, which seems to be a feature of the tolerance and not accumulated as a response to the stress.[22]

meso-Diaminopimelic acid predominates in the vegetative mycelium of the setamycin-producing actinomycete KM-6054, while the aerial mycelium contains predominantly the LL-diastereoisomer.[23]

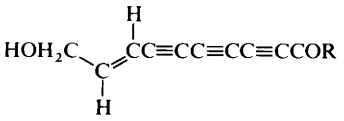
Unusual amino-acid residues in proteins include N^{ε}-trimethyl-lysine, in two histone-like proteins from wheat germ,[24] and dihydroxylysinonorleucine, shown to be present in collagen when the other well established crosslinking residue pyridinoline is also present.[25]

New Natural Amino-acids.—Several new amino-acids have been discovered in fungal and bacterial cultures, some with antibiotic properties [L-2-(1-methylcyclopropyl)glycine from *Micromonospora miyakonensis*[26] and 4-oxo-5-amino-6-hydroxyhexanoic acid from *Bacillus cereus* 102 804[27]]. Clithioneine (3) is a new amino-acid betaine from *Clitocybe acromelalga*;[28] polyacetylenic amino-acids (4)

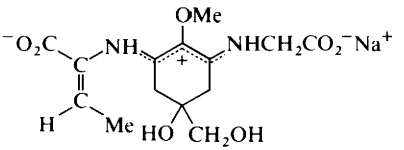


(3)

[16] E. A. Kean and C. E. Lewis, *Phytochemistry*, 1981, **20**, 2161.
[17] Q.-Z. Bao, H.-L. Zhang, S.-H. Xu, Y.-L. Hu, S.-X. Liu, and Y.-F. Ye, *Wei Sheng Wu Hsueh Pao*, 1981, **21**, 218 (*Chem. Abstr.*, 1982, **95**, 95 394).
[18] A. B. Bleecker and J. T. Romeo, *Phytochemistry*, 1981, **20**, 1845.
[19] H. C. Winter, G. K. Powell, and E. E. Dekker, *Plant Physiol.*, 1981, **68**, 588.
[20] S. Hatanaka and K. Takishima, *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo*, 1981, **31**, 33 (*Chem. Abstr.*, 1982, **95**, 129 385).
[21] E. A. Bell, L. K. Meier, and H. Sörensen, *Phytochemistry*, 1981, **20**, 2213.
[22] M. E. Farago and W. A. Mullen, *Inorg. Nucl. Chem. Lett.*, 1981, **17**, 275.
[23] S. Omura, Y. Iwai, Y. Takahashi, K. Kojima, K. Otoguro, and R. Oiwa, *J. Antibiot.*, 1981, **34**, 1633.
[24] K. Motojima and K. Sakaguchi, *FEBS Lett.*, 1981, **132**, 334.
[25] Y. Kuboki, M. Tsuzaki, S. Sasaki, C. F. Liu, and G. L. Mechanic, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 119.
[26] J. Shoji, R. Sakazaki, T. Kato, K. Tori, Y. Yoshimura, and S. Matsuura, *J. Antibiot.*, 1981, **34**, 370.
[27] K. L. Perlman, U. Schoemer, T. H. Williams, and D. Perlman, *J. Antibiot.*, 1981, **34**, 483.
[28] K. Konno, H. Shirahama, and T. Matsumoto, *Tetrahedron Lett.*, 1981, **22**, 1617.



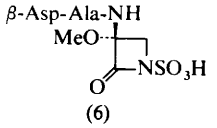
(4) R = OMe, Ala-OMe, or Gly-OMe



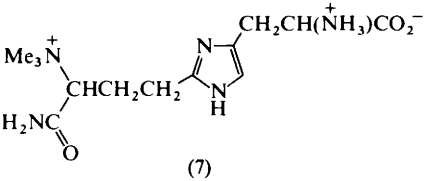
(5)

from *Fayodia bisphaerigera*,[29] L-α-amino-γ-nitraminobutyric acid from *Agaricus subrutilescens* (β-nitramino-L-alanine is also present),[30] and (5), a close relative of the palythene–mytilin group of amino-acid derivatives (see Vol. 12, p. 3, and Vol. 11, p. 4, respectively),[31] are also new natural products. Root nodule hydrolysates from *Lotus tenuis* (hosts to two strains of *Rhizobium* bacteria) contain 2,4-diamino-3-methylbutanoic acid, tentatively assigned the (2*R*,3*S*) configuration based on retention times on g.l.c. over a chiral stationary phase.[32] *N*-Acetyl-L-2-amino-3-(*o*-tolyloxy)propionic acid has been confirmed by synthesis as a metabolite of mephenesin.[33]

New Amino-acids from Hydrolysates.—The title of this section is to be interpreted this year to cover structure assignments to new amino-acid constituents of peptides and proteins. *Micromospora chalcea* produces the dipeptide *N*-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine,[34] while the novel antibiotic sulfazecin (from *Pseudomonas acidophila*) has the structure (6).[35]



(6)



(7)

The new amino-acid in baker's yeast protein elongation factor EF-2, which is ADP-ribosylated in the diphtheria toxin, has been christened diphthamide and assigned structure (7).[36] A protein containing hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] has been isolated from human lymphocytes treated with mitogen and grown in the presence of [^{3}H]putrescine or [^{3}H]spermidine.[37]

[29] M. Ahmed, M. Y. Jarrah, E. R. H. Jones, A. F. Magalhaes, M. G. Roberts, and V. Thaller, *J. Chem. Res. (S)*, 1981, 262.

[30] S. Hatanaka, *Nippon Kingakkai Kaiho*, 1981, **22**, 213.

[31] J. Kobayashi, H. Nakamura, and Y. Hirata, *Tetrahedron Lett.*, 1981, **22**, 3001.

[32] G. J. Shaw, P. J. Ellingham, and L. N. Nixon, *Phytochemistry*, 1981, **20**, 1853.

[33] N. Kosui, T. Kato, N. Izumiya, T. Kuhara, and I. Matsumoto, *Mem. Fac. Sci., Kyushu Univ., Ser. C*, 1981, **13**, 97.

[34] J. Shoji, H. Hinoo, T. Kato, K. Nakauchi, S. Matsuura, M. Mayama, Y. Yasuda, and Y. Kawamura, *J. Antibiot.*, 1981, **34**, 374.

[35] K. Kintaka, K. Kitano, Y. Nozaki, F. Kawashima, A. Imada, Y. Nakao, and M. Yoneda, *J. Ferment. Technol.*, 1981, **59**, 263; M. Asai, K. Haibara, M. Muroi, K. Kintaka, and T. Kishi, *J. Antibiot.*, 1981, **34**, 621.

[36] J. W. Bodley, B. G. Van Ness, and J. B. Howard, *Dev. Cell Biochem. (Amsterdam)*, 1980, **6**, 413; B. G. Van Ness, J. B. Howard, and J. W. Bodley, *J. Biol. Chem.*, 1980, **255**, 10710, 10717.

[37] H. L. Cooper and J. E. Folk, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 2869.

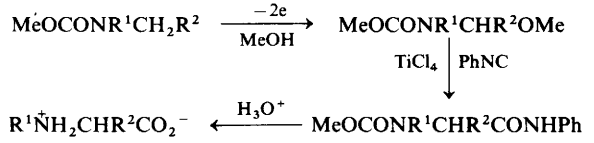
3 Chemical Synthesis and Resolution of Amino-acids

General Methods of Synthesis of α-Amino-acids.—Standard general syntheses and newer methods of potentially general character are collected in this section, although later sections also provide details of specific synthetic objectives achieved through the use of generally applicable methods.

Amino-acids may be synthesized in satisfactory yields from ammonia and the corresponding halogeno-acid, even though textbook warnings, that mixtures of amines result from this approach, linger in the minds of all organic chemists. DL-Alanine is obtained in 76% yield when a 10:1 molar ratio of NH_3 to α-chloropropionic acid is heated under pressure at 70 °C during 5 hours in aqueous solution.[38] A related approach, reaction of an alkali-metal cyanate with the halogeno-acid ester at 80—100 °C in dipolar aprotic solvents, has been developed further;[39] hydrolysis with dilute hydrochloric acid completes the synthesis in this variation.

Dialkyl acylamidomalonates $R^1CONHCH(CO_2R^2)_2$ continue to serve the need for reliable routes to α-amino-acids, in cases where the side chain to be introduced by alkylation of these substrates can withstand the conditions for cleavage and decarboxylation of the substituted malonate. Recent examples[40, 41, 46, 118, 123, 137] include a synthesis of 7-chloro-DL-tryptophan *via* $AcNHC(CO_2Me)_2CH_2CH_2CHO$[40] and of 4-methyleneglutamic acid from acetamidomalonate, HCHO, and diethyl oxalate.[41]

General routes to amino-acids could not reasonably be expected to develop from the reactions based on simple reactants, which have been studied as models of prebiotic synthesis. However, glow-discharge electrolysis of aliphatic amines[42] in aqueous solution gives amino-acids in addition to hydroxyalkylamines. While ethylamine gives glycine in this process, propylamine gives β-alanine and isoserine.[42] Reasonable yields of α-amino-acid amides are formed from urethanes, by anodic oxidation to give α-methoxyurethanes, followed by reaction with phenyl isocyanide, then hydrolysis:[43]



Ugi four-component condensation synthesis is gaining in favour because of variations that simplify the release of the amino-acid from the initial condensation product.[44,45,90] The product from *N*-benzyloxycarbonylglycine, 9-(aminomethyl)fluorene, benzaldehyde, and cyclohexylisocyanide carries the base-labile

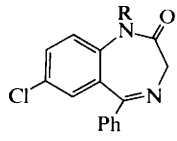
[38] Y. Ogata and M. Inaishi, *Kenkyu Hokaku-Asahi Garasu Kogyo Gijitsu Shoreikai*, 1980, **36**, 219 (*Chem. Abstr.*, 1982, **95**, 43613).
[39] F. Effenberger, K. Drauz, S. Förster, and W. Schöller, *Chem. Ber.*, 1981, **114**, 173.
[40] K. H. Van Pee, O. Salcher, and F. Lingens, *Liebigs Ann. Chem.*, 1981, 233.
[41] G. K. Powell and E. E. Dekker, *Prep. Biochem.*, 1981, **11**, 339.
[42] K. Harada, M. M. Nomoto, and H. Gunji, *Tetrahedron Lett.*, 1981, **22**, 769.
[43] T. Shono, Y. Matsumura, and K. Tsubata, *Tetrahedron Lett.*, 1981, **22**, 2411.
[44] C. F. Hoyng and A. D. Patel, *J. Chem. Soc., Chem. Commun.*, 1981, 491.
[45] P. Bukall and I. Ugi, *Heterocycles*, 1981, **15**, 381.

fluoren-9-ylmethyl group, removable with 1.1 equivalents 1,8-diazabicyclo-[5.4.0]undec-7-ene in pyridine.[44] Use of standard conditions for the four-component condensation in the synthesis of pyridylglycine derivatives has been described.[45]

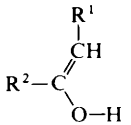
The azlactone synthesis has been used for the preparation of metallocene analogues of phenylalanine, starting from the corresponding metallocene aldehydes.[46] 4-Substituted oxazolin-5-ones give the corresponding 4,4-disubstituted analogues through Michael addition to electron-deficient alkenes.[47]

Schiff-base alkylation usually gives the α-substitution product, but the anion formed from *N*-benzylidenealanine benzyl ester in conditions established by Stork and co-workers[48] in their general synthesis based on the glycine analogue (−78 °C, HMPA–THF, LDA) leads to a γ-alkylation product with t-butyl 2-bromo-3-phenylpropionate.[49] Although the influence of steric hindrance, solvent, and other rate-controlling factors must account for the predominance of γ-alkylation in this case, this adds a further uncertainty to the method, which has already been shown to give αα-dialkylation products in various proportions, with the desired mono-alkylation product in the case of glycine Schiff bases.

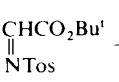
Like the oxazolinones and analogous thiazolines, when considered only as the Δ^2-isomer (other tautomeric forms do exist[50]), the benzodiazepinone (8) represents a cyclic Schiff base. Substitution products involving the methylene group have been reported,[51] including ^1H–^2H exchanged amino-acids obtained through alkaline hydrolysis of the substituted benzodiazepinone, apparently with no isomeric substitution product or di-substitution product.



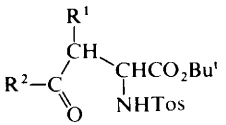
(8)



+



(9)



(10)

γ-Oxoalkyl-α-amino-acids (10) may be prepared through an ene reaction involving a *N*-toluene-*p*-sulphonylimide (9), followed by cleavage of the *N*-protecting group.[52]

General Methods of Synthesis of β- and Higher Homologous Amino-acids.—Routes to compounds carrying amino and carboxy groups separated by two or more carbon atoms are usually based on routine methods for introducing one of these functional groups into a compound already carrying the other. The Arndt–Eistert and Wolff rearrangement procedures provide an alternative approach, employing an α-amino-acid as starting material. New routes to β-amino-acids involve

[46] J. C. Brunet, E. Cuingnet, H. Gras, P. Marcincal, A. Mocz, C. Sergheraert, and A. Tartar, *J. Organomet. Chem.*, 1981, **216**, 73.
[47] H. Wegmann and W. Steglich, *Chem. Ber.*, 1981, **114**, 2581.
[48] G. Stork, A. Y. W. Leong, and A. Touzin, *J. Org. Chem.*, 1976, **41**, 3491.
[49] C. J. Harris, *Tetrahedron Lett.*, 1981, **22**, 4863.
[50] G. C. Barrett, *Tetrahedron*, 1980, **36**, 2023.
[51] E. Decorte, R. Toso, A. Sega, V. Sunjic, Z. Ruzic-Toros, B. Kojic-Prodic, N. Bresciani-Pahor, G. Nardin, and L. Randaccio, *Helv. Chim. Acta*, 1981, **64**, 1145.
[52] O. Achmatowicz and M. Pietraszkiewicz, *Tetrahedron Lett.*, 1981, **22**, 4323.

addition of a vinyloxyborane to a Schiff base,[53] aminomercuration of αβ-unsaturated esters followed by demercuration

$$R^1NHAr + Hg(OAc)_2 + R^2CH{=}CR^3CO_2R^4 \longrightarrow R^1NArCHR^2CR^3(HgOAc)CO_2R^4$$

with $NaBH_4$,[54] and condensation of *N*-thioacylurethanes with ethoxycarbonylmethylenetriphenylphosphorane

$$EtO_2CNHC(=S)R + Ph_3P{=}CHCO_2Et \longrightarrow EtO_2CNHCR{=}CHCO_2Et$$

followed by Pt-catalysed hydrogenation.[55] Yields in all these new methods are good or excellent. Wolff rearrangement of *N*-phthaloyl-D- or -L-valine (successive treatment with $SOCl_2$, diazomethane, and u.v. light) provides the β-leucine enantiomers in up to 68% yield.[56]

Asymmetric Synthesis of Amino-acids.—Extensions of established methods provide most of the references for this section. Enantioselective catalytic hydrogenation of α-acylamino-acrylates and -cinnamates continues to be studied by research groups already publishing prolifically in this area, which is also attracting newcomers.[57-59] Enantiomeric excess levels are remarkably uniform, around 75—80%, in all these reports in which open-chain substrates are used (although 84—99% levels were reported in one study[57]), in which there is the common feature of Rh–chiral phosphine complex homogeneous catalysis. More rigid systems (cyclodipeptides of α-aminoacrylates with an L-amino-acid) give 66—90% chiral induction of hydrogenation over palladized charcoal,[58] but reductive methanolysis of 2-methyl- and 2-phenyl-4-benzylideneoxazolin-5-ones in the presence of $PdCl_2$ and (*S*)-phenylethylamine gives less satisfactory results.[59] The *Z*-isomer leads predominantly to the L-phenylalanine derivative through this procedure, whereas hydrogenation of *Z*-α-benzamidocinnamic acid in the presence of (*S*)-phenylethylamine gives predominantly *N*-benzoyl-D-phenylalanine.[59] The chiral Schiff base formed between (*S*)-1-ferrocenylethylamine and pyruvic acid gives a mixture of *N*-substituted alanines in which the L-isomer predominates, through hydrogenation over palladized charcoal.[60] In an alternative approach based on Schiff bases, the glycine derivative (11) yields *threo*-β-hydroxy-α-amino-

[53] M. Otsuka, M. Yoshida, S. Kobayashi, M. Ohno, Y. Umezawa, and H. Morishima, *Tetrahedron Lett.*, 1981, **22**, 2109.

[54] J. Barluenga, J. Villamana, and M. Yus, *Synthesis*, 1981, 375.

[55] M. Slopianka and A. Gossauer, *Synth. Commun.*, 1981, **11**, 95.

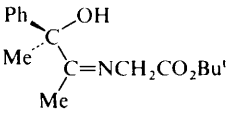
[56] S. R. Sylvester and C. M. Stevens, *Proc. West. Pharmacol. Soc.*, 1981, 24th Meeting, 117 (*Chem. Abstr.*, 1981, **95**, 13328).

[57] (*a*) H. Brunner, W. Pieronczyk, B. Schoenhammer, K. Streng, I. Bernal, and J. Korp, *Chem. Ber.*, 1981, **114**, 1137; (*b*) D. Lafont, D. Sinou, G. Descotes, R. Glaser, and S. Geresh, *J. Mol. Catal.*, 1981, **10**, 305; (*c*) G. L. Baker, S. J. Fritschel, J. R. Stille, and J. K. Stille, *J. Org. Chem.*, 1981, **46**, 2954; (*d*) P. A. MacNeil, N. K. Roberts, and B. Bosnich, *J. Am. Chem. Soc.*, 1981, **103**, 2273; (*e*) J. Irurre, A. Bosch, and J. Capdevila, *Afinidad*, 1981, **38**, 201; (*f*) U. Nagel, H. Menzel, P. W. Lednor, W. Beck, A. Guyot, and M. Bartholin, *Z. Naturforsch., Teil B*, 1981, **36**, 578; (*g*) D. Sinou, *Tetrahedron Lett.*, 1981, **22**, 2987; (*h*) J. Bakos, I. Toth, and L. Marko, *J. Org. Chem.*, 1981, **46**, 5427; (*i*) W. Bergstein, A. Kleemann, and J. Martens, *Synthesis*, 1981, 76.

[58] T. Kanmera, S. Lee, H. Aoyagi, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1980, **16**, 280.

[59] L. F. Godunova, E. S. Levitina, E. I. Kerpeiskaya, and E. I. Klabunovskii, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1981, 815.

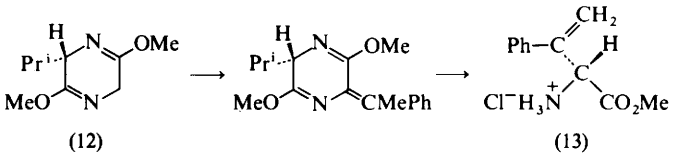
[60] A. Ratajczak and A. Czech, *Bull. Acad. Pol. Sci., Ser. Sci. Chim.*, 1979, **27**, 661.



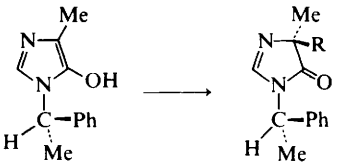
(11)

acids of high optical purity through addition of the derived anion to an aldehyde.[61]

Further uses of chiral dihydropyrazines (12) in the synthesis of a wide variety of α-amino-acids through enantioselective alkylation[62] and the methyl homologue of (12) in the synthesis of chiral α-methyl-α-amino-acids[63] have been reported (see also Vol. 13, p. 5). Condensation of (12) with PhCOMe and cleavage with HCl gives the βγ-unsaturated D-amino-acid (13) in 64% yield.[64] Extension of this approach to 1-chiral-substituted 4-methyl-2-imidazolin-5-ones (14), as a route to L-α-methylphenylalanine and analogues, achieves asymmetric induction at levels better than 95%.[65]



(12) (13)



(14)

A mixed-ligand cobalt(III) complex of D- or L-alanine with (6*R*,8*R*)-6,8-dimethyl-2,5,9,12-tetra-azatridecane, formed from the corresponding α-amino-α-methylmalonic acid complex through enantioselective decarboxylation (*cf.* ref. 66; Vol. 7, p. 5), has been shown[67] to release the amino-acid under conditions that do not cause racemization (a demerit of the earlier studies of this type).

Prebiotic Synthesis; Model Reactions.—Extensions of work featuring in all earlier volumes of this series are represented in near-u.v. irradiation of a suspension of Pt on TiO_2 in water under CH_4 and NH_3,[68] or Pt-catalysed decomposition of H_2O_2 in water under CH_4 and NH_3,[68] and hydrolysis of the reaction product of carbon

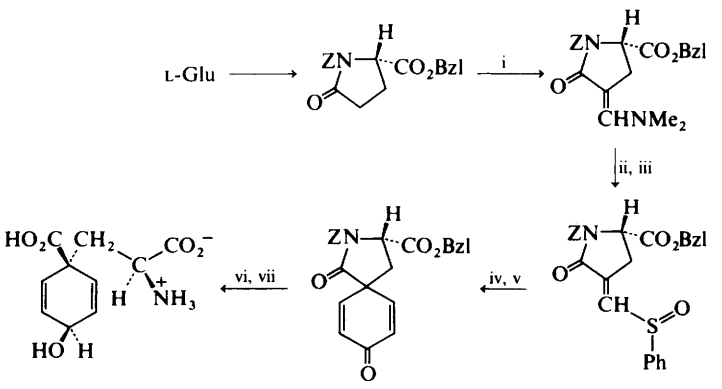
[61] T. Nakatsuka, T. Miwa, and T. Mukaiyama, *Chem. Lett.*, 1981, 279.
[62] U. Schöllkopf, U. Groth, and C. Deng, *Angew. Chem.*, 1981, **93**, 793.
[63] U. Schöllkopf, W. Hartwig, U. Groth, and K. O. Westphalen, *Liebigs Ann. Chem.*, 1981, 698.
[64] U. Schöllkopf and U. Groth, *Angew. Chem.*, 1981, **93**, 1022.
[65] U. Schöllkopf, H. H. Hausberg, M. Segal, U. Reiter, I. Hoppe, W. Sänger, and K. Lindner, *Liebigs Ann. Chem.*, 1981, 439.
[66] R. C. Job and T. C. Bruice, *J. Am. Chem. Soc.*, 1974, **96**, 809.
[67] M. Ajioka, S. Yano, K. Matsuda, and S. Yoshikawa, *J. Am. Chem. Soc.*, 1981, **103**, 2459.
[68] W. W. Dunn, Y. Aikawa, and A. J. Bard, *J. Am. Chem. Soc.*, 1981, **103**, 6893.

vapour (from a carbon-arc discharge) with ammonia.[69] The latter system creates glycine, alanine, β-alanine, *N*-methylglycine, serine, and aspartic acid, these last two amino-acids being formed with glycine and alanine in formaldehyde–hydroxylamine reaction mixtures [70] and arising from the reaction of the glycine with formaldehyde.[70]

Contact glow-discharge electrolysis of amines in aqueous solutions gives mixtures of amino-acids.[42]

Protein and Other Naturally Occurring Amino-acids.—The fermentative production and other biosynthetic routes to amino-acids continue to be well represented in the literature. Typical results cover the production of L-lysine by mutants of *Bacillus licheniformis*,[71] of L-arginine by mutant organisms resistant to L-arginine analogues,[72] and of L-aspartic acid by whole *Escherichia coli* cells immobilized on polyurethane, with ammonium fumarate as substrate.[73] *trans*-Cinnamic acid gives L-phenylalanine in nutrient media containing L-phenylalanine ammonia-lyase.[74] Familiar biosynthetic studies are represented in the establishment of L-pipecolic acid as an intermediate in the conversion of D-lysine into its enantiomer in *Nicotiana glauca*.[75]

A route from L-glutamic acid to L-tyrosine (Scheme 1) [76] involves a Diels–Alder addition to a vinyl sulphoxide as the key step and has been used in a total synthesis of pretyrosine (arogenic acid) and its epimer.[77]



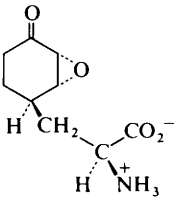
Reagents: i, HCHO, Me_2NH; ii, Ph_2S_2, Bu_3P; iii, *m*-chloroperbenzoic acid; iv, $MeOCH{=}CHC(=CH_2)OSiMe_3$; v, H_2O; vi, $NaBH_4$; vii, 2M NaOH–MeOH, 70 °C, 20—48 h

Scheme 1

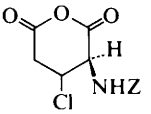
[69] P. B. Shevlin, D. W. McPherson, and P. Melius, *J. Am. Chem. Soc.*, 1981, **103**, 7006.
[70] F. Egami, *Origins Life*, 1981, **11**, 197.
[71] H. Hagino, S. Kobayashi, K. Araki, and K. Nakayama, *Biotechnol. Lett.*, 1981, **3**, 425.
[72] H. Yoshida, K. Araki, and K. Nakayama, *Agric. Biol. Chem.*, 1981, **45**, 959.
[73] M. C. Fusee, W. E. Swann, and G. J. Calton, *Appl. Environ. Microbiol.*, 1981, **42**, 672.
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[76] S. Danishefsky, J. Morris, and L. A. Clizbe, *Heterocycles*, 1981, **15**, 1205.
[77] S. Danishefsky, J. Morris, and L. A. Clizbe, *J. Am. Chem. Soc.*, 1981, **103**, 1602.
[78] B. C. Laguzza and B. Ganem, *Tetrahedron Lett.*, 1981, **22**, 1483.

The first chiral total synthesis of anticapsin from L-tyrosine has been announced.[78] Although this natural amino-acid (15) seems eminently approachable from L-tyrosine, its sensitive epoxycyclohexanone moiety rules out conventional protection strategies. The success of the present routine is due to the use of a new acid- and base-resistant N-protecting group that is inert to nucleophilic attack.[78]

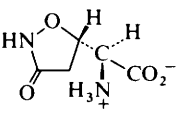
A simple synthesis of cysteine involves the condensation of chloroacetaldehyde with NH_3, acetone, and NaSH in aqueous solution at 0—10 °C, addition of HCN to the resulting 2-thiazoline, and hydrolysis with hydrochloric acid.[79] Another conversion of glutamic acid into proline *via* pyroglutamic acid employs successive treatment with P_4S_{10} and Raney nickel for the conversion of $>C{=}O$ to $>CH_2$;[80] it must be a relatively expensive operation for the purpose.



(15)



(16)



(17)

Routes to more distantly related naturally occurring amino-acids rarely involve the protein amino-acids as starting materials, though L-glutamic acid has been used[81] in a new stereospecific total synthesis of (α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazole-acetic acid, the anti-tumour compound AT-125 (see also Vol. 12, p. 9). The key step was Kollonitsch chlorination of the starting material to give β-chloroglutamic acid (as a 1 : 1 *erythro* : *threo* mixture) and conversion of the derived anhydride (16) into tricholomic acid (17), from which AT-125 has already been synthesized (Vol. 12, p. 9). Baldwin and co-workers have continued to explore synthetic routes to this amino-acid,[82] and an efficient synthesis starting from a protected dehydroglutamic γ-hydroxamate and ending eight steps later with hog kidney acylase I 'resolution' of the chloroacetylamino-acid has been reported.[82]

(−)-3-Aminocardicinic acid, a residue in the antibiotic nocardicin A, has been shown in two studies[83,84] to be accessible from an L-serine derivative through β-lactam formation using Ph_3P with CCl_4 and NEt_3 on L-serine *O*-benzylhydroxamate,[83] or Ph_3P and diethyl azodicarboxylate for the same purpose,[84] mimicking the biosynthesis of the four-membered ring. Homoserine derivatives feature in the synthesis of (+)-avenic acid A, $HO[CH_2CH_2CH(CO_2H)NH]_2CH_2CH_2CH(OH)CO_2H$,[85,86] and its azetidine analogue, 2′-deoxymugineic acid (2).[86,87] In these studies, L-*N*-(3-hydroxy-3-carboxypropyl)homoserine aldehyde was coupled

[79] J. Martens, H. Offermanns, and P. Scherberich, *Angew. Chem.*, 1981, **93**, 680.
[80] A. Kleemann, J. Martens, and K. Drauz, *Chem.-Ztg.*, 1981, **105**, 266.
[81] R. B. Silverman and M. W. Holladay, *J. Am. Chem. Soc.*, 1981, **103**, 7357.
[82] J. E. Baldwin, L. I. Kruse, and J.-K. Cha, *J. Am. Chem. Soc.*, 1981, **103**, 942.
[83] P. G. Mattingly and M. J. Miller, *J. Org. Chem.*, 1981, **46**, 1557.
[84] C. A. Townsend and L. J. Nguyen, *J. Am. Chem. Soc.*, 1981, **103**, 4582.
[85] Y. Ohfune and K. Nomoto, *Chem. Lett.*, 1981, 827.
[86] S. Fushiya, Y. Sato, S. Nakatsuyama, N. Kanuma, and S. Nozoe, *Chem. Lett.*, 1981, 909.
[87] Y. Ohfune, M. Tomita, and K. Nomoto, *J. Am. Chem. Soc.*, 1981, **103**, 2409.

with another homoserine derivative,[85, 86] while a longer route from L-malic acid to 2'-deoxymugineic acid (also accessible through the homoserine route[86]) has been described.[87]

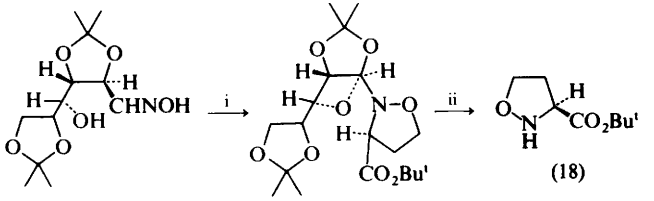
Standard routes have been employed in the syntheses of β-carboxyaspartic acid [from 2-bromo-1,1,2-tris(ethoxycarbonyl)ethane[88] and from 5-chlorohydantoin and diethyl malonate[89]], all isomers of furanomycin (2-methyl-2,5-dihydrofuran-5-ylglycine) through four-component condensation,[90] both diastereoisomers of (±)-2-amino-4-methyl-5-hexenoic acid from (2*R*,4*S*)-CH_2=CHCHMeCH$_2$-CHBrCO$_2$Me,[91] and (±)-dihydroviomycidine and (±)-viomycidine *via* alkylation of carboxylatoethylideneglycinatocopper with PhCH$_2$OCH$_2$CH(OEt)$_2$.[92]

An example of a new naturally occurring β-amino-acid derivative, and its synthesis by a straightforward method, is provided by 3-(*N*-methylamino)glutaric acid.[93]

α-Alkyl Analogues of Protein Amino-acids.—As well as the examples mentioned in the preceding paragraphs, α-alkylation of aspartic acid (protected as the *N*-formyl di-t-butyl ester) with an alkyl or allyl bromide or iodide in the presence of LDA in THF at −78 °C has been established.[94] Optical activity is preserved in this procedure, but substantial amounts of the β-alkylation product are also formed.

***C*-Alkyl and Substituted *C*-Alkyl Amino-acids.**—This section covers close relatives of the common amino-acids; several such examples have been included in other sections of this chapter. Hofmann rearrangement of Boc- or *Z*-L-asparagine using (CF$_3$CO$_2$)$_2$IPh gives L-2,3-diaminopropanoic acid.[95]

Several papers dealing with proline derivatives and analogues appear in the recent literature. *N*-Chlorination of esters of norvaline, *allo*-isoleucine, isoleucine, and leucine and base-induced Hofmann–Loeffler–Freytag cyclization give proline and *cis*- and *trans*-3- and -4-methylprolines.[96] 5-Oxaproline (18) has been obtained starting from D-mannose oxime (Scheme 2).[97] A 54% enantiomeric



(18)

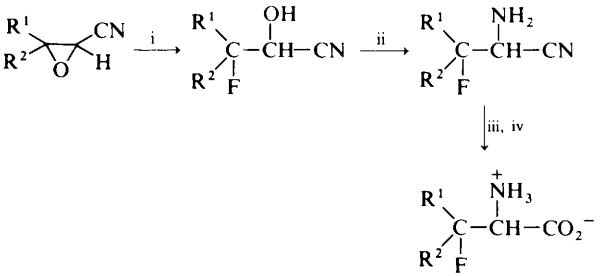
Reagents: i, Bu^{t}OCOCHO + CH$_2$=CH$_2$, 75 °C, 65 bar, 17 h; ii, H_3O^+

Scheme 2

[88] M. R. Christy, R. M. Barkley, T. H. Koch, J. J. Van Buskirk, and W. M. Kirsch, *J. Am. Chem. Soc.*, 1981, **103**, 3935.
[89] E. B. Henson, P. M. Gallop, and P. V. Hauschka, *Tetrahedron*, 1981, **37**, 2561.
[90] J. E. Semple, P. C. Wang, Z. Lysenko, and M. M. Jouillie, *J. Am. Chem. Soc.*, 1980, **102**, 7505.
[91] B. B. Snider and J. V. Duncia, *J. Org. Chem.*, 1981, **46**, 3223.
[92] T. Wakamiya, K. Konishi, H. Chaki, T. Teshima, and T. Shiba, *Heterocycles*, 1981, **15**, 999.
[93] R. E. Summons, *Phytochemistry*, 1981, **20**, 1125.
[94] D. Seebach and D. Wasmuth, *Angew. Chem.*, 1981, **93**, 1007.
[95] M. Waki, Y. Kitajima, and N. Izumiya, *Synthesis*, 1981, 266.
[96] S. L. Titouani, J. Lavergne, P. Viallefont, and R. Jacquier, *Tetrahedron*, 1980, **36**, 2961.
[97] A. Vasella and R. Voeffray, *J. Chem. Soc., Chem. Commun.*, 1981, 97.

excess of the L-isomer was obtained. 3-Methylglutamic acid diastereoisomers resulting from partial saponification and decarboxylation of diethyl 4-methylpyrrolidone-5,5-dicarboxylate have been described.[98]

Aliphatic Amino-acids Carrying Halogen Substituents in Side Chains.—First examples (Scheme 3) of uses of cyano-oxirans in the synthesis of β-fluoro-α-amino-acids have been described.[99] The route is analogous to that using aziridinecarboxylates (Vol. 12, p. 9); a further example is the synthesis of β-fluorophenylalanine from 2-phenyl-3-cyanoaziridine.[99]



Reagents: i, HF–py; ii, NH_3–MeOH; iii, H_3O^+; iv, MeOH–py

Scheme 3

γ-Fluoroisoleucine [prepared from MeCHFCOMe by Horner–Emmons–Wittig condensation with $(EtO)_2P(O)CH_2CO_2R$ followed by hydrogenation, α-bromination (Br_2/LDA) or α-iodination (I_2/LDA), substitution by azide ion, and hydrogenation of the derived α-azido-acid[100]] is a further close analogue of a protein α-amino-acid, which will be useful in metabolic studies. β-Fluoroalanine, already accessible through several routes, has been prepared as a test of a new general synthesis of α-amino-acids described earlier in this chapter.[51] There is no tradition in this Specialist Periodical Report of including citations from the patent literature since this is covered in concentrated form in Section 34 of *Chem. Abstr.* However, a route to β-chloroalanine, a compound first prepared around the turn of the century,[101] from the $ClCH_2CHO$–bisulphite adduct, HCN, and NH_3, is a typical example of continuing opportunities for patent applications in the amino-acid area.[102]

Aliphatic Amino-acids Carrying Hydroxy Groups in Side Chains.—Hydroxymethylation of benzylidene-amino-acid esters using HCHO/LDA followed by hydrolysis with boiling 6M hydrochloric acid proceeds in good yield.[103]

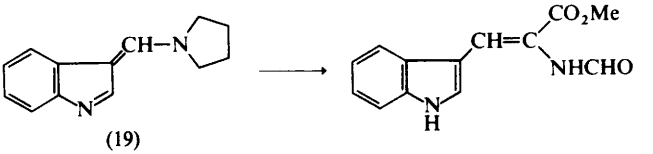
β-Hydroxyvaline has been prepared by a glycine-alkylation route, starting with $(PhCH_2)_2NCH_2CO_2Et$, with acetone and LDA/$MgBr_2$ as base.[104]

[98] A. B. Mauger, *J. Org. Chem.*, 1981, **46**, 1032.
[99] A. A. Ayi, M. Remli, and R. Guedj, *Tetrahedron Lett.*, 1981, **22**, 1505; *J. Fluorine Chem.*, 1981, **18**, 93.
[100] D. Butina and M. Hudlicky, *J. Fluorine Chem.*, 1980, **16**, 301.
[101] E. Fischer and K. Raske, *Berichte*, 1907, **40**, 3717.
[102] K. Nakayasu, O. Furuya, C. Inoue, and S. Moriguchi, *Ger. Offen.*, 3 021 566 (*Chem. Abstr.*, 1982, **95**, 25 629).
[103] A. Calcagni, D. Rossi, and G. Lucente, *Synthesis*, 1981, 445.
[104] A. I. Scott and T. J. Wilkinson, *J. Labelled Compd. Radiopharm.*, 1981, **18**, 347.

Reduction of γ-oxo-ornithine enantiomers, obtained from the corresponding histidines, gives all four stereoisomers of γ-hydroxyornithine, from which the corresponding γ-hydroxyarginines were obtained by standard methods.[105] Routes have been established to γ-hydroxyglutamic acid and analogous adipic acid and pimelic acid derivatives.[106]

2,3-*trans*-3,4-*trans*-3,4-Dihydroxy-L-proline and the 2,3-*cis*-epimer were obtained from the epoxide of *N*-Boc-L-Δ^3-pyrroline-2-carboxylic acid by conventional stages.[107] 3-Chloro- or -bromo-Δ^1-pyrroline-2-carboxylic acid has been used as the source of *cis*- and *trans*-3-acetoxyprolines and 3-heteroatom analogues.[108]

Unsaturated α-Amino-acids.—Conventional routes to αβ-unsaturated α-amino-acids are illustrated in condensation of a ketone with methyl isocyanoacetate and Bu^{t}OK [109] and in the equivalent dehydration of a β-hydroxy-α-amino-acid with disuccinimidyl carbonate [110] (the latter process conveniently provides the succinimido ester of the 'dehydroamino-acid'). Curtius rearrangement of ethyl (*E*)-2-azidocarbonylcarboxycinnamate has been applied for the synthesis of the *E*-dehydrophenylalanine derivative, although in the process a substantial amount of the *Z*-isomer was also formed.[111] Dehydrotryptophans can be obtained by the condensation of the enamine (19) with *N*-formylglycine methyl ester.[112] Deformylation was achieved by ethanolic HCl, a reaction that these authors have sought to optimize in the dehydroamino-acid series.[113]



(19)

Hydroxylamines $RCH_2CH(NHOH)CO_2Et$, formed by reduction of the corresponding oximes, give *N*-acetyldehydroamino-acid analogues through *N*-acetylation followed by treatment with base.[114]

Conversion of 2-isocyano-2-butenoates $MeCR^1{=}C(NC)CO_2R^2$ into the α-vinyl-α-isocyano-acids $CH_2{=}CR^1CR^3(NC)CO_2R^2$ through alkylation with R^3X following treatment with LDA in THF/HMPA provides a controlled route to βγ-unsaturated α-amino-acids.[115] γδ-Unsaturated analogues are obtained [116] through ene reaction of alkenes with (*E*)-$TsN{=}CHCO_2Bu$ [an equivalent route to γ-oxoalkyl α-amino-acids has been illustrated earlier in this chapter, (9) → (10) [52]].

[105] K. Mizusaki and S. Makisumi, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 470.
[106] E. P. Kristensen, L. M. Larsen, O. Olsen, and H. Sörensen, *Acta Chem. Scand., Ser. B*, 1980, **34**, 497.
[107] J.-U. Kahl and T. Wieland, *Liebigs Ann. Chem.*, 1981, 1445.
[108] J. Haeusler, *Liebigs Ann. Chem.*, 1981, 1073.
[109] U. Schöllkopf and R. Mayer, *Liebigs Ann. Chem.*, 1981, 1469.
[110] H. Ogura, O. Sato, and K. Takeda, *Tetrahedron Lett.*, 1981, **22**, 4817.
[111] T. J. Nitz, E. M. Holt, B. Rubin, and C. H. Stammer, *J. Org. Chem.*, 1981, **46**, 2667.
[112] T. Moriya, N. Yoneda, M. Miyoshi, and K. Matsumoto, *J. Org. Chem.*, 1982, **47**, 94.
[113] T. Moriya, K. Matsumoto, and M. Miyoshi, *Synthesis*, 1981, 915.
[114] J. D. M. Herscheid, H. P. H. Scholten, M. W. Tijhuis, and H. C. J. Ottenheijm, *Recl. Trav. Chim. Pays-Bas*, 1981, **100**, 73.
[115] I. Hoppe and U. Schöllkopf, *Synthesis*, 1981, 646.

Synthesis of Amino-acids with Aromatic and Heteroaromatic Side Chains.—Routes to L-tyrosine and L-dopa have been reviewed.[117] DL-2,5,6-Trifluorodopa has been synthesized starting from C_6F_6 (MeLi gives C_6F_5Me, then NaOMe gives 3,4-dimethoxytrifluorotoluene; photobromination gives the substituted benzyl bromide, used in a conventional acetamidomalonate preparation).[118] Reaction of dihydroxyphenylalanines with cystine in constant-boiling hydrobromic acid gave a variety of cysteinyldopas (*S*-cysteinylation of the phenolic moiety), while histidine gives the 2′-(*S*-cysteinyl)imidazole.[119] Other heteroaromatic amino-acids, 4′-pyridylglycines[45] and 7-chlorotryptophan,[40] were prepared by standard methods (Ugi four-component condensation and Fischer indolization, respectively).

α-Hydroxyamino-acids.—Alkylation of *N*-benzylidene-α-amino-acid methyl ester *N*-oxides $PhCH{=}N(=O)CHRCO_2Me$ with an alkyl or benzyl halide after carbanion formation with base gave the corresponding α-substituted amino-acid derivatives, which were converted into *N*-hydroxyamino-acids by hot ethanolic H_2NOH–HCl.[120] Details of the preparation of such compounds from oximes have been discussed in an earlier section.[114]

Synthesis of α-Amino-acids Containing Sulphur and Selenium.—Disulphide $AcSSCMe_2CH(NHAc)CO_2Me$, obtained from the DL-penicillamine derivative and $AcSCO_2Me$, gave the hydrodisulphide on treatment with methanol saturated with HCl.[121] Spontaneous dimerization gave the tetrasulphide, while the corresponding symmetrical trisulphide could be obtained through addition of penicillamine to the hydrodisulphide.[121]

S-Alkylation of cysteine derivatives may be an *in vivo* disposal mechanism for toxic arene oxides, and model studies involving styrene oxide and *N*-acetylcysteine show that attack at the most highly substituted oxiran carbon atom is preferred.[122]

β-(2-Amino-1,3-selenazol-4-yl)alanine has been prepared from 2-amino-4-chloromethylselenazole and diethyl formamidomalonate.[123]

Labelled Amino-acids.—This section covers syntheses of labelled amino-acids with some order imposed on the discussion by arrangement of the examples in order of increasing atomic number.

Synthetic routes allowing incorporation of ^2H have been described for [2,3-$^2H_2$]-L-glutamine [from 6-carboxy-3(2*H*)-pyridazinone],[124] (2*S*,5*R*)-[5-^2H]proline (from L-glutamic acid through enzymic decarboxylation in ^2H_2O, cyclization, condensation with diethyl oxalate, and conventional subsequent steps),[125] and [4,4′-$^2H_6$]-β-hydroxyvaline (synthesis from hexadeuterioacetone).[104]

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Bombardment of solid amino-acids with thermal (2000 K) and 0.6 keV-accelerated tritium atoms [126–129] causes 28% racemization of L-alanine accompanying 40% ^{3}H–^{1}H exchange at the α-carbon [126] and *ca.* 80% exchange at the β-carbon in phenylalanine.[127]

^{11}C-Labelled amino-acids continued to be prepared for tissue distribution studies. The short half-life of this isotope calls for rapid synthetic operations (Bücherer–Strecker synthesis within 40 min) for tryptophan [130] and for phenylalanine [131] (and a further 35 min for resolution using immobilized L- and D-amino-acid oxidases[131]). Even faster working (6—12 min) is needed for the synthesis of ^{13}N-labelled amino-acids using cyclotron-produced $^{13}NH_3$ and immobilized enzymes.[132] There is now a collection of reviews of ^{13}N-labelled [133] and ^{11}C- and ^{18}F-labelled amino-acids.[134]

(2*S*,4*S*)-[5-^{13}C]Leucine has been prepared using *E. coli* deficient in the synthesis of acetolactate and supplemented with (*RS*)-[2-$^{13}CH_3$]acetolactate;[135] silver picolinate oxidation to isovaleric acid, followed by conventional elaboration, leads to (2*RS*)-[4-^{13}C]valine.[135] ^{13}C-Labelled L-leucine has been synthesized [136] from $(^{13}CH_3)_2CO$ and $(EtO)_2P(O)CH_2{}^{13}CO_2Et$ by standard reactions ending with the use of the Strecker synthesis, and DL-[3-^{13}C,2-^{15}N]lysine has been obtained using ^{15}N-phthalimidomalonate and $Na^{13}CN$ at appropriate stages of conventional synthesis.[137] DL-Tyrosine-1-^{14}C,[138] 4-methylene-[2-^{14}C]-DL-glutamic acid,[41] β-*N*-phenyl-[U-^{14}C]-L-asparagine,[139] *O*-acetyl-[1-^{14}C]-L-serine,[139] *O*-benzoyl-[7-^{14}C)]-L-serine, benzyl-[7-^{14}C]- and [U-^{14}C]-glycinate toluene-*p*-sulphonate,[139] α-dimethylaminoisobutyric-[1-^{14}C]acid,[139] α-*N*-[methyl-^{14}C]-DL-asparagine,[140] and N^{G}-[monomethyl-^{14}C]-L-arginine [141] are likewise obtainable by standard methods.

Saponification of Boc-amino-acid methyl esters with NaOH–$^{17}H_2O$, followed by cleavage of the N-protecting group, gives ^{17}O-enriched amino-acids.[142]

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L-[^{35}S]Cysteinesulphinic acid,[143] ^{123m}Te-DL-α-amino-γ-(phenyltelluro)butyric acid,[144] [β-^{125}I]iodo-D- and -L-alanine,[145] and the introduction of a ^{125}I substituent into pteroyltyrosine (a folic acid analogue)[146] also appear in the recent literature.

Resolution of Amino-acids.—This section, as in previous volumes, covers the topic from the diverse viewpoints of theories of prebiotic enantioselection on the one hand and practical procedures for resolution on the other.

The origins of the exclusive dominance of L-amino-acids as protein constituents have been reviewed[147] with special reference to the putative role of chiral energy sources in this process. The Vester–Ulbricht theory (1959) proposing that the 'left-handed' electrons of naturally β-active elements (^{14}C and ^{40}K, for example) are the specific source of the asymmetry has received further support from theoretical analysis, which predicts a very small discrimination.[148] Other physical influences could also operate, and amino-acid solutions incubated with sodium montmorillonite at three different pH values lose slightly larger (0.5—2.0%) amounts of the L-enantiomer by adsorption onto this medium.[149] However, the selectivity does not seem to be conclusively demonstrated generally (it was not observed in some experiments[149]), and the observations of Bondy and Harrington (see Vol. 13, p. 17) on the selective bonding of L-amino-acids and D-sugars to bentonite, made using radioactively labelled amino-acids, may be better explained in terms of the effects of binding of the products of radiochemical decomposition.[150]

Resolution of *N*-benzyloxycarbonyl-DL-homoserine using L-tyrosine hydrazide has been carefully detailed;[151] the D-enantiomer is brought out of solution first when ethanol is used as solvent. Other conventional resolution procedures covered in the recent literature include a modified Pasteur method of visual sorting of crops, which separate from aqueous solutions of DL-amino-acids containing L-glutamic acid and other resolved amino-acids.[152] When DL-threonine separates from such mixtures, the D-enantiomer appears first in the form of long prisms, and the L-enantiomer follows in the form of a fine powder.[152] The preferential crystallization procedure has been studied further[153] with an assessment of the structural requirements for this phenomenon; these seem to be side chains of four atoms, as in methionine and norleucine.[153] Rates of crystallite growth in spontaneous resolution of L-glutamic acid are markedly suppressed by additions of impurities (L-aspartic acid[154]). A study of the characteristics of spontaneous crystallization of one enantiomer from a solution for DL-amino-acid arene-

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sulphonates as well as the influence of factors such as degree of supersaturation and temperature have been reported.[155]

Chromatographic methods hold considerable promise in this area, shown in circulation g.l.c. of amino-acid derivatives on *N*-stearoyl-L-valine t-butylamide [156] and in silica-gel separation of the diastereoisomers formed between DL-2-(2'-cyclopentenyl)glycine and (*R*)-2-acetamido-2-phenylethanol,[157] but particularly in the ligand-exchange procedure [L-hydroxyproline or L-phenylalanine modified polyacrylamide–polystyrene saturated with copper(II) ions [158] and similar systems on a silica support,[159] elution of L-enantiomers from DL-amino-acids adsorbed on reversed-phase silica-gel media by *NNN'N'*-tetramethyl-(*R*)-propane-1,2-diamine–copper(II) complex-containing eluant (H_2O:MeCN = 5:1),[160] and similar resolution of DL-dansylamino-acids using L-prolyl-n-octylamide–nickel(II) in the mobile phase [161]].

Classical traditions are represented also in the resolution of DL-β-(2-thienyl)-alanine as the Boc derivative using (*R*)- or (*S*)-phenylethylamine,[162] of γ-methyl-leucine using leucine aminopeptidase to provide the L-enantiomer from the partially resolved amide,[163] and the same principle for *N*-chloroacetyl-DL-amino-acids using hog renal acylase I.[82]

4 Physical and Stereochemical Studies of Amino-acids

Crystal Structures of Amino-acids and Their Derivatives.—Literature references are even more numerous for 1981, referring to *X*-ray analysis of amino-acids [DL-homocystine,[164] DL-proline hemihydrochloride,[165] L-thioproline hydrochloride,[166] *erythro*-β-fluoro-L-aspartic acid,[167] 5-nitro-L-histidine monohydrate,[168] 3,4-dehydro-L-proline and its *N*-Boc- and acetyl amide derivatives,[169] DL-β-(5-bromouracil-1-yl)alanine,[170] and the copper(II) complex of the novel metal-chelating mugineic acid (1) [171]] and of simple derivatives of amino-acids

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{*N*-Boc-DL-alanine,[172] *N*-Boc-D-glutamic acid monohydrate,[173] *N*-acetyl-L-tyrosine,[174] D-α-acetamidobutyric acid monohydrate,[175] *N*-acetyl-L-cystine,[176] *N*-acetyl-L-aspartic anhydride,[177] *N*-propionyl-L-proline,[178] di-*N*-benzyloxycarbonyl-δ-hydroxy-L-lysine lactone,[179] *N*-phosphonomethylglycine,[180] racemic *N*-[*S*-(methylethoxyphosphinyl)thioglycollyl]valine,[181] and L-proline benzyl ester hydrochloride[182]}. Points of interest from these studies include the revelation of a new self-association mode for *N*-Boc-DL-alanine in the solid state (ribbons of hydrogen-bonded cyclic dimers linked through carboxy groups)[172] and combination of the results of *X*-ray and of neutron diffraction to show the hydrogen-bonding patterns in *N*-acetyl-L-cystine (the —SH group is both donor —SH ... O and acceptor NH ... S).[176]

Crystal structures of amino-acids have been reviewed to study connections between side-chain conformations and the carboxy-group torsion angle χ_1.[183]

Nuclear Magnetic Resonance Spectrometry.—A review of the literature on n.m.r. spectroscopy of amino-acids, peptides, and proteins has appeared, covering the period 1977—1979.[184]

^{1}H n.m.r. studies of [β-^{2}H]-L-tyrosine and L-tryptophan derivatives (*N*-acetyl amino-acids and their esters and methylamides),[185] DL-tryptophan,[186] *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine,[187] L-histidine hydrochloride in water–DMSO,[188] L-histidine and its N^{α}-acetyl methylamide,[189] and *N*-acyl-L-phenylalanines[190] have been concerned with side-chain conformations,[185-187] effect of solvent on the chemical shifts of amide and α-protons in *N*-acylphenylalanines,[190] and proton-transfer studies.[188,189] When the mole fraction of DMSO in water–DMSO is greater than 42%, proton transfer from the imidazole NH group (N-3) to the carboxylate anion in L-histidine hydrochloride (the tautomerization $AH_2^{++-} \rightleftharpoons AH_2^{+}$) is facilitated.[188] The other study of histidine and its derivatives is also a good illustration of the unique contributions of n.m.r. spectroscopy, showing the pressure independence of imidazole p*K* values,

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and therefore suggesting that pressure-induced changes of catalytic activity or ligand-binding characteristic of proteins cannot be explained on this basis.[189] Lanthanide perturbations and nuclear Overhauser effects were part of one of these studies,[186] and complexation phenomena have also been the main feature of n.m.r. studies of Mg^{2+} and Ca^{2+} binding to aspartic acid and asparagine (only Mg^{2+} binds to the amino-group),[191] lanthanides to pyridoxalidene-aspartates,[192] aromatic side-chain complexation with cycloamyloses,[193, 222] and trifluoroacetic acid to *N*-acetyl-L-alanine methylamide (stoicheiometry 1 : 2).[194]

^{13}C and ^{15}N n.m.r. shows that a transition to *Z*/*E*-stereochemistry occurs on dissolution of (*Z*)-*N*-nitroso-*N*-alkyl-amino-acids.[195] Vicinal ^{15}N–^{13}C coupling constants of isotopically pure [^{15}N]-valine, -leucine, -isoleucine, and -threonine show a Karplus-type dihedral angle dependence for the γ-carbon atom, which may be applied to the conformational analysis of amino-acids and small peptides.[196] Other less conventional n.m.r. studies include solid-state quadrupole-echo ^{2}H n.m.r. of ring-deuteriated phenylalanine (showing rapid 180° flips of the phenyl group about the C^{β}–C^{γ} bond axis),[197] ^{13}C and ^{14}N n.m.r. of glycine (bond distances and bond angles from ^{13}C–^{13}C and ^{13}C–^{14}N dipolar splittings agree with neutron-diffraction results),[198] pulsed-n.m.r. study of ^{13}C,^{2}H-enriched glycine (longitudinal relaxation of the two ^{13}C nuclei),[199] and u.v.-excimer laser CIDNP of amino-acids histidine, tryptophan, or tyrosine at 360 MHz using quinoxaline and *p*-methoxyacetophenone as photoreagents (spin-polarized spectra may be interpreted in terms of molecular motion).[200]

^{17}O n.m.r. of four ^{17}O-enriched protein amino-acids is characterized by well resolved peaks.[142]

Optical Rotatory Dispersion and Circular Dichroism.—Use of these techniques to assign absolute configuration to α-amino-acids and small peptides has been reviewed.[201] Little scope remains for novel results on amino-acids themselves since all the common chromophores have been studied in this context. However, L-selenocystine shows Cotton effects centred at 320, 272, 232, and 211 nm, considered[202] to arise in the Se–Se chromophore. (*S*)-(+)-3-Amino-2-phenylpropionic acid shows o.r.d. and molecular-rotation changes accompanying variation in solvent composition, which follow well known empirical rules for α-amino-acids.[203]

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The semicarbazone chromophore in $Me_2C{=}NNHCONHCHRCO_2H$ is responsible for negative-Cotton-effect o.r.d. curves for compounds of the L-series.[204] This new example of the 'chromophoric derivative' approach must prove its worth against several well established alternatives, two of which are featured in configurational assignments to (2*S*,3*S*,4*S*)-2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid (the *N*-terminal amino-acid residue of the nikkomycins, showing a positive Cotton effect as its *N*-dithioethoxycarbonyl derivative;[205] *N*-dithioethoxycarbonyl-*N*-carboxymethyl-L-amino-acids also show positive Cotton effects[316]), *β*-amino-acids (as *N*-2,4-dinitrophenyl derivatives $DnpNHCHRCH_2CO_2H$; negative Cotton effect near 400 nm for *R*-configuration),[206] and 4-alkenyl and 4-alkynyl 2-amino-alkanoic acids (as Dnp derivatives; negative Cotton effect corresponds to the L-configuration).[207]

Calculated rotatory strengths of *N*-acetyl methylamides of L-alanine and L-serine as a function of conformation have been compared with measured c.d. spectra.[208]

Mass Spectrometry.—The major step forward represented by fast atom bombardment mass spectrometry has been demonstrated most spectacularly by spectra for amino-acids (glycine, L-valine, and phenylalanine) showing good $[M + 1]^+$ ions (a peak attributable to $[2M + 1]^+$ for valine) and detailed fragmentation patterns.[209] The study of thick layers of amino-acids (prepared by compression of the bulk solid on the probe, evaporation of a concentrated solution, or suspension of the solid in a liquid) by this technique and the excellent spectra contrast sharply with the well known problems of mass-spectrometric study of zwitterionic and involatile samples. Similar work[210] with leucine, either alone or in admixture with LiCl, on a silver probe, gives spectra containing $[M + 1]^+$, $[M + Ag]^+$, $[M + Li]^+$, $[M + 2Li - H]^+$, and $[M + Li + Ag - H]^+$ peaks, whose relative proportions depend on the structure of the amino-acid and other factors; 2.25 keV Ar^+ ion bombardment was used in this study.[210]

Chemical-ionization mass spectra of L-methionine and its derivatives in which the sulphide has been transformed into other simple sulphur functional groups have been fully discussed.[211]

The perils associated with thermal treatment of amino-acids on the probe in preparation for ionization are illustrated in the lack of reproducibility of isotopic abundances in [^{15}N]glycine spectra.[212]

Routine mass-spectrometric methods applied to the analysis of *γ*-aminobutyric acid as its trimethylsilyl derivative permit levels down to 25 pg to be reached.[213]

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[205] W. A. Koenig, K. P. Pfaff, H. H. Bartsch, H. Schmalle, and H. Hagenmaier, *Liebigs Ann. Chem.*, 1980, 1728.
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[210] W. Sichtermann and A. Benninghoven, *Int. J. Mass Spectrom. Ion Phys.*, 1981, **40**, 177.
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[212] J. R. Majer, B. I. Al-Ali, and A. S. P. Azzouz, *Org. Mass Spectrom.*, 1981, **16**, 147.
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Other Physical Studies.—This section covers other spectroscopic studies, as well as conventional physico-chemical techniques, applied to amino-acids.

Far-i.r. spectra (20—500 cm^{-1}) of solid layers of glycine and L-alanine represent one extreme application of this wavelength region,[214] while a combined i.r.–u.v. study of interactions between amino-acid esters and nucleotide bases in DMSO[215] represents the other extreme (with more directly usable information). In the latter study the orders (i) cytosine > adenine ≫ uracil ~ thymine and (ii) histidine > methionine > arginine > lysine > serine ~ glycine illustrate the relative hydrogen-bonding efficiencies. I.r. studies of *N*-acyl-α-amino-acids in $CHCl_3$ and CCl_4 to reveal hydrogen-bonding and solvation characteristics[172,216] continue long-established applications of this technique. The relative intensities of spectral lines in Raman spectra of phenylalanine and tyrosine have been compared, but attempts to determine the relative amounts of these amino-acids as residues in proteins, based on spectral lines specific to each, have low reliability.[217]

An i.r. spectral study of L-arginine adsorbed on apatitic calcium phosphate[218] is one of a number of reports of adsorption of L-amino-acids from aqueous solutions on to sodium montmorillonite,[149] α-zirconium phosphate,[219] and titanium(IV) oxide[220] or from aqueous NaF on to mercury.[221] Formation of inclusion complexes between phenylalanine and cyclohexa-, cyclohepta-, and cyclo-octa-amylose has received further detailed study.[193,222]

E.s.r. spectral characterization of semiquinone radicals generated by photolysis of dopa in aqueous solutions[223] and e.s.r.-established decay rates of radicals formed in glycine and β-alanine *X*-irradiated at 77 K[224] have been reported. The identification of alanine in fossil shells and bones is a novel application of e.s.r. in paleochemical studies.[225] Copper(II)-doped L-alanine single crystals at 4.2 K have been studied by the electron–nuclear double-resonance technique, to provide fundamental data, including assignment of the sp^2 state to the nitrogen atom in the amino-acid ligand.[226]

Potentiometric titration studies have been made of tyrosine and its *o*- and *m*-isomers,[227] histidine in water–DMSO,[228] and a series of amino-acids in CF_3CH_2OH.[229] Other physico-chemical studies deal with phase-equilibrium behaviour (solubilities of pairs of L-amino-acids in water[230] and distribution

[214] S. C. Chen, L. Santo, and L. Genzel, *Can. J. Spectrosc.*, 1981, **26**, 126.
[215] A. P. Gul'tyaev, S. A. Samoilenko, and N. V. Zheltovskii, *Mol. Biol. (Moscow)*, 1981, **15**, 1295.
[216] I. M. Ginzburg and G. Yu. Strebulova, *Zh. Obshch. Khim.*, 1981, **51**, 1907.
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[221] T. Kakiuchi and M. Senda, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 1354.
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[229] B. Carre and J. Devynck, *Bull. Soc. Chim. Fr.*, 1981, 309.
[230] C. E. Messer, G. Malakoff, J. Weil, and S. Labib, *J. Phys. Chem.*, 1981, **85**, 3533.

coefficients into 1-octanol–water [231]), viscosities of aqueous [232] and ethylene-glycol [233] solutions of amino-acids, osmotic coefficients of aromatic and hetero-aromatic amino-acids in water,[234] mechanism of reverse-osmosis separation of amino-acids through a cellulose acetate membrane,[235] partial molar volumes of α-amino-acids in water,[236] transport of benzoylamino-acids through a CH_2Cl_2 membrane separating two aqueous phases,[237] and molecular polarizabilities of α-amino-acids.[238] Thermodynamic studies cover enthalpies of combustion of L-cysteine and L-methionine,[239] integral enthalpies and densities of amino-acids in aqueous t-butanol,[240] and enthalpy and entropy of transfer of amino-acids from water to aqueous glycerol and its homologues.[241]

Molecular-orbital Calculations.—Solvation of glycine zwitterion in the gas phase [242] and interaction forces between a sodium ion and glutamate, aspartate, and alanine [243] and between purines and amino-acids [244] illustrate a continuing theme in theoretical studies of amino-acids. The last-mentioned study [244] suggests that both adenine and guanine preferentially hydrogen-bond to aspartic acid and asparagine; optimized geometries and conformational energies of these hydrogen-bonded complexes are given. Other conformational calculations reported deal with *N*-acetylglycine methylamide,[245] *N*-acetylglycine, glycine, and cysteine,[246] glycine methyl ester,[247] and L-histidine.[248]

Lattice energies of α-glycine, L-alanine, and DL-alanine crystals have been computed on the basis of a model which required modification after taking account of experimental data for these amino-acids.[249] Calculated proton-transfer energies and electrostatic-lattice energies of amino-acids indicate that lattice energies are greater for zwitterions than for non-zwitterions, but most of this energy surplus is cancelled by the proton-transfer energy. This explains why amino-acids and peptides often (but not always) crystallize as zwitterions.[250]

[231] L. M. Yunger and R. D. Cramer, *Mol. Pharmacol.*, 1981, **20**, 602.
[232] R. K. Wadi, A. Gupta, and D. V. S. Jain, *Indian J. Chem., Sect. A*, 1981, **20**, 21.
[233] O. P. Awasthi and P. P. Rastogi, *Bull. Soc. Chim. Belg.*, 1981, **90**, 139.
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[239] R. Sabbah and C. Minadakis, *Thermochim. Acta*, 1981, **43**, 269.
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[241] K. Gekko, *J. Biochem. (Tokyo)*, 1981, **90**, 1643.
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[250] J. Voogd, J. L. Derissen, and F. B. Van Duijneveldt, *J. Am. Chem. Soc.*, 1981, **103**, 7701.

5 Chemical Studies of Amino-acids

Racemization.—This topic is perhaps not a natural opening to this section at first sight, but it has occupied this position in this series from Volume 6 because it forms the basis of techniques providing age estimates of relatively young fossils and indications of average temperatures to which the fossils have been subjected. For these reasons mechanistic aspects of amino-acid racemization have been studied in some detail.

New examples have been published of applications of D : L ratios of leucine,[251] aspartic acid,[252-254] isoleucine,[152] and methionine[254] measured for quaternary molluscs,[251] fossil bones and teeth from the Olduvai Gorge,[252] from archaeological sites in Italy,[253] and fossil trees of ages 3710, 9000—10 000, and *ca.* 10^5 y.[254] Whereas good agreement between the dates indicated by both racemization and ^{14}C values were obtained in the Italian samples,[253] this is not so for the mollusc samples unless unlikely temperatures through the Pleistocene age are assumed.[251] Samples older than *ca.* 10^5 y contain significant amounts of aspartic acid that has diffused into the fossils since deposition, and the isoleucine : *allo*-isoleucine ratio is a better dating index for such samples.[252]

The uncertainties associated with the amino-acid dating technique (see preceding volumes) continue to be pointed out,[251] and a further source of error not previously taken into account is the ready formation of dioxopiperazines from dipeptides and the faster racemization rates of amino-acid residues in these compounds.[255]

Acid-catalysed racemization of protein amino-acids[254, 256] (*e.g.* under the normal protein hydrolysis regime[254]) is insignificant for isoleucine, valine, serine, and threonine but substantial for aspartic acid, cysteine, proline, glutamic acid, and methionine (rates decreasing in this order).[256]

N-Acylated α-amino-γ-lactones give fully racemized ring-opening products with aqueous NaOH,[257] and *N*-urethane-protected amino-acid anhydrides, previously thought to be immune from racemization during peptide-coupling operations, give significant (*ca.* 5%) amounts of epimers when coupled using *p*-dimethylaminopyridine as base.[258]

Racemization accompanying thermal polymerization of amino-acids is often faster than the peptide-formation process.[259]

Specific Reactions of Natural Amino-acids.—Some of the chemistry described in this section might also apply to amino-acids in general (and this should be borne in mind when reading the following section), but most of the work cited here deals with reactions of the side-chain functional groups of the protein amino-acids.

[251] J. F. Wehmiller, *Geochim. Cosmochim. Acta*, 1981, **45**, 261. Reply by K. A. Kvenvolden, D. J. Blunt, and H. E. Clifton, *ibid.*, p. 265..
[252] J. L. Bada, *Earth Planet. Sci. Lett.*, 1981, **55**, 292.
[253] G. Bellnomini, *Archaeometry*, 1981, **23**, 125.
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[256] H. Frank, W. Weiwode, G. Nicholson, and E. Bayer, *Liebigs Ann. Chem.*, 1981, 354.
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[259] K. Dose, *Origins Life*, 1981, **11**, 165.

Several papers have appeared describing uses of Ph_3P with diethyl azodicarboxylate to convert β-hydroxy-α-amino-acid amides into corresponding 3-amino-azetidin-2-ones[84, 260] (see also ref. 83). With *N*-benzyloxycarbonyl derivatives, no racemization accompanies this process,[260] but with other *N*-protecting groups aziridinecarboxylate derivatives may be obtained. *N*-Boc- and phthaloyl-serine and -threonine esters give corresponding dehydroamino-acids with Ph_3P and diethyl azodicarboxylate or, better, with a carbodi-imide.[261] Elimination on this basis is accompanied by reverse aldolization when β-hydroxyglutamic acid is in the presence of pyridoxal and metal ions.[262] α-Hydroxyglycine derivatives $R^1CONHCH(OH)CO_2R^2$ can be converted into α-chloro analogues using $SOCl_2$, thence into α-heteroatom analogues by nucleophilic substitution.[263]

Exposure of proline or lysine to superoxide anion H_2O_2 or $HO^{\cdot}$ leads to 3- and 4-hydroxyprolines (*cis–trans* mixtures) and 5-hydroxylysine, respectively.[264] Copper(I) autoxidation of cysteine in alkaline solutions gives cystine, H_2O_2, and H_2O, but no sulphur oxyacids.[265] The $Br_2^{-\cdot}$ ion, like $HO^{\cdot}$, gives a disulphide cation (λ_{max} 450 nm) with cystine and other disulphides;[266] electrochemical oxidation of cystine, cysteine, and methionine has been studied.[267] Irradiation with u.v. light ($\lambda < 320$ nm) converts a mixture of *NN'*-diacetylcystine and acetaldehyde into *NS*-diacetylcysteine.[268] The reversible Smiles rearrangement involving *S*- and *N*-Dnp-cysteines favours the *N*-substituted compound in MeOH in the presence of a base, but an equilibrium mixture is formed in DMF–DABCO.[269] Raney nickel desulphurization of common sulphur-containing amino-acids has received detailed study, methionine sulphone, cysteic acid, and homocysteinesulphonic acid being unaffected by the reagent whatever its method of preparation.[270] A novel sulphur-functional-group reaction of cysteine is represented by its conversion into tetrakis-(2-amino-2-carboxyethylmercapto)germane through heating with GeO_2 in water.[271] Protection of the cysteine SH group by the t-butyl group[272] or by using the diphenyl-4-pyridylmethyl group[273] has been described, the latter being particularly promising since it is cleaved easily using Zn–AcOH, $Hg(OAc)_2$, I_2, or electrochemical reduction.

N-Nitrosoproline is formed in solutions containing $NaNO_2$ and either L-citrulline or L-arginine under simulated human-stomach conditions; yields are 27.1 and 0.1%, respectively.[274] Proline, pipecolic acid, and 5-hydroxypipecolic acid are

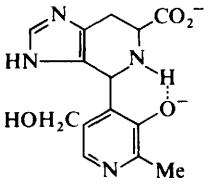
[260] A. K. Bose, D. P. Sahu, and M. S. Manhas, *J. Org. Chem.*, 1981, **46**, 1229.
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[271] Y. Yagi, *Yakugaku Zasshi*, 1981, **101**, 747.
[272] J. J. Pastuszak and A. Chimiak, *J. Org. Chem.*, 1981, **46**, 1868.
[273] S. Coyle, A. Hallett, M. S. Munns, and G. T. Young, *J. Chem. Soc., Perkin Trans. 1*, 1981, 522.
[274] T. Ishibashi and T. Kawabata, *J. Agric. Food Chem.*, 1981, **29**, 1098.

formed from the reaction of $Na_2Fe(CN)_5NO$ with ornithine, lysine, and 5-hydroxylysine, respectively.[275]

1,1-Diacetoxyiodobenzene reduces the amide group of N^{α}-benzyloxycarbonyl-asparagine to the primary amine, to an extent greater than 90%, but the possibility of using this reagent for protein modification is somewhat thwarted by the fact that tyrosine and the protein amino-acids carrying nitrogen or sulphur functional groups in the side chain all react with this reagent, as does the *N*-terminal NH_2 group.[276]

Glutamic acid γ-esters and *O*-acetylserine feature in studies of competitive hydrolysis and intramolecular aminolysis,[277] while ammonolysis of *N*-protected pyroglutamic acids to give glutamines is also in competition with cleavage of the *N*-protecting group.[278]

Apart from a study of *O*-alkylation of *N*-protected tyrosines by $MeSO_2OPr^i$ catalysed by 18-crown-6,[279] all the remaining papers in this section deal with heteroaromatic α-amino-acids. Histidine condenses with pyridoxal *via* the Schiff base to give (20);[280] full details of the proof that histidine decarboxylase catalysis

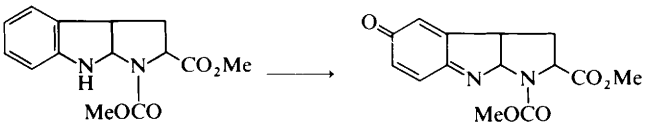


(20)

of the conversion of L-histidine in ^2H_2O to (1*R*)-[1-^{2}H]histamine involves retention of configuration have been published.[281] Protection of the histidine imidazole moiety by π-benzyloxymethylation or π-bromobenzyloxymethylation offers derivatives that are compatible with the stringent requirements of peptide synthesis.[282] Studies of reactions of tryptophan include sodium hypochlorite oxidation to 3-indoleacetaldehyde,[283] selective 5-hydroxylation *via* Fremy's salt oxidation of the cyclic tautomer (21) to the quinone-imine (22), and $NaBH_4$ reduction,[284] and attack by the trichloromethylperoxy radical to give a neutral tryptophan radical together with the radical adduct as major product.[285]

Pyridinoline, a crosslinking amino-acid residue in collagen, resists $NaBH_4$ reduction, and polarographic oxidation and reduction potentials indicate that its 3-hydroxypyridinium moiety is very difficult to reduce.[286]

[275] M. T. Beck, A. Katho, and L. Dozsa, *Inorg. Chim. Acta*, 1981, **55**, L55.
[276] L. A. Holt and B. Milligan, *Austral. J. Biol. Sci.*, 1981, **34**, 395.
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[280] L. Casella and M. Gullotti, *J. Am. Chem. Soc.*, 1981, **103**, 6338.
[281] E. Santaniello, A. Manzocchi, and P. A. Bondi, *J. Chem. Soc., Perkin Trans. 1*, 1981, 307.
[282] T. Brown and J. H. Jones, *J. Chem. Soc., Chem. Commun.*, 1981, 648.
[283] T. Rausch, F. Hofmann, and W. Hilgenberg, *Z. Naturforsch., Teil B*, 1981, **36**, 359.
[284] T. Hino, M. Taniguchi, and M. Nakagawa, *Heterocycles*, 1981, **15**, 187.
[285] J. E. Packer, J. S. Mahood, R. L. Wilson, and B. S. Wolfenden, *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem.*, 1981, **39**, 135.
[286] S. Sakura and D. Fujimoto, *J. Biochem. (Tokyo)*, 1981, **89**, 1541.



(21) (22)

The kinetic tritium isotope effect for the iodination of [5-^{3}H]-3-iodo-L-tyrosine at pH 7.5 is 4.29 for I_2 and 4.50 using Chloramine-T with KI.[287]

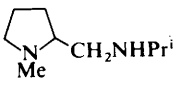
General Reactions.—KOBu^t-catalysed ^1H–^2H exchange of Piv-L-amino-acid dimethylamides in Bu^{t}OH occurs with small but real degree of retention and is the first example of stereospecific exchange at the α-carbon in an enolate-forming carbon acid.[288] Both ion pairing and the influence of the pivalyl group contribute to the stereospecificity.

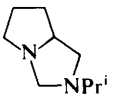
Further examples of well established reactions of amino-acids include a spectroscopic study of the formation of melanoidins from glycine with glucose or fructose (Maillard reaction),[289] non-random sequence oligopeptides from the thermal polymerization of glycine, glutamic acid, and tyrosine,[259, 290] hydroxymethylation of polyfunctional amino-acids (fast but incomplete reaction with NH_2, guanidinyl, OH, indole, and imidazole side chains; fast and complete with SH; slow and complete with the α-NH_2 group) and condensation of HCHO with asparagine, threonine, histidine, and tryptophan to form cyclic derivatives (see Vol. 12, p. 29),[291] replacement of the α-NH_2 group by F using $NaNO_2$ in HF in the presence of pyridine, with retention of configuration,[292] Friedel–Crafts reactions with *N*-methoxycarbonylamino-acids (benzyloxycarbonyl analogues proved unsuccessful)[293] or ethoxycarbonyl or benzenesulphonyl analogues,[294] Strecker degradation (oxidation by hypochlorite) under u.v. irradiation (which promotes the already rapid initial *N*-chlorination step),[295] and effects of structure on rates of hydrolysis of leucine esters[296] and their copper(II) iminodiacetate complexes.[297]

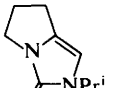
Reduction of amino-acid esters to α-amino-alcohols with $NaBH_4$ has been developed into a convenient procedure,[298] while BH_3 in THF was found to be the best reagent for the reduction of *N*-protected α-amino-acids to corresponding alcohols in terms of avoidance of racemization and lack of disruption of *N*-terminal and side-chain protection.[299] The Boc-amino-alcohols obtained in this way were conveniently oxidized to the corresponding aldehydes with pyridinium

[287] J. Baldas, S. Colmanet, and Q. N. Porter, *Aust. J. Chem.*, 1981, **34**, 1147.
[288] R. D. Guthrie and E. C. Nicolas, *J. Am. Chem. Soc.*, 1981, **103**, 4637.
[289] P. A. Bobbio, H. Imasoto, and S. R. de A. Leite, *An. Acad. Bras. Cienc.*, 1981, **53**, 83, 87.
[290] J. Hartmann, M. C. Brand, and K. Dose, *BioSystems*, 1981, **13**, 141.
[291] D. Tome and N. Naulet, *Int. J. Pept. Protein Res.*, 1981, **17**, 501.
[292] F. Faustini, S. de Munari, A. Panzeri, V. Villa, and C. A. Gandolfi, *Tetrahedron Lett.*, 1981, **22**, 4533.
[293] D. E. McClure, B. H. Arison, J. H. Jones, and J. J. Baldwin, *J. Org. Chem.*, 1981, **46**, 2431.
[294] T. F. Buckley and H. Rapoport, *J. Am. Chem. Soc.*, 1981, **103**, 6157.
[295] Y. Ogata, M. Kimura, and Y. Kondo, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 2057.
[296] T. Yamamoto, K. Ueda, S. Ando, H. Aoyagi, and N. Izumiya, *Mem. Fac. Sci., Kyushu Univ., Ser. C*, 1981, **13**, 81.
[297] R. W. Hay and P. K. Banerjee, *J. Inorg. Biochem.*, 1981, **14**, 147.
[298] M. Kubota, O. Nagase, and H. Yajima, *Chem. Pharm. Bull.*, 1981, **29**, 1169.
[299] C. F. Stanfield, J. E. Parker, and P. Kanellis, *J. Org. Chem.*, 1981, **46**, 4799.

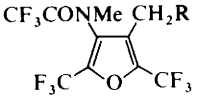
dichromate.[299] An unusual solvent effect on the reduction of *N*-benzyloxycarbonyl-L-proline isopropylamide by $LiAlH_4$ has been reported;[300] in ether the *N*-methyl analogue of the starting material is formed together with the fully reduced compound (23), while in THF (24) and (25) were formed.

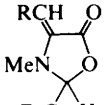

(23)


(24)


(25)

The preparation of *N*-(2,2,2-trichloroethoxycarbonyl)-L-amino-acids has been described,[301] and a polymer thiolacetate has been used for the synthesis of *N*-acetyl derivatives of amino-acids.[302] Instead of the expected *N*-trifluoroacetyl derivatives or mesoionic oxazolinones, *N*-methylamino-acids yield mainly furans (26) and the oxazolidinones (27) by reaction with trifluoroacetic anhydride.[303]


(26)


(27)

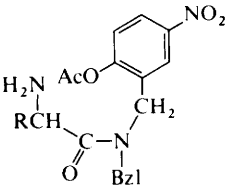
Further results (see Vol. 12, p. 26) on the cyclization of *N*-alkoxycarbonylamino-acids to 2-alkoxyoxazolin-5-ones, and the reaction of these with the starting material to give symmetrical anhydrides, have been reported;[304] it is the anhydride that is formed from this starting material, not the *O*-acylisourea, on treatment with di-isopropyl carbodi-imide.[305] The anhydrides can be converted into the oxazolinones through treatment with a tertiary amine or a carbodi-imide.[306] The oxidative decarboxylation of benzoylphenylglycine esters by O_2 in the presence of an alkali-metal alkoxide and HMPA or DMSO ($ArCONHCHArCO_2Et \rightarrow ArCONHCOAr$)[307] probably involves an oxazolinone intermediate.

Products are formed from amino-acids and ferrous salts in phosphate buffer, which yield a chromogen with thiobarbituric acid, similar to the chromogen formed with malondialdehyde, and iron(II)-catalysed aerial oxidative breakdown is assumed to be taking place;[308] clearly, further studies and rational confirmation of this conclusion are needed. Iridium(III) and iridium(III)–manganese(II)-catalysed oxidative decarboxylation and deamination by cerium(IV) have been achieved for representative amino-acids.[309]

[300] S. Kiyooka, F. Goto, and K. Suzuki, *Chem. Lett.*, 1981, 1429.
[301] J. F. Carson, *Synthesis*, 1981, 268.
[302] M. Gosselet and B. Sebille, *J. Chem. Technol. Biotechnol.*, 1981, **31**, 341.
[303] U. Hess and W. A. König, *Liebigs Ann. Chem.*, 1981, 1606.
[304] N. L. Benoiton and F. M. F. Chen, *Can. J. Chem.*, 1981, **59**, 384.
[305] N. L. Benoiton and F. M. F. Chen, *J. Chem. Soc., Chem. Commun.*, 1981, 543.
[306] N. L. Benoiton and F. M. F. Chen, *J. Chem. Soc., Chem. Commun.*, 1981, 1225.
[307] C. Yijima, F. Hino, and K. Suda, *Synthesis*, 1981, 610.
[308] J. M. C. Gutteridge, *FEBS Lett.*, 1981, **128**, 343.
[309] Y. R. Serma and P. K. Saiprakash, *Indian J. Chem., Sect. A*, 1981, **19**, 1175.

Mutagenic *N*-acetyl-*N*-nitroso-α-amino-acids formed from an *N*-acetylamino-acid and $NO^+BF_4^-$ rearrange into α-diazoalkanoic acids and give 2-methoxy- and 2-hydroxy-alkanoic acids in MeOH and H_2O, respectively.[310]

Reactions through the amino group are involved in: diazo transfer from $CF_3SO_2N_3$ to give corresponding α-azido-acids of high optical purity,[311] stereo-selective addition of amino-acids to chiral *N*-acylimines to give αα-diamino-acid derivatives,[312] reaction of α-amino-acids[313] and esters[314] with malondialdehyde to give enaminals, formation of *N*-phosphovaline in 3.2% yield by reaction of L-valine with cyclotetraphosphate,[315] preparation of *N*-(carboxymethyl)amino-acids using bromoacetic acid,[316] and nine- and twelve-membered ring formation followed by intramolecular *ON*-acyl transfer in (28).[317]



(28)

Enantioselectively catalysed hydrolysis of *N*-protected D- or L-amino-acid *p*-nitrophenyl esters (D:L = 5.68) has been demonstrated, using an L-α-aminohydroxamate as catalyst.[318] Full details of enantioselective deacylation of long-chain acylated α-amino-acid *p*-nitrophenyl esters using co-micelles of *N*-acyl-L-histidine and a cationic surfactant have been published.[319]

Following similar studies with β-hydroxy-α-amino-acid amides reported in 1981,[83, 84, 260] β-amino-acids have been shown to give high yields of β-lactams using Ph_3P and 2,2′-dipyridyl disulphide.[320]

Effects of Electromagnetic Radiation on Amino-acids.—As in all previous volumes, the photochemistry of tryptophan provides most of the papers eligible for this section. Flash photolysis of aqueous tryptophan at 265 nm with simultaneous excitation at other wavelengths causes ionization from a short-lived pre-fluorescent state.[321] Radical cations formed in similar studies[322] in aqueous acidic solutions were the mono- and di-protonated species. Dye-sensitized photo-oxidation of tryptophan in aqueous micellar dispersions[323] and in aqueous

[310] Y. L. Chow and J. Polo, *J. Chem. Soc., Chem. Commun.*, 1981, 297.
[311] J. Zaloom and D. C. Roberts, *J. Org. Chem.*, 1981, **46**, 5173.
[312] C. Shin, H. Ohmatsu, Y. Sato, and J. Yoshimura, *Chem. Lett.*, 1981, 701.
[313] V. Nair, D. E. Vietti, and C. S. Cooper, *J. Am. Chem. Soc.*, 1981, **103**, 3030.
[314] D. J. Pietrzyk and J. Stodola, *Anal. Biochem.*, 1981, **117**, 245.
[315] M. Tsuhako, N. Fujita, A. Nakahama, T. Matsuo, M. Kobayashi, and S. Ohashi, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 289.
[316] T. Miyazawa, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 2555.
[317] D. S. Kemp, D. J. Kerkman, S.-L. Leung, and G. Hanson, *J. Org. Chem.*, 1981, **46**, 490.
[318] S. Ono, H. Shosenji, and K. Yamada, *Tetrahedron Lett.*, 1981, **22**, 2391.
[319] K. Ohkubo, K. Sugahara, H. Ohta, K. Tokuda, and R. Ueoka, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 576.
[320] S. Kobayashi, T. Iimori, T. Izawa, and M. Ohno, *J. Am. Chem. Soc.*, 1981, **103**, 2406.
[321] L. I. Grossweiner, A. M. Brendzel, and A. Blum, *Chem. Phys.*, 1981, **57**, 147.
[322] B. Finnstroem, S. Claesson, and E. A. Cherniak, *Chem. Scr.*, 1980, **16**, 65.
[323] E. Rossi, A. Van de Vorst, and G. Jori, *Photochem. Photobiol.*, 1981, **34**, 447.

solutions[324] gives 3a-hydroperoxypyrroloindole as labile precursor to the eventual product, *N*-formylkynurenine. Fluorescence-decay kinetics as a function of pH[325] and emission wavelength[326] (collisional quencher H^+ and $H_2C{=}CHCONH_2$, respectively) and temperature dependence of tryptophan and tyrosine luminescence in poly(vinyl alcohol) films[327] have also been studied. Fluorescence analysis confirms that γ-radiolysis of aqueous phenylalanine gives dityrosine, from tyrosine, in amounts determined by the interplay of many factors.[328] Pyridoxal amino-acid methyl esters undergoing transamination can photorearrange to a *p*-quinonoid structure whose lifetime and other parameters have been determined.[329]

6 Analytical Methods

Gas–Liquid Chromatography.—This technique is the basis of most of the data discussed earlier in this chapter concerning enantiomer ratios for amino-acids.[149, 251–255] The technique has also been studied for its own sake, with the intention to optimize the derivatization preliminaries[330] and to investigate new instrumentation.

Derivatives employed in recent reports are all familiar combinations of *N*-trifluoroacetyl,[331–335] *N*-pentafluoropropionyl,[335] *N*-heptafluorobutyryl,[335, 336] or *N*-isobutoxycarbonyl,[337] as acylating group, which is introduced after esterifying the amino-acids with methanol[335–337] (esterification achieved using BF_3–MeOH[336]), n-propanol,[332] isopropanol,[333] n-butanol,[331, 334, 335] or hexafluoropropanol.[335] Trimethylsilylation has been used for derivatization of ^{14}C-labelled amino-acids for g.l.c. analysis.[338] Glass-capillary-column g.l.c. has been advocated for this purpose,[333, 339] including whisker-walled columns (one column coated with a chiral stationary phase followed by another coated with the enantiomer of the same chiral stationary phase) for the analysis of enantiomer ratios.[333] Several other papers cited in this chapter describe the use of g.l.c. for the analysis of partly racemized amino-acids.[340]

[324] M. Nakagawa, S. Kato, S. Kataoka, S. Kodato, H. Watanabe, H. Okajima, T. Hino, and B. Witkop, *Chem. Pharm. Bull.*, 1981, **29**, 1013; M. Nakagawa, S. Kato, K. Nakano, and T. Hino, *J. Chem. Soc., Chem. Commun.*, 1981, 855.

[325] E. Gudgin, R. Lopez-Delgado, and W. R. Ware, *Can. J. Chem.*, 1981, **59**, 1037.

[326] M. R. Eftink and C. A. Ghiron, *Photochem. Photobiol.*, 1981, **33**, 749.

[327] R. Sakúrovs and K. P. Ghiggino, *Aust. J. Chem.*, 1981, **34**, 1367.

[328] G. Boguta and A. M. Dancewicz, *Nukleonika*, 1981, **26**, 11.

[329] J. W. Ledbetter, J. M. Hankel, and T. J. Cornish, *Photochem. Photobiol.*, 1981, **34**, 115.

[330] J. Drozd, 'Chemical Derivatization in Gas Chromatography', Elsevier, Amsterdam, 1981, p. 126.

[331] F. Boudah, K. Abdeddaim, and M. H. Guermouche, *J. High Resolut. Chromatogr., Chromatogr. Commun.*, 1981, **4**, 247.

[332] E. Mussini, L. Cotellessa, L. Colombo, D. Cani, P. Sfondrini, F. Marcucci, and F. Poy, *J. Chromatogr.*, 1981, **224**, 91.

[333] T. Hobo, M. Yamada, S. Suzuki, S. Araki, A. Shimoyama, and C. Ponnamperuma, *Bunseki Kagaku*, 1981, **30**, T71 (*Chem. Abstr.*, 1982, **95**, 220 275).

[334] A. L. Lapidus, Y. B. Yan, and S. Ya. Grabovenko, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1981, 429.

[335] K. Murayama, N. Shindo, R. Mineki, and K. Ohta, *Biomed. Mass Spectrom.*, 1981, **8**, 165.

[336] Y. Okano, M. Kataoka, T. Miyata, H. Morimoto, K. Takahama, T. Hitoshi, Y. Kase, I. Matsumoto, and T. Shinka, *Anal. Biochem.*, 1981, **117**, 196.

[337] S. Matsuura, S. Yamamoto, and M. Makita, *Anal. Biochem.*, 1981, **114**, 371.

[338] C. Pertzsch, L. Bender, and K. H. Neumann, *Z. Pflanzenernaehr. Bodenkd.*, 1981, **144**, 231.

[339] I. M. Moodie and J. Bunger, *J. High Resolut. Chromatogr., Chromatogr. Commun.*, 1981, **4**, 218.

[340] M. H. Engel, T. K. Sawyer, M. E. Hadley, and V. J. Hruby, *Anal. Biochem.*, 1981, **116**, 303.

It is rare to find a report in which some of the available variations of the g.l.c. technique are compared, and an attempt to simplify g.l.c. analysis (omitting desalination in analysis of glutamic acid and γ-aminobutyric acid at the femtomole level in Ringer's solution) has been described.[335] Dimethyl *N*-pentafluoropropionyl-L-glutamate and hexafluoropropyl *N*-trifluoroacetyl γ-aminobutyrate were found to be superior combinations in terms of sensitivity at these levels, compared with the other groups mentioned in the preceding paragraph. The effects of various salts in either facilitating or inhibiting the esterification and acylation steps are discussed in this report.[335] The objectives of the other reports mentioned in this section were comparisons of different poly(siloxane) stationary phases using amino-acid derivatives,[331] analysis of N^{π}-methylhistidine in physiological fluids,[332] estimation of pipecolic acid and proline at picomole levels,[336] estimation of γ-carboxyglutamic acid,[337] and analysis of alkali-treated frog skins to test whether the observed increase in melanotropic activity is related to partial racemization of amino-acid residues in the constituent proteins.[340]

Ion-exchange Chromatography.—The well established procedures represented in estimations of methylated basic amino-acids in urine [341] and of N^{τ}-methylhistidine (using either fluorescamine[342] or the ninhydrin–*o*-phthalaldehyde reagent after separation from other amino-acids [343]) are typical of a much larger number of more routine studies that have been excluded by considerations of economy of space. New internal standards for routine amino-acid analyser use, γ-carboxyglutamic acid and [^{14}C]cysteic acid, have been proposed.[344]

Thin-layer Chromatography.—The coverage is restricted to representative examples illustrating trends in favour of particular techniques, as well as novel procedures.

Polyamide t.l.c. features in several studies,[345-347] being particularly suitable for separation of dansyl derivatives at low levels;[345, 346] samples extracted from regions of the polyamide layer can be identified by mass spectrometry.[347]

Conventional t.l.c. techniques (including two-dimensional t.l.c.[348]) have been applied to diazoaminobenzenesulphonylthiohydantoins [348] and *N*-diphenylindenonesulphonyl amino-acids.[349]

Novel materials for t.l.c. include activated carbon (visualization involving ninhydrin colour formation on 'prints' taken by pressing filter paper on the developed plates) [350] and ammonium tungstophosphate and molybdophosphate (mainly adsorption but partly an ion-exchange medium).[351]

[341] M. F. Lou and M. Siena, *Biochem. Med.*, 1981, **25**, 309.
[342] A. J. Murray and F. J. Ballard, *Anal. Biochem.*, 1981, **116**, 537.
[343] H. Vielma, J. Mendez, M. Druckenmiller, and H. Lukaski, *J. Biochem. Biophys. Methods*, 1981, **5**, 75.
[344] Y. Ishikawa and R. E. Wuthier, *Anal. Biochem.*, 1981, **114**, 388.
[345] G. Ulmar, *Adv. Biochem. Psychopharmacol.*, 1981, **29**, 105.
[346] R. Roeser, *Mikrokosmos*, 1981, **70**, 152.
[347] R. Kraft, A. Otto, A. Makower, and G. Etzold, *Anal. Biochem.*, 1981, **113**, 193.
[348] C. Marriq, M. Rolland, and S. Lissitzky, *Anal. Biochem.*, 1981, **116**, 89.
[349] Yu. Vladovska, *Dokl. Bolg. Akad. Nauk*, 1981, **34**, 51.
[350] H. Isono, S. Miyaura, and R. Mikawa, *Eisei Kagaku*, 1981, **27**, 38 (*Chem. Abstr.*, 1981, **95**, 20603).
[351] L. Lepri, P. G. Desideri, and D. Heimler, *Ann. Chim. (Rome)*, 1981, **71**, 89.

High-performance Liquid Chromatography.—More space is provided for this topic, which, though developing routine aspects, continues to provide opportunities for pioneering studies.

Electrochemical detection methods lower the sensitivity limits in several areas, for example to 500 and 50 pg levels for dopa and 5-hydroxytryptophan, respectively, in brain tissue [352] (this particular area has been reviewed [353]). Use of a copper tubular electrode as a potentiometric detector for h.p.l.c. of amino-acids has been described.[354]

Most procedures now use fluorescence detection, illustrated in the assay of dansyl thyroxine at femtomole levels,[355] but particularly in the use of *o*-phthalaldehyde–thiol reagents for pre-column fluorescent labelling of amino-acid mixtures.[356–359] Analysis at the 25 picomole level is feasible using this approach.[359] 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole has been used as a pre-column fluorescent labelling reagent for amino-acids[360] including proline and hydroxyproline,[360, 361] which can be estimated in fluids containing 0.08 and 0.04 nmol ml^{-1}, respectively. The ninhydrin–*o*-phthalaldehyde reagent system, with effluent monitoring at 405 nm, has been applied to the assay of N^{τ}-methyl histidine.[362]

Standard procedures have been followed in h.p.l.c. analysis of tryptophan and its metabolites,[363] α-methyldopa in plasma,[364] and serotonin and related compounds.[365] A review of h.p.l.c. of amino-acids has appeared.[366] Most of the h.p.l.c. studies in the amino-acid area have been concerned with derivatives, and current examples are *N*-dimethylaminoazobenzenesulphonylamino-acids (one study [367] claiming 0.3 picomole detection limits, another from the same research group [368] claiming 2—5 picomole limits), dansyl amino-acids,[369] phenylthiohydantoins,[370] and diphenylindenoylthiohydantoins.[371] The h.p.l.c. behaviour of particular derivatives [*N*-acetylaspartic acid [372] and *cis*- and *trans*-$CF_3CONHC(CO_2Et)_2CH{=}CHCO_2Et$ [373]] has been described.

[352] S. P. Arneric, D. B. Goodale, J. R. Flynn, and J. P. Long, *Brain Res. Bull.*, 1981, **6**, 407.
[353] G. C. Davis, D. D. Koch, P. J. Kissinger, C. S. Bruntlett, and R. E. Shoup in 'Liquid Chromatography in Clinical Analysis', ed. P. M. Kabra and L. M. Marton, Humana, Clifton, New Jersey, 1981, p. 253.
[354] P. W. Alexander, P. R. Haddad, G. K. C. Low, and C. Maitra, *J. Chromatogr.*, 1981, **209**, 29.
[355] R. Bongiovanni, K. D. Burman, R. K. Garis, and J. Boehm, *J. Liq. Chromatogr.*, 1981, **4**, 813.
[356] M. Kuwada and K. Katayama, *Anal. Biochem.*, 1981, **117**, 259.
[357] M. J. Drescher, J. E. Medina, and D. G. Drescher, *Anal. Biochem.*, 1981, **116**, 200.
[358] B. N. Jones, S. Paabo, and S. Stein, *J. Liq. Chromatogr.*, 1981, **4**, 565.
[359] B. R. Larsen and F. G. West, *J. Chromatogr. Sci.*, 1981, **19**, 259.
[360] Y. Watanabe and K. Imai, *Anal. Biochem.*, 1981, **116**, 471.
[361] K. Imai and Y. Watanabe, *Anal. Chim. Acta*, 1981, **130**, 377.
[362] L. C. Ward, M. Miller, and S. Hawgood, *J. Chromatogr.*, 1981, **223**, 417.
[363] J. B. Tarr and J. Arditti, *New Phytol.*, 1981, **88**, 621.
[364] D. A. Jenner, M. J. Brown, and F. J. M. Lhoste, *J. Chromatogr.*, 1981, **224**, 507.
[365] D. E. Mais, P. D. Lahr, and T. R. Bosin, *J. Chromatogr.*, 1981, **225**, 27.
[366] K. Hara, E. Uenishi, C. Ishii, H. Egawa, and K. Murata, *Rinsho Byori*, 1981, **29**, 868 (*Chem. Abstr.*, 1982, **96**, 31012).
[367] J.-Y. Chang, R. Knecht, and D. G. Braun, *Biochem. J.*, 1981, **199**, 547.
[368] J.-Y. Chang, P. Martin, R. Bernasconi, and D. G. Braun, *FEBS Lett.*, 1981, **132**, 117.
[369] S. Weiner and A. Tishbee, *J. Chromatogr.*, 1981, **213**, 501.
[370] F. Lottspeich, *Hoppe-Seyler's Z. Physiol. Chem.*, 1980, **361**, 1829.
[371] I. Mancheva, R. Nikolov, and J. Pfletschinger, *J. Chromatogr.*, 1981, **213**, 99.
[372] K. Lenda, *J. Liq. Chromatogr.*, 1981, **4**, 863.
[373] R. Westwood and P. W. Hairsine, *J. Chromatogr.*, 1981, **219**, 140.

Two studies aimed at the determination of enantiomer ratios through conversion of partly racemic amino-acids into diastereoisomeric dipeptides (by reaction with *N*-carboxyanhydrides derived from L-phenylalanine or L-leucine)[374] or into *N*-Boc derivatives by reaction with the *N*-hydroxysuccinimide ester of *N*-Boc-L-leucine[375] are, respectively, a broad study of 20 common amino-acids (except proline and hydroxyproline)[374] and a specific application to the analysis of thyroxine in serum.[375]

A porous polystyrene–divinylbenzene copolymer has been found to be comparable with alkyl-modified silicas in the liquid chromatography of amino-acids and their derivatives.[376]

Other Analytical Methods.—Fluorescence spectrometry has been used for estimation of total amino-acids in serum, after treatment with *o*-phthalaldehyde–mercaptoethanol,[377] a widely used fluorogenic reaction that has been studied by the fluorescence stopped-flow technique.[378]

Several reviews in a symposium proceedings volume[2] cover the more esoteric variations of standard techniques, for example[379] derivative spectroscopy in the analysis of aromatic amino-acids.

Determination of Specific Amino-acids.—Whereas most papers considered for this section describe colorimetric assays and other methods based on long-established analytical techniques, much routine work is excluded. Enzymatic methods, although also based on familiar principles, are given more space here.

Analysis of mixtures of methionine and cysteine can be achieved in two stages.[380] First, reaction with formaldehyde converts cysteine into thiazolidinecarboxylic acid, which does not interfere with the assay for methionine based on K_2PtI_6 complex formation and colorimetry; the cysteine content is determined using 3,3′-dithio-bis(5-nitrobenzoic acid). Nitroprusside colorimetry is used in an assay for γ-carboxyglutamic acid after conversion into a yellow proline derivative by reaction with acetaldehyde.

N-Hydroxyproline can be assayed at levels of $4\,\mu mol\,l^{-1}$ after alkaline hydrolysis and spectrophotometric estimation of derivatives of products of the resulting reverse aldol reaction.[382] Estimation of *N*-hydroxymethylmethionine similarly involves hydrolysis and separate assays for formaldehyde (chromotropic acid) and methionine (iodometric titration).[383]

Enzymatic methods have little competition in terms of their selectivity, illustrative examples being: an assay of L-cysteine based on catalysis by L-methionine γ-lyase (from *Pseudomonas putida*) of its conversion into pyruvate, estimated colorimetrically (this reaction can also be exploited in an assay of L-

[374] T. Takaya, Y. Kishida, and S. Sakakibara, *J. Chromatogr.*, 1981, **215**, 279.
[375] G. Leb, E. P. Lankmayr, R. Goebel, H. Pristantz, F. Nachtmann, and G. Knapp, *Klin. Wochenschr.*, 1981, **59**, 861.
[376] Z. Iskandarani and D. J. Pietrzyk, *Anal. Chem.*, 1981, **53**, 489.
[377] O. F. Velikanova and Yu. V. Galaev, *Lab. Delo*, 1981, 701 (*Chem. Abstr.*, 1982, **96**, 16869).
[378] E. Trepman and R. F. Chen, *Arch. Biochem. Biophys.*, 1980, **204**, 524.
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methionine),[384] radiochemical assay of *S*-adenosyl-L-homocysteine and L-homocysteine using *S*-adenosyl-L-homocysteine hydrolase and labelled adenosine,[385] and assay of L-serine based on its conversion into phosphoserine, using pyrophosphate and L-serine *O*-phosphotransferase, and spectrometric or fluorimetric estimation of the product.[386] The 0.2 nanomole detection limit compares favourably with that which can be achieved with the amino-acid analyser.[386]

Details of the assembly and use of a tyrosinase electrode for estimation of L-dopa have been published.[387] The potentiometric technique has also been used for estimation of L-phenylalanine in serum, based on a lactate electrode with immobilized *Leuconostoc mesenteroides*.[388]

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2
Structural Investigations of Peptides and Proteins

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PART IA: Protein Isolation and Characterization *By M. D. Scawen, R. J. Sherwood, P. M. Hammond, A. Electricwala, and T. Atkinson*

1 Introduction

Two major books of interest to those involved in protein purification were published in 1981. The first is the Proceedings of the 4th International Symposium on Affinity Chromatography and Related Techniques,[1] which covers many theoretical and practical aspects of affinity chromatography. The second is a volume of 'The Enzymes' [2] devoted to the enzymes that act on DNA, covering their purification and properties.

Several other review articles covering specific applications of affinity chromatography to the purification of hormonal proteins,[3] glycosyl transferases,[4] and lectins,[5] the application of carbohydrate ligands to the purification of a variety of macromolecular substances,[6] and the purification of many enzymes on heparin-Sepharose [7] have been published.

2 Protein Isolation Methodology

Affinity Chromatography.—*General Comments.* During 1981 there were four examples of researchers developing their own specialized affinity matrix for adsorption of a particular enzyme: cross-linked polyuronides for pectin esterases,[8] gelatin-agarose copolymers for fibronectin,[9] acid-treated Sepharose CL-6B for a mitogenic lectin glycoprotein,[10] and a poly(hydroxybutyrate) gel for D-3-hydroxybutyrate dimer hydrolase.[11] A novel affinity technique has also been described for the rapid purification of actin from non-muscle cell homogenates.[12]

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The cell homogenate is mixed with a preparation of myosin thick filaments and actin is allowed to bind. After washing, the actin is specifically eluted with ATP.

There have been numerous examples in the literature of the use of phosphocellulose for the adsorption of nucleic acid-binding proteins; although this may be regarded as a form of affinity chromatography, most people do not classify it as such. Therefore we have omitted it from this review except where some form of biospecific elution procedure has been employed.

The theory and prediction of neutral salt elution profiles for enzymes on affinity matrices have been studied, and the relative elution potencies of various salts have been shown to be independent of the protein or affinity ligand in the system. The absolute salt concentration necessary to elute any given protein was shown to be proportional to the algebraic sum of the elution coefficients determined for the separate ions present in the solution. This has implications for large-scale application where expensive biospecific eluants may be replaced by neutral salts.[13]

Two other interesting applications of affinity chromatography are the specific removal of pyrogenic endotoxins from human α-galactosidase A by affinity chromatography on α-D-galactosylamine linked to aminohexyl-Sepharose[14] and the conversion of native Glu-plasminogen to Lys-plasminogen using matrix-bound plasmin.[15]

Coupling Techniques. The most widely used activation procedure still employs cyanogen bromide, and Kohn and Wilchek [16] have described an analytical method for the selective quantitation of the active groups, either imidocarbonates or cyanate esters, introduced into agarose or dextran by cyanogen bromide. Various other coupling methodologies have been described. 2,2,2,-Trifluoroethanesulphonyl chloride (tresyl chloride) has been proposed for the facile activation of a variety of hydroxyl-containing supports, including h.p.l.c. matrices. Both high- and low-molecular-weight ligands could be coupled at neutral pH values. The resulting conjugates were highly resistant to leaching. Tresyl chloride is superior to tosyl chloride for the immobilization of pH-sensitive ligands.[17]

A comparison of the use of various carbonylating reagents for the coupling of amine ligands to agarose has been carried out. 1,1′-Carbonyldi-imidazole was found to be the most generally useful, although 1,1′-carbonyldi-1,2,4-triazole gave a more reactive matrix suitable for the rapid coupling of very labile ligands.[18] Two new methods for preparing immobilized carbohydrate adsorbents for lectins have been described. Epoxy-activated Sepharose can be aminated and oligosaccharide ligands coupled by reductive amination with $NaCNBH_3$; alternatively, epoxy-activated Sepharose can be succinylated and amino-sugars coupled by using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide.[19] Both methods gave superior yields of stable matrix compared with either CNBr-activated Sepharose or by direct coupling to epoxy-activated Sepharose.

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Optimum conditions for the coupling of small, aromatic and aliphatic amines to epoxy-activated Sepharose have been determined.[20]

A new method for the preparation of polyacrylhydrazido-agarose using periodate oxidation followed by reaction with polyacrylhydrazide and $NaBH_4$ has been described. The resulting gel was useful for the immobilization of amino- and hydroxyl-containing ligands of high or low molecular weight.[21]

Inman[22] has described the use of heterobifunctional reagents for ligating peptides or proteins through thioether or disulphide bonds. Improved methods have been developed for introducing sulphydryl groups into amino functions in proteins or supports. These can be allowed to react together to form disulphide linkages or with derivatives of maleic anhydride to form thioether bonds.

Affinity Elution. The technique of affinity elution from ion-exchange materials continues to be studied. Quantitative studies of the binding and elution of a wide range of enzymes to CM-cellulose have been carried out.[23] The method has been used in the purification of eight mitochondrial enzymes from beef heart.[24] The crude enzymes were adsorbed onto CM-cellulose and sequentially and specifically eluted by the application of their substrates. Another example from the same laboratory is the elution of rabbit muscle or yeast pyruvate kinase from CM-cellulose by phosphoenolpyruvate.[25]

Biospecific desorption has also been studied in relation to aspartate trans-carbamoylase from wheat germ.[26] This enzyme was found to be specifically desorbed by one of its substrates, carbamoyl phosphate, from a variety of adsorbents including hydroxyapatite, Cibacron Blue 3-GA Sepharose, Coomassie Blue R-250 Sepharose, *N*-(3-carboxypropionyl)amino-octyl-Sepharose, and 10-carboxydecylamino-Sepharose. In a purification procedure devised for the enzyme a 5460-fold purification factor was achieved by adsorption and desorption from 10-carboxydecylamino-Sepharose.

Dye-affinity Chromatography. Preparative and analytical applications of triazine dyes in both affinity chromatography and protein studies have been recently reviewed.[27, 28]

Metal-ion-promoted adsorption of proteins to immobilized dyes is a novel technique for influencing protein–dye interaction in affinity chromatography; zinc-promoted binding of carboxypeptidase G2, alkaline phosphatase, and yeast hexokinase to immobilized Procion Red H8-BN, Procion Yellow H-A, and Cibacron Blue 3-GA has been demonstrated.[29] Metal-ion-bound enzymes were subsequently eluted by appropriate chelating agents. In addition, magnesium has been shown to promote the binding of yeast hexokinase to immobilized Procion

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Green H-4G, with elution being effected by omission of magnesium from the column buffer or by inclusion of Mg-ATP or D-glucose in the buffer.[30]

Studies on the interaction between yeast RNA polymerase 1 and Cibacron Blue have shown that during chromatography two polypeptides are dissociated from the enzyme, destroying its activity. The inclusion of ATP in the elution buffer prevented both this dissociation and the subsequent inactivation of the enzyme.[31]

An example of the resolving power of affinity chromatography on immobilized Cibacron Blue is the separation of the two closely related bovine kidney enzymes propionyl CoA carboxylase and 3-methylcrotonyl CoA carboxylase in high yield in a single step.[32]

The significance of ligand presaturation and its effects on the interaction of both serum albumin and 6-phosphogluconate dehydrogenase with immobilized triazine dyes has been recently studied.[33, 34]

It was inevitable that with the widespread use of triazine dyes in affinity chromatography research workers would examine the potential of other dyes. In an elegant example of this approach Tucker *et al.*[35] have demonstrated the potential of immobilized tetraiodofluorescein for the affinity chromatography of proteins, which had not previously been found amenable to dye-affinity chromatography.

Other Applications and Techniques. Two novel uses of affinity techniques have been described. An acetylcholine receptor covalently attached to nylon tubes has been used for the assay of anti-receptor immunoglobulins in rabbit blood. After perfusion of a length of tube containing a known density of receptor sites, the remaining sites were determined by competitive binding of ^{125}I-labelled α-bungarotoxin.[36] Non-specific binding of antibody-coated lymphocytes to Staphylococcal Protein A-Sepharose could be greatly reduced by reacting CNBr-activated Sepharose sequentially with Protein A and Blue Dextran. Before using the resulting gel to trap lymphocytes the Blue Dextran was saturated with albumin.[37]

Tables 1 and 2 list proteins purified during 1981 by affinity chromatography and dye-affinity chromatography, respectively.

Hydrophobic Interaction Chromatography and Covalent Chromatography.—The separation of proteins on the basis of their ability to form hydrophobic bonds of differing strength with a stationary support matrix has become a powerful tool amongst those techniques available for protein purification. Ionic interaction between protein and matrix was thought, until recently, to be an influencing factor in the binding of many proteins to hydrophobic matrices. Halperin *et al.*,[141] however, have examined relative binding of several proteins to charged and electrically neutral hydrophobic matrices. They conclude that, although many

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Table 1 *Proteins purified by affinity chromatography*

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Hypoxanthine-guanine phospho-ribosyl transferase	*Saccharomyces cerevisiae*	Sepharose	GMP	1 mM 5-phosphoribosyl 1-pyrophosphate	38
Hypoxanthine-guanine phospho-ribosyl transferase	Human erythrocytes	Sepharose	GMP	5 mM GMP	39
Phosphate carrier protein	Pig-heart mitochondria	AM-Ultrogel A-4-R	Mersalyl	30 mM dithiothreitol	40
D-Glucose transport protein	Human erythrocytes	Sepharose	Wheatgerm lectin	50 mM and 500 mM *N*-acetyl glucosamine	41
Cytidine 3′:5′-monophosphate phosphodiesterase	Pig liver	Sepharose	3′:5′-cyclic AMP	10 mM cyclic AMP	42
Cyclic AMP phosphodiesterase	Rat liver	Sepharose	Theophylline	500 μM isobutylmethyl xanthine	43
Double-stranded RNA-activated eIF-2 kinase	Rabbit reticulocyte-ribosomes	Sepharose	Histone	1 M KCl, 0.2 mM ATP, 2 mM $Mg(MeCOO)_2$	44
α-Mannosidase	Rabbit liver	Sepharose	Concanavalin A	0.75 M methyl-α-D-mannopyranoside, 10% glycerol, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% Triton X-100	45
Plasminogen activator	Human melanoma cells	Sepharose	(i) Zn^{2+}-chelate (ii) Concanavalin A	(i) 0—0.05 M imidazole gradient (ii) Linear gradient of 0—0.4 M α-D-methylmannoside, 2 M KSCN	46
Troponin T kinase	Dog heart	Sepharose	De-phosphorylated Troponin T	0—0.5 M KCl gradient	47
Myosin light-chain kinase	Turkey smooth muscle	Sepharose	Calmodulin	2 mM EGTA	48
Polyadenosine-diphosphoribo-sylated proteins	Hamster liver	BioGel P-300	*m*-Aminophenyl boronic acid	pH shift, 8.2—4.0	49

cAMP-dependent protein kinase (separation of catalytic and regulatory subunits)	Bovine liver	Sepharose	8-(6-Aminohexyl)-amino-cAMP	Catalytic subunit does not bind. Regulatory subunit eluted by 3 h incubation with 10 mM cAMP	50
Alkaline protease and neutral protease	Rat chromatin	Sepharose	Sequential use of: (i) soy-bean trypsin inhibitor (ii) casein (iii) histone	(i) 50 mM Na acetate–HCl, 50 mM NaCl, 2 M urea, pH 1.5 (ii) 50 mM Na acetate–HCl, 50 mM NaCl, 2 M urea, pH 4.0 Alkaline protease does not bind. Neutral protease eluted in low yield by 50 mM Na acetate–HCl, 50 mM NaCl, 2 M urea, pH 3.0	51
Rifamycin-binding protein	*E. coli*	Sepharose	*N*-Demethyl rifampicin	0.1—1.5 mg ml^{-1} rifampicin gradient	52
D(−)-3-Hydroxybutyrate dimer hydrolase	*Zoogloea ramigira*	—	Poly(hydroxy-butyrate gel	0.5% sodium cholate	11

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Table 1 (*cont.*)

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Glycoproteins	Rat brain	Agarose	Wheatgerm agglutinin Concanavalin A *Ulex europaeus* lectin *Ricinus sanguinis* lectin	Various eluants used in presence of sodium dodecyl sulphate to prepare 9 glycoprotein fractions	53
Cobalamin-binding protein	Rabbit serum, human saliva, and human gastric juice	Sephacryl S-200	3-Aminopropyl cobalamin	Photolytic hydrolysis to release protein-bound aquocobalamin	54
Aspartate transcarbamoylase	Wheatgerm	Sepharose 4B	10-Carboxydecyl-amine	1 mM carbamoyl phosphate	26
Amylase	Rat pancreas and salivary gland	Aminohexyl-Sepharose	α-Glucohydrolase inhibitor (Bay g5421)	0.1% glycogen	55
Thyroid hormone-receptor protein	Rat liver	Aminohexyl-Sepharose	L-Tri-iodothyronine	0·1 μM tri-iodothyronine	56
3-Methyladenine-DNA-*N*-glycosylase	Rat liver	Cellulose	DNA	0.04—0.6 M NaCl gradient	57
Aldose reductase	Human placenta	Aminohexyl-Sepharose	4-Carboxybenz-aldehyde	0.1 M Na, K phosphate buffer, pH 6.2	58
Salicylate hydroxylase	*Pseudomonas* sp.	Aminohexyl-Sepharose	*p*-Aminosalicylate	0—60 mM salicylate gradient	59
Dihydropteridine reductase	Human liver	Aminohexyl-Sepharose	1,2-Naphthoquinone	2 mM dithiothreitol	60
Riboflavin kinase	*Phaseolus aureus*	Sepharose	(i) Riboflavin (ii) ATP	(i) Binds in 0.5 M phosphate. Elute with H_2O (ii) Binds in 0.5 M phosphate. Elute with H_2O	61
Cathepsin D	Pig myometrium	Sepharose	(i) Concanavalin A (ii) Pepstatin	0.2 mM α-methylmannoside 0.05 M $NaHCO_3$, 0.6 M NaCl	62

Aldehyde reductase	Rat brain	Sepharose	NADP	0—0.1 M KCl gradient	63
RNase U_2	*Ustilago sphaerogena*	Aminohexyl-Sepharose	AMP	pH 5.3—6.5 gradient	64
RNase T_2	Taka-diastase powder	Aminohexyl-Sepharose	AMP	2.5 M NaCl, 1 mM 2′(3′)-AMP	65
Tryptophan 5-mono-oxygenase	Rabbit brain	AffiGel 202	Dimethyltetrahydro-pteridine	10 mM $NaHCO_3$–NaOH, pH 10.3	66
Ca^{2+}-activated neutral protease	Chicken muscle	Carboxyhexyl-Sepharose	Casein	Binds in presence of 25 mM Ca^{2+}. Eluted by buffer containing 2 mM EGTA	67
ADP glucose synthetase	*Rhodospirillum tenue*	Aminohexyl-Sepharose	N^6-AMP	3 mM AMP, 0.5 mM ATP	68
Glutathione *S*-transferase	Rat liver	Aminohexyl-Sepharose	Cholic acid	5 mM cholate	69
Glutathione *S*-transferase	Rat liver	Lysyl-Sepharose	Reduced glutathione	5 mM GSH	70
Glutathione *S*-transferase μ	Human liver	Epoxy-activated Sepharose	*S*-Hexyl glutathione	0.2 M NaCl, 5 mM *S*-hexyl glutathione	71

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Table 1 (*cont.*)

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Calmodulin	Bovine brain	AffiGel 10	2-Chloro-10-(aminopropyl) phenothiazine	Binds in presence of 1 mM Ca^{2+}. Elute with 10 mM EGTA	72
Calmodulin	Bovine brain	Sepharose	*N*-(6-Aminohexyl)-5-chloro-1-naphthalene sulphonamide	Binds in presence of 0.1 mM Ca^{2+}. Elute with 4 mM EGTA	73
Glutathione reductase	Calf liver	Sepharose	2′,5′-ADP	Wash with 0.4 M phosphate, pH 7.5. Elute with either 0.5 M KCl or 0—0.5 mM NADPH gradient	74
α-Glucosidase	Yeast	Agarose	*m*-Aminophenyl boronic acid (Matrex Gel PBA-60)	Binds in presence of 100 mM maltose. Elute with equilibration buffer.	75
α-Glucosidase	Yeast	Sepharose	3-Nitro-4-(6-amino-hexylamido)phenyl boronic acid	Binds in presence of maltose. Elute with 0.1 M sorbitol	75
β-Glucosidase	Human placenta	Sepharose	Dextran sulphate	3 mg ml^{-1} sodium taurocholate	76
Lactate dehydrogenase C_4	Rat testes	Aminohexyl-Sepharose	N^6-AMP	Gradient of pyruvate–NAD^+ adduct separates LDH-C from other isoenzymes	77
Lactate dehydrogenase	Soy bean	Sepharose	ATP	1 mM NADH	78
Hyaluronidase	Bovine testes	Aminohexyl-Sepharose	Dermatan sulphate	0—1.0 M NaCl gradient	79
Xanthine oxidase	Cow's milk	Aminohexyl-Sepharose	Folate	0.5 mM hypoxanthine	80
Orotate phospho-ribosyl transferase	*E. coli*	Sepharose	Orotidine 5′-monophosphate	17 mM 5′-phosphoribosyl-β-D-ribose diphosphate, 17 mM $MgCl_2$	81
Malic enzyme	*Clostridium thermocellum*	Agarose	NADP	2 mM $NADP^+$	82
Haemagglutinin II	*Ulex europaeus*	Sepharose	Chitobiose	1 M MeCOOH	83

Glucocorticoid receptor	Rat liver	Sepharose	17β-carboxamide derivative of dexamethasone	10^{-6} M triaminolone acetonide	84
Dexamethasone-binding protein	Rat thymus	Aminohexyl cystaminium-Sepharose	Dexamethasone	Elute receptor–dexamethasone complex using 100 mM 2-mercaptoethanol	85
Methionyl-tRNA synthetase	*Bacillus stearothermophilus*	Aminohexyl-agarose	AMP	Binds in buffer containing 1 mM $MgCl_2$, 10 mM L-methioninol, 20 mM D-Met. Elute by substituting 20 mM L-Met	86
Isoleucyl-tRNA synthetase	*E. coli*	Aminohexyl-agarose	AMP	Binds in buffer containing 20 mM L-isoleucinol, 3 mM $MgCl_2$, 2 mM D-Ile. Elute by substituting 2 mM L-Ile	86
Valyl-tRNA synthetase	*E. coli*	Aminohexyl-agarose	AMP	Binds in buffer containing 3 mM $MgCl_2$, 20 mM L-valinol, 20 mM D-Val. Elute by substituting 20 mM L-Val	86
Tryptophanyl-tRNA synthetase	*E. coli*	Aminohexyl-agarose	AMP	Binds in buffer containing 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM tryptamine, 20 mM D-Trp. Elute by substituting 20 mM L-Trp	86

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Table 1 (*cont.*)

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Tyrosyl-tRNA synthetase	*E. coli*	Aminohexyl-agarose	AMP	Binds in buffer containing 0.1 mM L-tyrosinol, 1 mM D-Tyr. Elute by substituting 1 mM L-Tyr	86
Lysozyme	Hen egg white	Beaded cellulose	$(Glc\text{-}NAc)_3$	0.1 mM MeCOOH	87
Lysozyme	Hen egg white	Ultrogel A4R	Heparin	0—0.8 M NaCl gradient, pH 7.4	88
Dihydrofolate reductase	HeLa BU 25 cells	Sepharose	Methotrexate	1 mM folic acid	89
Lipoprotein lipase	Rat heart	Sepharose	Heparin	1.2 M NaCl	90
Lipase	*Rhizopus japonicus*	Sepharose	Heparin	0—1.0 M NaCl gradient	91
Plasma-membrane fractions	Bovine thymus lymphocytes	Sepharose	Concanavalin A	0.1 M α-methyl α-mannoside	92
Haptoglobulin	Rat (and other species	Sepharose	Haemoglobin	8 M urea	93
Trypsin	Bovine pancreas *Streptomyces griseus*	Sepharose	Gly-Gly-L-arginal	0.1 mM leupeptin	94
Phosphoribulo-kinase	*Alcaligenes eutrophus*	Aminohexyl-agarose	AMP	Column washed with buffer containing 100 mM KCl, 5 mM ATP. Enzyme eluted with 15 mM ATP	95
Cholesterol ester hydrolase	Rabbit aorta	Sepharose	Concanavalin A	0.7 M α-methyl mannoside	96
Antigen	*Trypanosoma conglolense*	Sepharose	Concanavalin A	Protein desorbed electrophoretically	97
N-Acetyl glutamate *S*-phosphotrans-ferase	Pea	Agarose	ATP	50 mM ATP	98
S-Adenosyl-L-homocysteine hydrolase	Rat liver	Sepharose	8-(3-Aminopropyl-amino) adenosine	0.4 M KCl, 0.2 mM adenosine	99

Eukaryotic mRNA cap-binding protein	Rabbit reticulocytes	Aminohexyl-Sepharose	2′,3′-O-[1-(2-carboxyethyl) ethylidene]-7-methyl guanosine diphosphate or 7-(5-carboxypentyl) guanosine diphosphate	0.07 mM 7-methyl guanosine diphosphate	100
Carboxypeptidase A	*Streptomyces griseus*	Sepharose	*p*-Aminobenzyl succinic acid	0.55—1.0 M NaCl gradient	101
Heparin	Bovine plasma	Silica (macroporous)	Antithrombin III	3 M NaCl	102
Antithrombin III	Bovine plasma	Silica (macroporous)	Heparin	4 M NaCl and pH change	102
Trypsin	Bovine pancreas	Spheron (Separon)	Aminocaproyl L-Phe-D-Phe-OMe	1 M NaCl	103
Albumin	Human serum	Silica	Anti-human serum albumin	0.2 M glycine, pH 2.2	104
Pectin esterases	Onion	Crosslinked polyuronides		0—0.5 M NaCl gradient	8

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Table 1 (*cont.*)

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Endopoly-galacturonases	*K. marxianus*	Crosslinked polyuronides		pH change	8
Plasminogen	Human serum	Agarose	Lysine	0.1 M amino-caproate	15
Antithrombin	Human serum	Sepharose	Heparin	1 M NaCl or heparin (2 mg ml^{-1})	105
Heparin	Human serum	Sepharose	Concanavalin A	0.2 M α-methyl D-glucoside	105
Polio virus	Virus suspension	Sepharose	D-Antigen type I	7 M ammonium thiocyanate	106
Pyruvate dehydro-genase E2 protein	Several sources	Sepharose	Ethanol	0—0.25 M KCl gradient	107
α-Ketoglutarate dehydrogenase	Several sources	Sepharose	Ethanol	0—0.2 M KCl gradient	107
Pepsin	Porcine	Spheron	Aminocaproyl L-Phe-D-Phe-OMe	1 M NaCl	108
		Separon	Aminocaproyl L-Phe-D-Phe-OMe	1 M NaCl	
Anti-glyco sphingo-lipid antibody	Anti-sera	Sepharose	Sphingolipid glycerol oligosaccharide	0.5 M or 3 M sodium thiocyanate	109
FSH testicular-membrane receptors	Calf testes	Agarose	Human chorionic gonadotropin	FSH or NaCl	110
Interferon	Human amniotic fluid	Agarose	(i) Bovine plasma albumin (ii) Cibacron Blue (iii) Concanavalin A (iv) L-Trp-L-Tyr-carboxypentyl	50% ethylene glycol or 1 M NaCl	111
Mitogenic lectin glycoprotein	*Nura crepitans* seeds	—	Acid-treated Sepharose CL-6B	D-Galactose	10
Glucose 6-phosphate dehydrogenase	Yeast	Agarose	$NADP^+$ complex with aminophenyl-boronic acid	Salt, diols, monosaccharides, cofactor, or pH	112

Hexokinase	Yeast	Agarose	ATP complex with aminophenyl-boronic acid	Enzyme retarded	112
Steroid 12α-mono-oxygenase	Rabbit liver	Sepharose	Cholate	0.4% cholate in 300 mM phosphate	113
α-1,4-Glucan phosphorylase	Spinach	Sepharose	Starch	Soluble glucan/NaCl	114
Herpes simplex virus type I specific g C glycoprotein	*Herpes simplex*	Sepharose	Soy-bean lectin	*N*-Acetyl galactosamine	115
Galactose specific lectin	*M. charantia* seeds	—	Crosslinked arabinogalactan	0.1 M lactose	116
Opiate receptor	Rat brain	Sepharose	14β-Bromo-acetamido-morphine	1 μM levorphanol or etorphine	117
Thiamin-binding protein	Chicken egg yolk	Sepharose	Thiamin pyrophosphate	10 μM thiamin, pH 7.0	118

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Table 2 *Proteins purified by affinity chromatography on immobilized dyes*

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
NAD glycohydrolase	*B. fasciatus* venom	Agarose	Cibacron Blue 3G-A	NAD	119
Formamido-pyrimidine-DNA glycosylase	*E. coli*	Agarose	AffiGel Blue (Cibacron Blue 3G-A)	0.1—0.8 M KCl gradient	120
Acyl coenzyme A synthetase	*E. coli*	Sepharose	Blue Dextran	0.05—1.0 M KCl gradient	121
Deoxycytidylate deaminase	Human spleen	Sepharose	Blue Sepharose (Cibacron Blue 3G-A)	2 mM ATP, 2 mM $MgCl_2$, followed by 2.5 mM dCMP	122
Phosphatidyl inositol-specific phospholipase C	Rat liver		AffiGel Blue (Cibacron Blue 3G-A)	1.2 M NaCl	123
α-1-Antitrypsin	Rat serum	Sepharose	Blue Sepharose (Cibacron Blue 3G-A)	Negative purification to remove albumin and other serum protein	124
Nucleoside diphosphate kinase	Pig heart	Sepharose	Cibacron Blue 3G-A	0.3 M NaCl, 1.5 mM ATP	125
Aldose reductase	Human placenta	Crosslinked agarose	Matrex Orange A (Procion Yellow HA)	0.6 mM NAD, followed by 0.5 mM digitonin, followed by 0.1 mM NADP to elute enzyme	58
Ferrochelatase	Rat liver	Sepharose	Blue Sepharose (Cibacron Blue 3G-A)	1% sodium cholate, 1.5 M NaCl	126
β-Glucosidase	Human liver	Sepharose	Blue Sepharose (Cibacron Blue 3G-A)	Negative purification to remove albumin	127
Glutamine synthetase	*Anabaena* 7120	Sepharose	Cibacron Blue 3G-A	0—70 μM ADP gradient	128
Short-chain and general acyl Co A dehydrogenases	Rat liver	Sepharose	Blue Dextran (Cibacron Blue 3G-A)	20 μM butyryl Co A, followed by 20 μM decanoyl Co A	129
Asparagine synthetase	Rat liver	Agarose	Reactive Blue 2	1 mM L-aspartate, 0.1 mM ATP	130
Adenylyl sulphate: ammonia adenyl-transferase	*Chlorella*	Agarose	Reactive Blue 2	Enzyme does not bind but 2—3-fold purification is achieved	131

Cyclic AMP phosphodiesterase	Rat liver	Agarose	AffiGel Blue (Cibacron Blue 3G-A)	0.5 M NaCl	43
Riboflavin-kinase	*Phaseolus aureus*	Sepharose	Cibacron Blue 3G-A	Binds in 1.0 M phosphate. Elute with H_2O	61
Carnosine synthetase	Chicken muscle	Agarose	Cibacron Blue 3G-A	50 mM carnosine	132
Phosphoglycerate mutase	*Leuconostoc dextranicus*	Sepharose	Blue Dextran (Cibacron Blue 3G-A)	0—0.5 mM gradient of 2,3-bisphosphoglycerate	133
Phytochrome	Rye (*Secale cereale*)	Agarose	AffiGel Blue (Cibacron Blue 3G-A)	10 mM FMN	134
Phytochrome	Oat (*Avena sativa*)	Agarose	AffiGel Blue (Cibacron Blue 3G-A)	10 mM FMN (purified free of lumichrome)	135
17-β-Hydroxysteroid dehydrogenase	Porcine testes	Agarose	Procion Red HE-3B	0—1.5 M NaCl gradient	136
Phosphoribulo-kinase	*Alcaligenes eutrophus*	Agarose	Matrex Blue A (Cibacron Blue 3G-A)	Column washed sequentially with buffer containing 100 mM KCl and 2 mM NAD; 3 mM ATP. Enzyme eluted with 15 mM ATP	95

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Table 2 (*cont.*)

Protein	*Source*	*Matrix*	*Ligand*	*Eluant/Comment*	*Ref.*
Lactate dehydrogenase	Soy bean	Sepharose	Cibacron Blue 3G-A	1 mM NADH	78
Albumin	Human serum	Trisacryl	Cibacron Blue 3G-A	0.35 M NaCl	137
Lactate dehydrogenase isoenzymes	Heart and muscle	Silica	Cibacron Blue 3G-A	NADH gradient	27
Tryptophanyl tRNA synthetase	*Bacillus stearothermophilus*	Sepharose	Procion Brown MX-5BR	10 mM tryptophan	28
		Silica	Procion Brown MX-5BR	10 mM tryptophan, 10 mM ATP	
Pyruvate kinase	*Aspergillus nidulans*	Sepharose	Mikacioi Yellow 6GS	0.15 M NaCl	107
			Blue Dextran (Cibacron Blue 3G-A)	0.15 M NaCl	
Dopamine β-mono-oxygenase	Bovine adrenal medulla	Sepharose	Cibacron Blue 3G-A	0.2 M NaCl	138
Dopamine β-mono-oxygenase	Bovine adrenal medulla	Agarose	Procion Red HE-3B	0.8 M NaCl	138
Adenylosuccinate synthetase	*E. coli*	Sepharose	(i) Procion Red H-3B (ii) Procion Blue H-B	(i) 0—1 M KCl gradient (ii) 4 mM IMP, 2 mM GTP, 10 mM L-aspartate	139
Alkaline phosphatase	Calf intestine	Sepharose	Cibacron Blue 3G-A	α-Naphthyl phosphate	140

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such matrices may contain a weak electrical charge, this charge does not play a significant role in protein binding.

Agarose still remains the most popular support matrix, octyl- and phenyl-Sepharose being those matrices most frequently used. The nature of the ligand continues to diversify, however, to include Spheron P300[142] and octanoic acid hydrazide.[143]

A recent major advance in the use of hydrophobic interaction chromatography has been its application to h.p.l.c. An investigation has recently been made using a series of alkylsilane bonded silica packings with different carbon-chain lengths varying from C_1 to C_{18}.[144] These authors found that reversed-phase h.p.l.c. using packings with a chain length of $<C_6$ was most effective. A reversed-phase procedure employing LiChroprep RP-8 has also been used for peptide fractionation,[145] although solute ionization was also found to be an influencing factor, resulting in decreased retention.

A new method, using reductive alkylation, for immobilizing enzymes on hydrophobic matrices has been described.[146] The procedure is mild and does not reduce the catalytic activity of many enzymes. It may therefore be of use for enzymes which may otherwise be irreversibly bound to hydrophobic gels.

Table 3 lists examples from the literature of 1981 of protein materials isolated by use of hydrophobic interaction chromatography.

The purification of proteins by means of covalent chromatography has been limited to the isolation of thiol-containing proteins[147] and peptides[148] and a study of the cysteine proteinases actinidin and papain[149] that examines the differences in the interactions of the catalytic groups of their active centres.

Immunoaffinity Chromatography.—Immunoaffinity chromatography continues to be used as one of the many techniques in the field of protein purification.

The literature survey has shown that the conditions for the binding and specific elution of proteins from immunoadsorbents vary considerably with different proteins. For example, barley β-amylase[179] can be desorbed from the immunosorbent with water whereas elution of isocitrate dehydrogenase[180] and platelet membrane proteins[181] requires harsh conditions like 4 M guanidine–HCl buffer and 8 M urea, respectively. The elution of proteins can also be governed by controlling the pH of the eluting buffer, as is the case of isolation of neurofilament proteins,[182] or by increasing the ionic strength of the buffer by addition of NaCl.[183] It has also been reported that, with certain proteins, addition of poly(ethylene glycol)6000[184] or EDTA to the eluting buffer increased the yield considerably.

Immunoaffinity chromatography has also been used successfully, on a preparative scale, for the isolation of α-fetoprotein (α-AFP) from partially purified

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Table 3 *Proteins purified by hydrophobic interaction chromatography**

Protein	*Source*	*Ligand and support*	*Loading buffer*	*Elution buffer*	*Ref.*
D(−)-3-Hydroxy butyrate dimer hydrolase	*Zoogloea ramigera*	Octyl-Sepharose	0.1 M Tris–HCl, pH 7.5	Linear gradient, 0—2% Na-cholate in loading buffer	53
α-Trypsin and β-trypsin		Spheron P300			142
Caseins	Bovine milk	Octyl-Sepharose	0.1 M Na_2HPO_4, pH 7.0	Linear gradient, 0—6 M urea in loading buffer	150
Egg-yolk apoproteins	Hen egg	Phenyl-Sepharose	Not stated	Linear gradient, 2—9 M urea	151
Galactosyl-ceramidase	Murine intestine	Octyl-Sepharose	10 mM NaP, pH 7.2	Linear gradient, 0—1% Triton X-100 in loading buffer	152
Calmodulin	Bovine brain	Phenyl- and octyl-Sepharose	50 mM Tris–HCl, pH 7.5, 1 mM MSH, 0.1 M $CaCl_2$	50 mM Tris–HCl, pH 7.5, 1 mM MSH, 1 mM EGTA	153
Phenylalanine hydroxylase	Rat liver	Phenyl-Sepharose	Not stated	Inclusion of glycerol. Little detail given	154
Cytochrome P-450	*Lodderomyces elongisporus*	ω-Amino-octyl-Sepharose	10 mM phosphate, pH 7.25, 20% glycerol, 1 mM EDTA, 0.5 mM DTT, 1.2% Na-cholate	10 mM phosphate, pH 7.25, 35% glycerol, 0.5 mM DTT, 0.6% Na-cholate, 0.5% Tween 20	155
Unidentified Scrapie infectivity protein	Scrapie virus	Phenyl-Sepharose	50 mM Tris–HCl, pH 7.8, 1 mM DTT, 10% glycerol, 1 M KCl	8.5 M ethanediol, 4% NP40 detergent, 2% Sarkosyl	156
Collagenase-stimulating factor	Human-blood monocyte macrophages	Phenyl-Sepharose	10 mM NaP, pH 6.8, 0.8 M $(NH_4)_2SO_4$	10 mM NaP, pH 6.8, 50% ethanediol	157
Delta toxin	*Staphylococcus aureus*	Phenyl- and octyl-Sepharose	50 mM NH_4OH, no buffer present	Distilled H_2O, followed by linear gradient, 0—100% ethanol	158

Histone-binding polypeptide	Mouse liver	ω-Aminobutyl-agarose	10 mM Tris–HCl, pH 7.3, 5 mM MSH, 70% $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$ in loading buffer	159
Ribosomal proteins	*E. coli*	Octyl-Sepharose	10 mM Tris–HCl, pH 7.5, 10 mM Mg-acetate, 60 mM NH_4Cl	1% Brij 35 in loading buffer	160
Sphingomyelinase	Human placenta	Octyl-Sepharose	10 mM maleic acid, pH 6.5, 10% $(NH_4)_2SO_4$	10 mM maleic acid, pH 6.5, 0.5% Triton X-100	161
		Hexyl-agarose	10 mM maleic acid, pH 6.5, 0.5% Triton X-100	10 mM maleic acid, pH 6.5, 0.5% Triton X-100, 0.5 mM NaCl	
Adenylate cyclase	Rat brain	Dodecyl-Sepharose	10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA	0.5% Lubrol PX in loading buffer	162
Tryptic peptides of lysozyme	Hen egg	LiChroprep RP-8	0.1% HCl, no buffer present	Linear gradient, 5—50% ethanol in 0.1% HCl	145
Cobalamine-binding protein	Human tissues	Phenyl-Sepharose	50 mM NaP, pH 7.4, 150 mM NaCl	IF-Cbl and R-Cbl complexes: not retarded Tc II-Cbl Intrinsic Factor: 50% glycerol in loading buffer	163

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Table 3 (*cont.*)

Protein	*Source*	*Ligand and support*	*Loading buffer*	*Elution buffer*	*Ref.*
Antigen TR-d	*Treponema pallidum*	Phenyl-Sepharose	50 mM Tris–HCl, pH 8.0, 1 M $(NH_4)_2SO_4$	Loading buffer	164
Prolactin	Human pituitary	Phenyl-Sepharose	5 mM ammonium acetate, pH 8.5, 1 mM NaN_3	Linear gradient, 0—30% acetonitrile in loading buffer	165
Nuclear androgen receptor	Rat prostate	Aminoalkyl-agarose	10 mM TES, pH 7.0, 0.6% NaCl	Not stated	166
Soluble egg-antigen glycoproteins	*Schistosoma japonicum*	Phenyl-Sepharose	1 M $(NH_4)_2SO_4$	20 mM glycine–NaOH, pH 9.8, 50% ethanediol	167
NADH dehydrogenase	*Bacillus caldotenax*	Phenyl-Sepharose	10 mM phosphate, pH 7.0, 20% methanol, 0.1 mM DTT, 3% Triton X-100	Linear gradient, 0—0.6 M NaSCN in loading buffer	168
Colon-stimulating factor and erythroid-enhancing factor	Human-cell line GcT	Phenyl-Sepharose	30 mM Tris–HCl, pH 7.4, 1 M $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$, followed by 50% ethanediol, both in loading buffer	169
Porcine kallikrein	Porcine urine	Octyl-Sepharose	2 M ammonium phosphate–H_3PO_4, pH 7.5	Linear gradient, 2.0—0.05 M ammonium phosphate, pH 7.5	170
Phosphatidyl-inositol kinase	Rat brain	Hexyl-agarose	10 mM Tris, 1% (w/v) Triton X-100	(i) 10 mM Tris, 10% (w/v) Triton X-100, 0.5 mM DTT (ii) 10 mM Tris, 0.5 mM DTT (iii) Linear gradient, 0—0.7 mM DCHO	171
		Dodecyl-Sepharose	10 mM Tris, 1% (w/v) Triton X-100	10 mM Tris, 8 mM DCHO, 0.5 mM DTT	
Coagulation Factors II, VII, IX, and X (Technique used to separate proteins from hepatitis B virus)	Human	Octanoic acid hydrazide-Sepharose	0.75 M NH_4HCO_3	Not retarded	143
Heparin	Hog mucosa	Phenyl-Sepharose	3.8 M $(NH_4)_2SO_4$ in 0.01 M HCl	Linear gradient, 3.8—2.0 M $(NH_4)_2SO_4$ in 0.01 M HCl	172

Globular proteins (unspecified)	Sunflower seeds	Octyl-Sepharose	20 mM borate, pH 7.4, 10% NaCl, 20% $(NH_4)_2SO_4$	Decreasing gradients of constituents of loading buffer	173
Gonadotropin	Pike eel	Phenyl-Sepharose	0.1 M acetic acid	Subunit SI: not retarded Subunit SII: 0.1 M ammonium acetate	174
Lipase	*Staphylococcus aureus*	Octyl-Sepharose	0.05 M Tris–HCl, pH 8.0	Linear gradient, 0—1% DCHO in loading buffer, followed by linear gradient, 0—1% Triton X-100 in loading buffer	175
Heat-stable enterotoxin	*E. coli*	Octyl-Sepharose	Phosphate-buffered saline containing $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$, increasing gradient of ethanediol	176
Collagenase	*Clostridium histolyticum*	CC-Sepharose C_7NH_2	50 mM Tris–HCl, pH 7.4	Loading buffer with 150 mM NaCl	177
Ornithine decarboxylase	*Saccharomyces cerevisiae*	Phenyl-Sepharose	25 mM Tris–HCl, pH 7.6, 5 mM DTT, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 M $(NH_4)_2SO_4$	Decreasing gradient of $(NH_4)_2SO_4$ in loading buffer	178

*Abbreviations used in this Table: NADH nicotinamideadenine dinucleotide (reduced), NaP sodium phosphate buffer, KP potassium phosphate buffer, DCHO deoxycholate, DEAE diethylaminoethyl, CM carboxymethyl, TGEDP 50 mM Tris–acetate, pH 8.2, 25% (v/v) glycerol, 1 mM EDTA, 10 mM DTE, 0.5 mM PMSF, PBS phosphate-buffered saline, CMBE {[*p*-(chloromercuri)benzamido]ethyl}, EDTA ethylenediaminetetra-acetate, EGTA ethyleneglycolbis(aminoethylether)tetra-acetate, DTT dithiothreitol, MSH β-mercaptoethanol, DTE dithioerythritol, PMSF phenylmethylsulphonyl fluoride, SDS sodium dodecyl sulphate, TES 30 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM Na_2-EDTA, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid).

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human-cord serum.[185] The procedure does not require an anti-AFP immunosorbent, but instead the protein is purified on a column filled with anti-cord-serum-coupled Sepharose from which most of the anti-albumin has beeen removed.

Recently an immunosorbent has been used in a novel way to assay radiolabelled carboxylated protein, the reaction product of vitamin K-dependent carboxylase and $^{14}CO_2$.[186]

Table 4 lists several proteins isolated, during 1981, by means of immunoaffinity chromatography.

Table 4 *Proteins purified by immunoaffinity chromatography*

Protein	*Source*	*Eluant/Comment*	*Ref.*
NADP-specific glutamate dehydrogenase	*Chlorella* cell homogenate	Isolation of radiolabelled enzyme by use of rabbit anti-GDH IgG	187
CBA thymocyte receptors	Mouse	Isolation of active proteins by use of immobilized anti-CBA brain antibody	188
β-Amylase	Barley	Elution with water after interruption for a few hours	179
Factor V	Human blood	1.2 M NaCl at pH 6.5	183
Platelet membrane proteins	Human blood	Proteins were labelled with reagents containing haptenic group prior to chromatography. Elution with 8 M urea of 10% formic acid	181
Vitamin K-dependent carboxylase	Bovine liver	Radiolabelled carboxylated proteins were assayed by immunoaffinity chromatography. The bound protein was eluted with 6 M urea in 2% SDS	186
α_1-Protease inhibitor	Rat	Elution with 3 M NaSCN	189
Neurofilament proteins	Human/cattle/ rabbit/rat brain spinal cord and sciatic nerve	Elution of tightly and less tightly bound material with buffer of pH 2.9 and 6, respectively	182

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Table 4 (*cont.*)

Protein	*Source*	*Eluant/Comment*	*Ref.*
Egg antigens	*Schistosoma japonicum*	0.05 M glycine–HCl, pH 2.8	190
DNA polymerase	Bacteriophage T7	Elution of active enzyme by a pulse of 0.1 M Sorensen buffer, pH 12	191
Complement attack complexes ($SC5_b$-9 and $MC5_b$-9)	Human serum and erythrocyte membrane	2 M guanidine–HCl in phosphate-buffered saline	192
Alpha-fetoproteins (AFP)	Human-cord serum	Purification by anti-cord serum (devoid of anti-albumin) coupled-Sepharose column. Does not require specific anti-AFP IgG	185
Prekallikrein	Rat pancreas	Elution with 0.1 M sodium acetate–0.5 M NaCl–0.66% PEG 6000, pH 4.0. Absence of PEG decreased the yield considerably	184
NADP-specific isocitrate dehydrogenase (IDH)	*E. coli*	Anti-IDH IgG cross-linked to Protein A-agarose was used as immunoadsorbent. Enzyme eluted with 4 M guanidine–HCl	180
α-Fetoprotein	Rat amniotic fluid	Removal of adult serum proteins by direct negative immunoadsorption and excess antibodies by ion-exchange chromatography	193
$SP_1\alpha$- and $SP_1\beta$-antigens	Human	Purification by positive and negative affinity columns	194
Immunoglobulin M	Human serum	Use of Protein A-Sepharose as an immunoadsorbent with 50% recovery	195
Anti-Protein I antibody	Bovine brain	Elution with 0.1 M citrate, pH 2.5	196
Monoclonal antibodies to phenylalamine hydroxylase	Hybrid cell lines	Elution with 50 mM diethylamine, pH 11.5	197

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Metal Chelate Chromatography.—Conventional metal chelate chromatography, using zinc chelate agarose, has been used to purify a plasminogen activator secreted by a human melanoma cell line.[46] The enzyme from 10 litres of culture medium was adsorbed onto zinc chelate agarose and eluted with a 0—0.05 M imidazole gradient, giving a 66-fold concentration and 11-fold purification.

Copper chelate chromatography has been used to purify non-histone proteins from rat-liver nuclei in the presence of 0.5 M NaCl to prevent aggregation of the proteins. The proteins were preferentially eluted using a 0—10 mM histamine gradient.[198]

In other cases advantage was taken of the metal-binding properties of the proteins of interest. Thus the calcium-containing seed lectin from *Dolichos biflorus* was purified by chromatography on calcium chelate agarose and eluted by EDTA.[199] A calcium-activated protease from chicken muscle bound to immobilized casein and could be eluted with EGTA.[67] The calcium-binding protein calmodulin has been purified by adsorption, in the presence of Ca^{2+}, onto the immobilized anatagonists 2-chloro-10-(aminopropyl)phenothiazine[72] or 5-chloro-1-naphthalene sulphonamide.[73] In both cases the proteins were eluted with EGTA.

A procedure utilizing copper chelate chromatography[200] has been described for the purification of human serum albumin. The authors investigated the behaviour of a weakly chelating protein on a variety of chelating matrices using Zn^{2+} or Cu^{2+} as metal ions. The effects of a range of buffers, acetate, phosphate, or amine, on adsorption and desorption were studied.

Phase Partition and Partition Chromatography.—Phase partition in aqueous systems has found particular application in the preparation of sub-cellular organelles and membrane fractions. The use of a partition procedure often means that there is no need for ultracentrifugation. Thus mitochondria from rat brain were prepared rapidly and in high yield by partitioning a crude homogenate in a mixture containing 6.4% dextran T500 and 6.4% poly(ethylene glycol)4000.[201] A detergent-containing two-phase system has been used to prepare chloroplast thylalkoid membranes. By using a mixture containing dextran T500 and poly(ethylene glycol)6000 and adding increasing concentrations of Triton X-100 it was possible to produce upper phases that were progressively enriched in chlorophyll *a* protein followed by chlorophyll *a*/*b* protein complexes. This procedure was completed in 3 hours.[202]

Affinity partitioning has been used to purify discrete membrane domains by means of quaternary ammonium compounds, choline analogues, bound to poly(ethylene oxide)6000 in conjunction with dextran T500. This powerful technique was combined with counter-current distribution and biospecific elution of the cholinergic receptors from the poly(ethylene oxide)[203] with decamethonium.

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Dye-affinity partition has recently been explored for combining the advantages of phase separation and dye-affinity chromatography. Both Cibacron Blue and Procion Red HE-3B have been covalently bound to poly(ethylene glycol) and used to raise the partition-coefficient values of glucose 6-phosphate dehydrogenase from <1 to >20.[204] Although extraction of the enzyme by affinity partition is only possible at low ionic strength this technique holds great promise for large-scale affinity separations.

High-performance Liquid Chromatography.—With the advent of new, macroporous, rigid supports h.p.l.c. is now becoming an accepted and viable technique for the purification of proteins (as opposed to peptides and other low-molecular-weight compounds). Hydrophobic peptides, which can be readily separated by chromatography on conventional supports, can also be purified by chromatography on reversed-phase h.p.l.c. matrices. For example, human fibrinopeptides have been purified by acetonitrile elution on C_{18}-silica[205] and rat pituitary corticotropin has been purified by an acetonitrile gradient in trifluoroacetic acid on C_{18}-micro Bondapak,[206] as has a melanotropin-related peptide from the same source.[207] Reversed-phase h.p.l.c. has also been used for the separation and identification of human haemoglobin variants,[208] for the separation of human haemoglobin subunits,[209] and particularly for the rapid diagnosis of β-thalassemia.[210]

Gel-permeation chromatography on diol-coated silica supports has been used to fractionate microsomal P450,[211] tyrosine hydroxylase,[212] and sea-worm chlorocruorin.[213] The rapidity of this technique has been used to advantage for the purification of the unstable enzyme carbamoyl phosphate synthase.[214]

High-concentration agarose gels (12%) in the form of 2—100 μm spheres, fractionating up to a molecular weight of 200 000, have been proposed for molecular seiving of proteins, *e.g.* thyroglobulin, ferritin, and phycoerythrin.[215] Agarose gels have the advantages of being naturally hydrophilic and showing little non-specific adsorption of proteins. They are also readily derivatized for hydrophobic interaction or affinity chromatography.

High-speed gel-permeation chromatography has also been performed using a new, semi-rigid hydrophilic polymer. Toyapearl HW55F has been used to separate proteins over the molecular-weight range 10 000—1 000 000.[216]

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A new technique described in last year's report, high-performance liquid-affinity chromatography (h.p.l.a.c.), has been more widely applied in the current year. Triazine-dye-substituted silica has been used for the purification of a wide range of proteins, including several dehydrogenases, kinases, and isoenzymes,[217] alkaline phosphatase, and tryptophanyl t-RNA synthetase.[218]

Heparin and antithrombin III silica have been used for the industrial-scale preparation of antithrombin III and heparin, respectively,[102] and anti-human serum albumin-silica for the purification of human serum albumin by glycine elution at pH 2.0.[104]

Hydroxyalkyl methacrylate gels, crosslinked with epichlorohydrin (Separon), have also been derivatized for h.p.l.a.c. ε-Amincaproyl-L-Phe-D-Phe-OMe derivatives have been used for the purification of porcine pepsin, proteinases from *Aspergillus oryzae*,[108, 219] and bovine pancreatic trypsin.[103]

Other Chromatographic Techniques and Applications.—Salting-out chromatography on agarose has been used for the purification of a number of proteins, especially from halophilic micro-organisms, for example malate dehydrogenase and glutamate dehydrogenase from *Halobacterium marismortui*[220] and 2-oxoacid : ferridoxin oxidoreductase from *H. halobium*[221] as well as mouse transferrin.[222]

A method has also been described for the complete removal of sodium dodecyl sulphate from peptides and proteins by gel filtration on Sephadex G-25 in a propionic acid–formic acid–water mixture (2 : 1 : 2 v/v). The method is claimed to give complete separation for peptides containing 25—30 residues.[223]

3 Isolation of Specific Classes of Proteins

Membrane Proteins.—As has been the case in the past, Triton X-100 continues to be the most commonly used detergent for the solubilization of membrane proteins. Other agents, some of which are being used with increasing frequency, include Brij,[224, 225] digitonin,[226] Empigen BB,[227] sulphobetaine 3-14,[228] and octyl-β-D-glucopyranoside.[229, 230] The latter agent, however, is expensive compared to others and is unlikely to find widespread application in large-scale preparations. The use of such detergents generally necessitates their removal at a subsequent stage. In many cases (*e.g.* octyl glucopyranoside) dialysis is sufficient, but the removal of Triton X-100 in particular has often proved either difficult or

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expensive. Lukacovic *et al.*[231] summarize an effective procedure for Triton removal from human-erythrocyte preparations that uses a lecithin/toluene extraction, and this may be suitable for preparations containing proteins that are unaffected by toluene.

After protein solubilization, the majority of membrane-protein purifications reported in 1981 were carried out using conventional chromatographic techniques. Many such protocols include an affinity-chromatography column, although such a step has been less frequently used than in previous years.

Although the investigation of membrane proteins from microbial sources has increased, animal tissues remain the most commonly studied source of these proteins. The range of proteins studied, however, has become more diverse and the number of clinically important membrane proteins investigated has increased. These include a study by Lee *et al.*[224] that reports the first purification and partial characterization of an enzyme from human brain that degrades the putative neurotransmitter, substance P; they also include a study of detergent-solubilized insulin receptors.[232] Table 5 lists the investigations of membrane proteins described in the literature during 1981.

Plasma Proteins.—Owing to a wide spectrum of proteins present in plasma, this section has been broadly divided into four parts: (i) proteins involved with coagulation and fibrinolysis, (ii) plasma fibronectin, (iii) complement and associated proteins, and (iv) other plasma proteins, namely enzymes, inhibitors, binding proteins, *etc.* Table 6 lists some of the proteins isolated during 1981 under each subsection, together with their major purification procedures.

The majority of plasma proteins are initially partially purified by either poly(ethylene glycol) precipitation,[264-267] salt precipitation,[268, 269] and/or heat denaturation.[268] The material is then subjected to either affinity chromatography, gel filtration, ion-exchange chromatography, or a combination of these techniques to yield homogeneous protein. Recently, use of sensitive techniques like high-pressure liquid chromatography (h.p.l.c.), preparative isoelectric focusing, and preparative electrophoresis has meant that some of the plasma proteins present in trace amounts can be isolated in pure form and in large enough quantity for further biological and structural studies.

Proteins Involved with Coagulation and Fibrinolysis. The purification of some of the blood-coagulation factors continues to be of major interest. Recently the trend has been towards the use of affinity chromatography over the conventional purification procedures. Kane and Majerus[265] have purified human Factor V using ion-exchange chromatography and gel filtration with a 21% yield. However, the same factor when purified using monoclonal antibodies and immunoaffinity chromatography resulted in a higher yield.[264] Affinity chromatography was also used in the purification of coagulation Factors II, VIII, IX, and X.[270]

Plasminogen activator, a component of the fibrinolytic system, has been studied in some depth with regard to its purification and characterization. It has been purified from various sources in the past and the list continues to grow. Allen and

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Table 5 *Purification of membrane proteins*

Protein	*Source*	*Solubilizing agent*	*Purification matrix*	*Eluant*	*Ref.*
NADH oxidase	*Acholeplasma laidlawii*	Triton X-100	Sephadex G-150	Tris, pH 7.4, Triton X-100	233
			DEAE-Sepharose CL-6B	Tris, pH 8.2, NaCl, Triton X-100	
			Sephacryl S-300	Tris, pH 8.2, NaCl, Triton X-100	
Platelet membrane glycoproteins IIb and IIIa	Human platelets	DCHO	Lentil lectin-Sepharose 4B	α-Methylmannoside, DCHO	234
Complement receptor C3d	Human lymphocytes	C3d shed during membrane turnover	DEAE-Sephacel	NaP, pH 7.5, NaCl gradient	235
			Sephadex G-150	PBS	
			C3d-Sepharose	Phosphate, pH 7.5, NaCl, NP40	
Acetylcholine receptor	*Torpedo californica*	Octyl-β-D-gluco-pyranoside	Purified by α-cobratoxin chromatography (see Sator *et al.*, *Biochemistry*, 1980, **18**, 1200)		230
Iodothyronine deiodinase	Rat kidney	DCHO	Chromatographed on Dowex 50W-X2, Sephadex G-200, and Sepharose CL-6B		236
Phosphatidic acid phosphohydrolase	Rat lung	Triton X-100	Purified by $(NH_4)_2SO_4$ fractionation and chromatography on Biogel A-5m		237
Unidentified, similar to Harvey sarcoma virus src gene product	Rat brain	Triton X-100	Con A-Sepharose	Not retarded	238
			DEAE-cellulose DE23	Not retarded	
			Procion Red agarose	Not retarded	
Sulphydryl oxidase, γ-glutamyl transferase	Rat kidney	Trition X-100	Biogel P-200	Tris, pH 7.6, glycerol	239
Substance P degrading enzyme	Human brain	Brij 35	DEAE-cellulose	KP, pH 6.5, Brij, KCl	224
			Hydroxyapatite	KP, pH 7.5, Brij	
			DEAE-cellulose	KP, pH 6.5, Brij, KCl	
			Sephadex G-200	KP, pH 7.5, Brij	
			Sephadex G-100	KP, pH 7.5, Brij	
Platelet membrane glycoproteins	Human platelets	Triton X-100	Proteins isolated by purification using affinity chromatography on anti-dinitrophenyl antibody-Sepharose		240
Protease IV	*E. coli*	DCHO	DEAE-cellulose DE52	NaCl gradient in Tris, pH 7.5, DCHO	241

Protein kinase	Human erythrocytes	NaCl	CM-Sephadex A50 Histone-Sepharose 4B	NaP, pH 6.7, NaCl NaCl in NaP, pH 7.6, $MgCl_2$	242
Aminopeptidase	Rat brain	Triton X-100	DEAE-cellulose DE52	NaCl gradient in Tris, pH 7.5	243
Band 3 protein	Human erythrocytes	Triton X-100	DEAE-cellulose CMBE-agarose	NaP, pH 7.5, NaCl, Triton X-100 NaP, pH 7.5, cysteine, Triton X-100	231
Oxaloacetate decarboxylase	*Klebsiella aerogenese*	Brij 58	Ca-phosphate gel	KP, pH 7.0	225
Platelet membrane glycoprotein V	Human platelets	Extraction with HEPES/NaCl	Sephacryl S-200 Hydroxyapatite DEAE-cellulose DE52 CM-cellulose CM52	KP, pH 6.8, EDTA KP gradient, pH 6.8, EDTA Not retarded Na-acetate, pH 4.5, NaCl gradient	244
Demosine isomers	Hen egg	Alkali extraction	Sephadex G-75, G-100	Pyridine acetate	245
Dolichylphosphate phosphatase	*Tetrahymena pyriformis*	Extraction with succinate–KCl, pH 6.0	Biogel A-1.5 Ca-phosphate cellulose	Succinate-KCl, pH 6.0 Phosphate, pH 6.7	246
H^+-translocating ATPase	Rat liver	Extraction with ethanediol–$CHCl_3$	Preparation by glycerol gradient centrifugation and polyacrylamide gel electrophoresis		247

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Table 5 (*cont.*)

Protein	*Source*	*Solubilizing agent*	*Purification matrix*	*Eluant*	*Ref.*
Aldehyde oxidase	Guinea-pig granulocytes	Extracted in isotonic KCl	Ammonium sulphate fractionation followed by chromatography on Sepharose CL-6B		248
Unidentified, membrane protein I	*Neisseria gonorrhoeae* P9	Na-cholate–empigen BB	Sephadex G-200	Empigen–glycine	227
Transferrin receptor complexes	Rabbit reticulocytes	Triton X-100 or sulphobetaine 3-14	Biogel A-5	Tris, pH 7.6, SDS	228
Alkaline phosphatase	*Saccharomyces cerevisiae*	Triton X-100	DEAE-cellulose	NaCl gradient	249
NADPH: protochloro-phyllide oxidoreductase	Oat etioplast	Triton X-100	Sucrose gradient centrifugation Sephadex G-150	Tricone–HEPES, pH 8.0, KCl, $MgSO_4$, EDTA, Triton X-100, glycerol, cysteine	250
Muscarinic acetylcholine receptor	Mammalian brain (rat, dog, cat, calf)	Digitonin	Sephadex G-50	NaP, pH 7.2, NaN_3, Digitonin	226
			Ammonium sulphate fractionation Sepharose CL-4B	NaP, pH 7.2, NaN_3, Digitonin	
Formate dehydrogenase	*E. coli*	—	Purification by sucrose gradient centrifugation		251
Sindbis glycoprotein	Sindbis virus	—	Purified by isopycnic centrifugation and polyacrylamide gel electrophoresis. Peptides also analysed by chromatography on hydroxyapatite and Ultrogel AcA44		252
Metalloendo-peptidase	Bovine pituitaries	DCHO	Sephadex G-200	Tris, pH 8.0, NaCl	253
			DEAE-cellulose DE52	Tris, pH 8.0, gradient with NaCl	
			CM-cellulose CM52	Not retarded	
Pyridine dinucleotide transhydrogenase	Bovine heart	Triton X-100	Purified by immunoexclusion chromatography on immunoaffinity Sepharose columns		254
Uridylyl transferase	*Vignia unguiculata*	TGEDP + K-acetate	DEAE-Sepharose CL-6B	TGEDP, pH 8.2, K-acetate	255
			Poly(U)-Sepharose 4B	TGEDP, pH 8.2, K-acetate	

NADH dehydrogenase	*Bacillus caldotenax*	Sodium lauroyl-sarcosinate	Ammonium sulphate fractionation		168
			Phenyl-Sepharose	NaSCN gradient in phosphate, pH 7.0, DTT, methanol, Triton X-100	
			TEAE-cellulose	KCl gradient in phosphate, pH 7.0, DTT, methanol, Triton X-100	
DT-diaphorase	Rat liver	DCHO	Azodicoumarol Sepharose 6B	Tris, pH 7.5, sucrose, NADH	256
			Sephadex G-100	Tris, pH 7.5, sucrose	
Peptide activator of pyruvate dehydrogenase	Rat adipocytes	Released in response to insulin	Sephadex G-25 and G-50	KP, pH 7.4	257
			AG 1X8 resin	KP, pH 7.4, NaCl gradient	
			Hydroxyapatite	KP, pH 7.4, gradient	
Adenosine triphosphatase	*Zea mays*	Octyl-β-D-glucopyranoside	Ammonium sulphate fractionation		229
Phosphatidyl-inositol kinase	Rat brain	Triton X-100	Dodecyl-Sepharose	10 mM Tris, DCHO, DTT	171
Alkaline phosphatase	*E. coli*	Triton X-100	Ammonium sulphate fractionation		258
			DEAE-cellulose	NaCl gradient in Tris–HCl, pH 7.4	
			Sephadex G-150	Tris–HCl, pH 7.4	
NADH dehydrogenase	*E. coli*	Potassium cholate	Hydroxyapatite	KP gradient, pH 7.5	259

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Table 5 (*cont.*)

Protein	*Source*	*Solubilizing agent*	*Purification matrix*	*Eluant*	*Ref.*
Aspirin carboxylesterase	Guinea-pig liver	Triton X-100	Preliminary electrophoretic studies		260
Ca^{2+}–Mg^{2+}-ATPase	Pig stomach	Triton X-100	Calmodulin-Sepharose	HEPES, pH 7.4, KCl, $MgCl_2$, DTT, EDTA, asolectin, Triton	261
Cathepsin	Rat liver	Triton X-100	DEAE-Sephadex	NaCl gradient in KP, pH 6.0	262
			Sephadex G-100	Acetate buffer, pH 5.0, NaCl, MSH	
Acetylcholine-esterase	Rat brain	Triton X-100	Con A-Sepharose	Saline buffer, α-methyl-D-mannoside, Triton X-100	263
			Dimethylaminobenzoic acid-Sepharose	Saline buffer, Triton X-100, tetraethylammonium bromide	
			DEAE-cellulose	NaCl gradient in phosphate	

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Table 6 *Purification of plasma proteins**

Protein	Source	Major procedures: Affinity chromatography	Ion exchange	Gel filtration	Other	Ref.
Proteins Involved with Coagulation and Fibrinolysis						
Factor V	Human	Immunoaffinity chromatography			PEG ppt. phenyl-Sepharose	264
Factor V	Human		DEAE-Sepharose	Ultrogel AcA34	PEG ppt.	265
Factor VII	Human		DEAE-Sephadex		Preparative p.a.g.e.	285
Factor VIII	Human				NaCl ppt. and heat denaturation	268

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Table 6 (*cont.*)

Protein	*Source*	*Major procedures*				*Ref.*
		Affinity chromatography	*Ion exchange*	*Gel filtration*	*Other*	
Factor IX	Human	Heparin-agarose	DEAE-cellulose		Preparative p.a.g.e.	286
Factors II, VIII, IX, and X	Human	Agarose-bound phospholipid residues				270
Plasminogen activator	Human	*p*-Aminobenzamidine-Sepharose		Ultrogel AcA44		271
Plasminogen activator	Human	Lysine-Separose Fibrin-Sepharose		Sepharose CL-6B Sephadex G-200	PEG ppt. hydroxyapatite	266
Anti-haemorrhagic factor	Rat		DEAE-Sephadex		Flat-bed i.e.f.	287
Anti-haemophilic factor	Human			Sepharose CL-4B Sepharose G-100	PEG ppt.	288
Plasma Fibronectin						
Fibronectin	Rat	Gelatin-Sepharose				275
Fibronectin	Human	Gelatin-Sepharose	DEAE-cellulose			276
Fibronectin	Bovine and human plasma	Gelatin-agarose co-polymer				9
Complement and Associated Proteins						
C1q	Mouse		DEAE-Sephadex A50	Sepharose 6B		289
C3	Rabbit	Lysine-Sepharose	DEAE-Sepharose QAE-Sepharose A25	Sepharose CL-6B	PEG ppt.	267
C3	Human				Sulphated Sepharose	278
C5	Rabbit		TEAE-Sepharose	Sepharose 6B Biogel P-200	PEG ppt. hydroxyapatite	267
C5	Rainbow trout		DEAE-Sepharose CM-Sepharose	Sephadex G-200	PEG ppt.	279

C5	Guinea pig		DEAE-cellulose	Sephadex G-200 ppt. hydroxy-apatite	Ammonium sulphate	269
C3, C5, C4, C7, C8, C9, C1E1, C3b1NA	Human	Lysine-Sepharose	DEAE-Sepharose			280
C4a anaphyla-toxin	Human		CM-Sephadex C-50	Sephadex G-50		281
C4b binding protein	Human		DEAE-Sephacel	Sepharose 4B	Barium citrate adsorption	290
Other Plasma Proteins						
Apolipoproteins	Human				Ion-paired reversed-phase h.p.l.c.	291
Lipoprotein subfractions HDL_2 and HDL_3	Human				Ppt. with heparin, and $MnCl_2$ ultracentrifugation	284
Lipoproteins	Human				Density-gradient ultracentrifugation	292
Lipoproteins	Human				Ppt. with phospho-tungstate and $MgCl_2$	293
Kininogen	Horse	*p*-Chlorobenzylamine-Sepharose	DEAE-Sephadex A50 CM-Sephadex C50	Sephadex G-150		282

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Table 6 (*cont.*)

Protein	*Source*	*Major procedures*				*Ref.*
		Affinity chromatography	*Ion exchange*	*Gel filtration*	*Other*	
Kininogen	Human	Con A-Sepharose RCA-Sepharose	DEAE-cellulose DEAE-Sephadex	Sephadex G-200		294
Galactosyl-transferase	Human	α-Lactalbumin-Sepharose				295
α-Antichymo-trypsin	Human	Thiopropyl-Sepharose Thiol-Sepharose Immunoaffinity chroma-tography				296
Thiol proteinase inhibitor	Human	Trypsin/chymotrypsin Chromogel A2	DEAE-cellulose	Sephadex G-150	Preparative p.a.g.e.	297
α_1-Protease inhibitor	Rat	Affigel Blue Immunoaffinity chromato-graphy	DEAE-cellulose	Sephadex G-100		298
Esterase 1F	Mouse		DEAE-Sepharose	Sephacryl S-200	Preparative i.e.f.	299
Eosinophil peroxidase	Human	Sulphopropyl-Sephadex (C-50)				300
Elastase-like enzyme	Human			Sepharose 4B		301
Pregnancy-asso-ciated α_1-macro-globulin	Human				Flat-bed i.e.f.	302
Insulin-binding α_1-globulin	Cat	Immunoaffinity chromato-graphy				303
Immunoglobulin M	Human	Protein A-Sepharose		Sephadex G-200		304

Sex-steroid-binding proteins	Rat/rabbit	Steroid-Sepharose 4B			Hydroxyapatite	283
Monoglyceride hydrolase	Rat			Agarose gel		305
Pregnancy-associated Protein A	Human	Immunoaffinity chromatography				306
α-Fetoprotein	Chicken	Con A-Sepharose		Sephadex G-200	Preparative i.e.f.	307
α-Fetoprotein	Human	Con A-Sepharose Immunoaffinity chromatography	DEAE-cellulose	Sephadex G-200 Blue Sepharose		308
α-L-Fucosidase	Human		DEAE-Sephacel			309

*Abbreviations used in this Table: p.a.g.e. polyacrylamide gel electrophoresis, PEG poly(ethylene glycol), i.e.f. isoelectric focusing, h.p.l.c. high-performance liquid chromatography.

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Pepper[266] have isolated human vascular plasminogen activator using lysine-Sepharose or fibrin-Sepharose, whereas Aasted[271] has used *p*-aminobenzamidine-Sepharose for its purification. The isolation of this protein has also been reported from human milk[272] and from cultured bovine endothelial cells.[273] A recent review describes the purification of various components of coagulation and fibrinolytic systems by affinity chromatography.[274]

Plasma Fibronectin. Weiss and Reddi[275] isolated plasma fibronectin from rat using affinity chromatography on a gelatin-Sepharose column. Fibronectin was eluted with 4.0 M urea; another protein of 70 000 molecular weight was eluted with 8.0 M urea. The latter protein was identified as a collagen-binding molecule and was shown to be a normal constituent of plasma and not a proteolytic degradation product formed during isolation, as suggested previously. Antibodies raised against rat fibronectin weakly crossreacted with plasma fibronectin of chicken, horse, and human. Two recent reviews summarize the characteristics of fibronectin from various sources in terms of their structural and biological activity.[276, 277]

Complement and Associated Proteins. Some of the nine components of the classical complement system have been isolated and studied from several animal sources. Complement component protein C3 was purified from rabbit and human plasma by affinity and ion-exchange chromatography.[267, 278] Discipio *et al.*[278] have shown that trace contaminants, namely C3b, C3u, and haemolytically inactive C3, present in C3 preparations can be separated from each other and from the active protein by chromatography on sulphated Sepharose.

Complement protein C5 has also been purified to homogeneity from rabbit, rainbow trout, and guinea pig by 3—4-step purification procedures.[267, 269, 279] Hammer *et al.*[280] describe a preparative scheme for the isolation of several human complement proteins in purified form and with full haemolytic activity.

Studies on C4a anaphylatoxin have shown 25% homology of the *N*-terminal sequence of C4a with C3a and 13.3% homology with C5a.[281] This is taken as evidence for the evolutionary relationship between anaphylatoxin C3a, C4a, and C5a, and it is suggested that a similar relationship may exist for precursor proteins.

Other Plasma Proteins. A wide selection of proteins have been isolated from plasma, a few examples of which are listed in Table 6. Some of these proteins, which have been studied in detail in the past, are isolated and purified from various other sources for comparative studies. For example, kininogen purified from horse and bovine plasma shows similar physico-chemical, but different immunological, properties,[282] whereas rat and rabbit sex-steroid-binding proteins exhibit similar characteristics.[283] Some of the purification procedures for proteins, which have been time-consuming and expensive, are now being replaced by more sensitive, rapid and simple methods, as in the case of the isolation of high-density lipoprotein subfractions HDL_2 and HDL_3.[284]

4 Protein Characterization

Protein Determination in Solution.—The continued trend has been the modification of existing procedures, such as the Lowry method and the dye-binding assay based on a Coomassie Blue reagent, either to achieve a general improvement in

sensitivity or, more often, to allow determinations against a background of interfering substances. The latter is of particular concern when measuring protein in complex biological mixtures, and quantitative precipitation of protein by 10% trichloroacetic acid in the presence of yeast-soluble ribonucleic acid as a carrier, followed by solution in alkali prior to the Lowry procedure, has been used to determine protein at concentrations in the range 5—25 $\mu g\,ml^{-1}$.[310] A particular problem with the Lowry method is the interference by sulphydryl groups. Although this problem may be overcome by prior precipitation of the protein from the sample as described above, this usually requires addition of several extra reagents, and recoveries of protein may not always be quantitative. An alternative is to use a chloroamide compound for the oxidative removal of sulphydryl groups, often arising from thiol reagents present in the protein solution. Such a compound, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril, has been used in an immobilized form to remove —SH groups prior to the Lowry assay.[311] A similar approach has been to alkylate irreversibly protein samples containing a variety of thiol reagents, using *N*-ethylmaleimide.[312]

Determination of low levels of protein in Percoll isopycnic density gradients can be made directly using the Lowry method by estimating protein on the basis of differences between absorbance at 750 nm and 420 nm, following reaction with the Folin–Ciocalteau reagent, to overcome interference by Percoll.[313]

A significant modification to the Coomassie Blue G dye-binding assay for protein has removed much of the variation observed when different proteins are tested. Increasing the dye concentration or decreasing the phosphoric acid concentration increased the colour yield of the dye–protein complex of a range of proteins close to that of the common standard, bovine serum albumin.[314] The dye-binding assay has also been successfully modified for the assay of biliproteins, which are particularly difficult to assay by other methods owing to a strong natural absorbance in the visible region.[315] The method relies on the separation of the dispersed dye–protein complex by centrifugation and solution in Tris–methanol. In this solvent the complex gives maximum absorbance, but although highly sensitive it is only linear up to 5 $\mu g\,ml^{-1}$.

The dye-binding assay is also likely to find increasing use in automated protein analysis systems. Two systems recently described use microtitre plates and automatic recording spectrophotometers of the type used for ELISA. The first system relates to the estimation of soluble proteins[316] and the second more ambitiously addresses the problem of measuring protein concentration in sub-cellular membrane fractions that must first be solubilized with NaOH solution.[317] Both methods, as expected, only show linearity over a very limited range, although sensitivity below 5 $\mu g\,ml^{-1}$ is good. The above systems to a large extent overcome the problems of protein-bound dye adhesion to the walls of flow cells and tubes in

[310] I. Polacheck and E. Cabib, *Anal. Biochem.*, 1981, **117**, 311.
[311] R. W. McClard, *Anal. Biochem.*, 1981, **112**, 278.
[312] J. Hughes, S. Joshi, and D. Ascoli, *Anal. Biochem.*, 1981, **117**, 1.
[313] M. N. Khan, R. J. Khan, and B. I. Posner, *Anal Biochem.*, 1981, **117**, 108.
[314] S. M. Read and D. H. Northcote, *Anal. Biochem.*, 1981, **116**, 53.
[315] R. Almog and D. S. Berns, *Anal. Biochem.*, 1981, **114**, 336.
[316] D. B. Rylatt and C. R. Parish, *Anal. Biochem.*, 1982, **121**, 213.
[317] I. A. Simpson and O. Sonne, *Anal. Biochem.*, 1982, **119**, 424.

more conventional automated analyses. This problem has been discussed in relation to the automated assay of protein in urine samples.[318]

Protein Detection in Gels.—Silver staining of protein bands in one- and two-dimensional electrophoresis gels has become a popular replacement for the Coomassie Blue stain, exhibiting up to 200 times greater sensitivity and detecting as little as 0.01 ng protein mm^{-2}.[319] This paper claims linearity over a 40-fold range in protein concentration, but in a critical analysis of silver staining[320] the non-stoicheiometric nature of the protein–silver interaction is demonstrated together with the differing sensitivity of various proteins to silver stain. Silver-staining techniques are numerous,[321-323] and a comparison of six methods[324] points out the differences found with gels of varying thickness. For detection of proteins in 4—20% (w/v) polyacrylamide gradient gels of 3 mm thickness, methylamine can be substituted for ammonia in the diamine preparations to reduce the background of ultrasensitive silver stains.[325]

A rapid and sensitive fluorescent stain based on *o*-phthaldialdehyde can be used for proteins,[326] with the major bands visualized within 1—2 min and with maximum sensitivity (in the same order as Coomassie Blue) within 15 min. The reaction is in neutral or slightly alkaline pH with aqueous buffers, and enzymes may retain their activity. Fluorescence staining was also described using *o*-phthalaldehyde[327] and 4-(3′-phenyl-2′-pyrazolin-1′-yl)benzenesulphonyl chloride.[328]

Rapid (3—5 min) fixation and staining of isoelectric focusing gels have been achieved by staining the gel dried on silanized supports.[329] Gel thickness is reduced to 50 μm and using Serva-violet 17 or 49, with low affinity for Servalyt carrier ampholyte, sensitivity is 100—200 ng protein mm^{-2} on a 10 cm gel.

Radiolabelling of tissue proteins by reductive methylation has been used for precision two-dimensional p.a.g.e.[330] Labelling was achieved by the use of sodium borohydride with ^{14}C- or ^{3}H-formaldehyde. The same isotopes were used for protein determination in a method by which slab gels were dried onto a filter paper under vacuum and, following autoradiography, were sectioned, digested in 50% H_2O_2, and counted in an aqueous scintillator.[331]

An improved method for the detection of weak β-emitting radioisotopes in agarose and polyacrylamide media has been described,[332] and the distribution pattern of ^{3}H-labelled proteins in polyacrylamide gels has been recorded with a

[318] K. Sano, K. Kanamori, A. Shiba, and M. Nakao, *Anal. Biochem.*, 1981, **113**, 197.
[319] C. R. Merril, D. Goldman, and M. L. Vankeuren, *Electrophoresis*, 1982, **3**, 17.
[320] H.-M. Poehling and V. Neuhoff, *Electrophoresis*, 1981, **2**, 141.
[321] D. W. Sammons, L. D. Adams, and E. E. Nishizawa, *Electrophoresis*, 1981, **2**, 135.
[322] W. Wray, T. Boulikas, V. P. Wray, and R. Hancock, *Anal. Biochem.*, 1981, **118**, 197.
[323] J. H. Morrissey, *Anal Biochem.*, 1981, **117**, 307.
[324] D. C. Ochs, E. H. McConkey, and D. W. Sammons, *Electrophoresis*, 1981, **2**, 304.
[325] T. Marshall and A. L. Latner, *Electrophoresis*, 1981, **2**, 228.
[326] M. D. Taylor and A. T. Andrews, *Electrophoresis*, 1981, **2**, 76.
[327] T. Yajima, T. Itoh, S. Mutom, and H. Yuki, *Chem. Pharm. Bull.* (*Tokyo*), 1980, **28**, 3696.
[328] M. A. Tshabalala and H. W. Latz, *Anal. Biochem.*, 1981, **111**, 343.
[329] M. D. Frey and B. J. Radola, *Electrophoresis*, 1982, **3**, 27.
[330] J. M. Finger and K. H. Choo, *Biochem. J.*, 1981, **193**, 371.
[331] L. Chao and J. Chao, *Electrophoresis*, 1981, **2**, 60.
[332] D. E. Pulleybank and G. M. Booth, *J. Biochem. Biophys. Methods*, 1981, **4**, 339.

'Betacamera', which has the advantage of viewing the whole gel and avoids scanning.[333]

Trypsin isozymes have been visualized in two-dimensional gels by irradiation with monochromatic light and measurement of light absorption or fluorescent emission.[334] The method may be applicable in general for hydrolases and oxidases, enabling visualization without the use of histochemical stains or radioisotopes.

A number of enzymes have been detected in gels on the basis of their biological activity, including proteases,[335] α-glucosidase,[336] and DNA polymerases and exonucleases.[337] Diaminobenzidine has been used as the electron acceptor to detect cytochrome *f*[338] and β-fructosidase[339] activity.

Rapid removal of SDS from polyacrylamide gels prior to staining for the carbohydrate moiety of glycoproteins can be achieved by transverse electrophoresis in isopropanol : acetic acid : water.[340] Isopropanol can also be used before activity staining of nucleolytic enzymes.[341] Proteins may also be removed from gels prior to estimation, either continuously during the electrophoretic separation[342] or, more usually, following separation by electrophoretic transfer to a second supporting medium such as an ion-exchange paper[343] or by elution from sectional gel pieces.[344-346]

Electrophoretic Techniques.—Electrophoresis has continued to be a front-line technique for the characterization of proteins and has been increasingly applied to protein separation on a preparative scale. It is convenient to divide electrophoretic techniques into a number of subsections and discuss in each section theory and concepts, advances in technique, analytical and diagnostic application, and preparative use.

One-dimensional Electrophoresis. The new journal *Electrophoresis* (1981, Volume 2) has provided an outlet for papers that reassess many of the basic concepts of electrophoresis.[347] This has led to a number of papers that review the classical theories of electrophoresis in the light of modern knowledge[348, 349] and attempt to define in greater mathematical detail parameters that influence electrophoretic separation.[350]

[333] J. Kruppa and A. Bauche, *Electrophoresis*, 1980, **1**, 177.
[334] R. J. Klebe, M. G. Mancuso, C. R. Brown, and L. Teng, *Biochem. Genet.*, 1981, **19**, 655.
[335] K. R. Lynn and N. A. Clevette-Radford, *Anal. Biochem.*, 1981, **117**, 280.
[336] L. L. Spielman and D. B. Mowshowitz, *Anal. Biochem.*, 1982, **120**, 66.
[337] A. Spanos, S. G. Sedgwick, G. T. Yarranton, U. Hubscher, and G. R. Banks, *Nucleic Acids Res.*, 1981, **9**, 1825.
[338] A. McDonnel and L. A. Staehelin, *Anal. Biochem.*, 1981, **117**, 40.
[339] L. Faye, *Anal Biochem.*, 1981, **112**, 90.
[340] H. M. Phillips, *Anal. Biochem.*, 1981, **117**, 398.
[341] A. Blank, R. H. Sugiyama, and C. A. Dekker, *Anal. Biochem.*, 1982, **120**, 267.
[342] M. Faupel, M. de Gasparo, and E. von Arx, *J. Chromatogr.*, 1982, **238**, 226.
[343] T. McClellan and J. A. M. Ramshaw, *Biochem. Genet.*, 1981, **19**, 647.
[344] D. Every and R. S. Green, *Anal. Biochem.*, 1982, **119**, 82.
[345] D. A. Hager and R. R. Burgess, *Anal. Biochem.*, 1980, **109**, 76.
[346] G. Scheele, J. Pash, and W. Bieger, *Anal. Biochem.*, 1981, **112**, 304.
[347] L. M. Hjelmeland and A. Chrambach, *Electrophoresis*, 1981, **2**, 1.
[348] L. M. Hjelmeland and A. Chrambach, *Electrophoresis*, 1982, **3**, 9.
[349] L. Rosenfeld, 'Origins of Clinical Chemistry. The Evolution of Protein Analysis', Academic Press, New York, 1982.
[350] A. Marmur, *J. Colloid Interface Sci.*, 1982, **85**, 556.

Electrophoresis in gels containing zwitterionic groups demonstrated a retardation of the absolute migration velocities of proteins and dyes.[351] Anionic species were more strongly retarded than cationic species on zwitterionic methacrylamide derivatives, *e.g.* (3-sulphopropyl)dimethyl(3-methacrylylamidopropyl) ammonium inner salt (MAPS) copolymerized with *NN'*-methylene bisacrylamide (BIS). Results indicated a lowering of the available net charge on the protein because of interaction with the zwitterionic charge on the gels. The conclusions drawn were that isoelectric zones of carrier ampholytes or proteins in isoelectric focusing systems can be viewed as thin layers of zwitterionic species, and if these zones are more retarding for anionic species then this may explain the 'cathodic drift' observed during electrofocusing. Agarose gels neutralized by incorporation of positively charged groups could also be expected to retard proteins in an analogous manner to that observed in MAPS gels.

A study has been made on the use of different crosslinking reagents and conditions of the polymerization kinetics of polyacrylamide gels.[352-354] Crosslinking reagents other than BIS were sought to effect a reversible polymerization of gels and included *NN'*-bisacrylylcystamine (BAC), *NN'*-(1,2-dihydroxyethylene bisacrylamide) (DHEBA), and ethylene diacrylate (EDT). The reaction of these compounds was mainly measured spectrophotometrically at 283 nm (disappearance of double bond), and the order of reactivity was BIS = DHEBA > EDT = BAC. Polymerization at 0—4 °C produced porous, turbid, and inelastic gels and was best achieved at 25—30 °C. Homogeneous gels were obtained in the presence of 8 M urea or in formamide as solvent owing to disruption of H bonds. BAC produces disulphide bridges capable of cleavage by thiols under mild conditions, EDA crosslinks contain ester bonds capable of base-catalysed hydrolytic cleavage, and DHEBA produces a 1,2-diol structure susceptible to oxidation with periodic acid.

The effects of the catalysts riboflavin and riboflavin 5'-phosphate (FMN) were also studied at 283 nm. At least 8 hours of light exposure are needed to ensure 95% conversion of monomer to polymer, not the one hour often reported.

Several modifications to standard one-dimensional electrophoresis systems have been made to achieve improved separations of new or difficult classes of proteins. A Triton X-100–acid–urea system was modified to allow separation of low-specific-activity globins,[355] having been originally developed for the analysis of histone proteins.[356]

High-temperature electrophoresis has been used to study the thermal denaturation of proteins such as beta-lactamase[357] and cytochrome b_5.[358] It was also applied to the electrophoresis of mucins, where an elevated temperature

[351] L. M. Hjelmeland, S. Allenmark, B. An der Lan, B. A. Jackiw, N. Y. Nguyen, and A. Chrambach, *Electrophoresis*, 1981, **2**, 82.
[352] C. Gelfi and P. G. Righetti, *Electrophoresis*, 1981, **2**, 213.
[353] C. Gelfi and P. G. Righetti, *Electrophoresis*, 1981, **2**, 220.
[354] P. G. Righetti, C. Gelfi, and A. B. Bosisio, *Electrophoresis*, 1981, **2**, 291.
[355] V. A. Mezl, G. N. Oakes, G. W. Wilshire, and J. A. Hunt, *Anal. Biochem.*, 1981, **117**, 452.
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[357] D. R. Thatcher and B. Hodson, *Biochem. J.*, 1981, **197**, 105.
[358] P. Bendzko and M. Strauss, *Anal. Lett.*, 1981, **14**, 1233.

increased the migration rate and apparently separated additional components by dissociation of macromolecular aggregates.[359]

The separation of pH-labile proteins such as bovine cytochrome C oxidase was attempted using low-pH lithium dodecyl sulphate polyacrylamide gradient gels, and migration order was compared with the conventional high-pH gel systems.[360] An alternative approach to the separation of bovine cytochrome C oxidase is to incorporate chloral hydrate into the gel.[361] Low-pH polyacrylamide gels (pH 2.7) were also used recently for the preparative electrophoresis of histone P3 protein with total histone as starting material.[362]

Alterations in the running time and migration distance of proteins were found to be related to pH in non-detergent polyacrylamide gels when one of several amino-acids was used in place of glycine in the electrode-chamber buffer.[363] In an empirical approach to discontinuous buffer systems, several amino-acids with pK values ranging from 9.1 to 10.6 were substituted for glycine. This resulted in a more rapid migration of tadpole haemoglobin, proportional to the pK value of each amino-acid, when the latter was divided by the volume conductivity for the tank buffer measured prior to the run. Following general criteria for the design of new discontinous electrophoresis systems rather than resorting to the computer-generated systems derived from theory is also advocated by Thomas and Kodes.[364] They describe the use of MOPS [3-(*N*-morpholine)propanesulphonic acid] buffer for the separation of cationic proteins at near-neutral pH where the leading ion is K^+ and the trailing ion histidine. The application of a discontinuous electrophoresis system on a preparative scale was demonstrated by the purification of tetanus toxin and its peptide components.[365]

A technique for free-zone electrophoresis in open tubular glass capillaries has been described for amino-acids and dipeptides,[366, 367] but separation of proteins is only mentioned as a possibility. Although the system overcomes interaction of the separated species with the support medium, *e.g.* gel or paper, the glass capillaries used are untreated and exhibit surface adsorbtion. A more inert surface would be necessary for proteins. The described system operates with capillaries of 75 μm inside diameter with an applied voltage of up to 30 kV. A strong electro-osmotic flow in the capillary allows both positive and negative ions of a variety of sizes to be analysed in a single run in less than 30 minutes and has been applied to the separation of fluorescent derivatives of amino-acids and peptides in urine.

Linear density gradients of metrizamide have been used in place of sucrose for zone electrophoresis of viral capsid–DNA complexes.[368] Metrizamide is a non-ionic, water-soluble molecule that has a low ratio of viscosity to density; viscous

[359] S. Kashket and J. M. Ciocolo, *Electrophoresis*, 1981, **2**, 55.
[360] G. D. Jones, M. T. Wilson, and V. M. Darley-Usmar, *Biochem. J.*, 1981, **193**, 1013.
[361] D. C. Griffin and M. Landon, *Biochem. J.*, 1981, **197**, 333.
[362] A. Lipinska and L. Klyszejko-Stefanowicz, *Int. J. Biochem.*, 1981, **13**, 859.
[363] A. M. Parkinson, A. R. Dorn, P. B. Maples, and R. H. Broyles, *Anal. Biochem.*, 1981, **117**, 6.
[364] J. M. Thomas and M. E. Hodes, *Anal. Biochem.*, 1981, **118**, 194.
[365] S. J. Dimari, M. A. Cumming, J. H. Hash, and J. P. Robinson, *Arch. Biochem. Biophys.*, 1982, **214**, 342.
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[367] J. W. Jorgenson and K. D. Lukacs, *Clin. Chem.*, 1981, **27**, 1551.
[368] P. Serwer and R. H. Watson, *Anal. Biochem.*, 1981, **114**, 342.

slowing of samples during electrophoresis is therefore minimized compared with sucrose. The determination of protein in the presence of metrizamide has been described in a previous volume of this Report (Volume 13, Chapter 2, Part IA).

Isoelectric Focusing. One of the recurrent themes in 1981 has been the study of isoelectric focusing (i.e.f.) in ampholine-free systems. This is particularly relevant to the separation of proteins on a preparative scale, mainly because of the prohibitive cost of ampholines, but also in view of the problem of ensuring complete removal of ampholines from protein preparations.

Computer simulations have been attempted to derive models of the structure of concentration, pH, conductivity, and protein gradients arising from the focusing of electrochemically defined ampholytes.[369, 370] Binary systems were investigated and scored as 'good' (symmetrical concentration profile and useful pH gradient projected), *e.g.* β-Ala-His/Arg and Tyr-Arg/Orn, or 'poor', *e.g.* β-Ala/His. A more practical approach was taken using natural pH gradients formed on polyacrylamide gel by amino-acid mixtures Glu/Lys and Glu/His/Lys.[371] Glu (anodic) and Lys (cathodic) focus as single peaks adjacent to a steep pH gradient of linear step function. His focuses at the centre of the gradient and the system has been used to electrofocus haemoglobin.

In an attempt to move away from the use of carrier ampholytes completely a method termed 'rheoelectrolysis' has been described that uses buffer solutions comprising a weak acid and strong base[372] and a weak acid and weak base.[373] Assuming no liquid flow in the electrolyser, a pH span of 2.6 units is claimed for the rheoelectrolysis of an ordinary buffer solution. The pH course is sigmoidal in shape and may be useful for large-scale protein separations, but there are practical problems due to electro-osmotic effects, gas bubbles and anodic oxidation of buffer components, such that only multi-membrane types of apparatus are envisaged. Such an apparatus consisting of 22 compartments has been described for use with two simple component buffers.[374] Choice of separation membrane presents difficulties: as with permeable membranes small differences in hydrostatic pressure cause liquid flow, which disrupts the pH gradient, and flow-tight membranes give rise to electro-osmosis. The best choice was polyacrylamide membranes, but there are problems of low mechanical strength and reproducibility.

Returning to the use of the major commercially available ampholytes, a comparison made between Pharmalyte (pH 3—10), Ampholine (pH 3.5—9.5), and Servalyt (pH 2—11) shows only very minor differences in performance.[375] Proteins with pI values above 8 focus slightly slower in terms of volt hours (V h) on Servalyt. Contamination of protein preparations has to be considered during preparative i.e.f., and a method for detecting microgram quantities of carrier ampholytes by chromatography on thin-layer cellulose sheets has been used to

[369] M. Bier, R. A. Mosher, and O. A. Palusinski, *J. Chromatogr.*, 1981, **211**, 313.
[370] O. A. Palusinski, T. T. Allgyer, R. A. Mosher, M. Bier, and D. A. Saville, *Biophys. Chem.*, 1981, **13**, 193.
[371] B. A. Jackiw and A. Chrambach, *Electrophoresis*, 1980, **1**, 150.
[372] H. Rilbe, *Electrophoresis*, 1981, **2**, 261.
[373] H. Rilbe, *Electrophoresis*, 1981, **2**, 268.
[374] M. Jonsson and S. Fredriksson, *Electrophoresis*, 1981, **2**, 193.
[375] T. Laas and I. Olsson, *Electrophoresis*, 1981, **2**, 235.

measure removal of ampholytes by electrodialysis in cases where separation by mixed-bed ion-exchange chromatography was not suitable.[376]

Agarose has become a widespread alternative support matrix for i.e.f., particularly for preparative work (see later). The macroporous structure of agarose minimizes molecular-sieving effects, and the availability of i.e.f.-grade agarose, with its extremely low content of negatively charged groups counterbalanced by positively charged groups that are covalently bound to the matrix, gives a gel with practically no electroendo-osmosis or central pH-gradient drift. I.e.f. in agarose under denaturing conditions can be carried out using urea, mercaptoethanol, and a non-ionic detergent,[377] and an agarose–urea system has been applied to the separation of tubulin.[378]

I.e.f. of serum proteins has continued to find application in the diagnosis of human-disease conditions such as cystic fibrosis,[379] multiple sclerosis,[380] and, more notably, β-thallasaemia.[381, 382] Ultra-thin layers of agarose and polyacrylamide have been used for the improved analysis of haemoglobins[383, 384] and haem-free globins.[385]

The heterogeneity of apparent single bands after i.e.f. of antibodies was demonstrated empirically using light chains from antilactose immunoglobin M and predicted by use of a computer analysis based on the theoretical isoelectric points of various amino-acid compositions in the range found in antibody Vh regions.[386] Two problems associated with the analysis of immunoglobulins by electrophoretic methods are the inability of conventional buffer systems to spread polyclonal immunoglobulin across the entire separation path length and the appearance of distinct bands in conventional i.e.f. instead of the continuous zones expected from a polyclonal mixture.[387] The acidic side of the pI of immunoglobulins can be chosen to exploit their relative basicity. At pH 5.32 a discontinuous buffer system was capable of stacking all the immunoglobulins present in a mixture. In order to demonstrate immunoglobulin heterogeneity the same mixture was fractionated by preparative isotachophoresis and then subjected to p.a.g.e. at pH 4.85, which achieved a complete spread of proteins (*i.e.* relative mobilities 0.1 to 0.9), and i.e.f. with a pH 6—9 ampholine solution plus 0.01 M amino-acid mixtures to show continuous polyclonal immunoglobulin patterns.

Scale-up of the i.e.f. technique from analytical- to gram-scale separation of protein has continued to be a goal, and the use of agarose as supporting medium

[376] T. G. Bloomster and D. W. Watson, *Anal. Biochem.*, 1981, **113**, 79.
[377] I. Olsson and T. Laas, *J. Chromatogr.*, 1981, **215**, 373.
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has been described for serum proteins and red-cell haemolysates.[388] The agarose gels gave linear gradients in the pH range 3—8, and up to 1 g serum proteins were loaded on the gel with recoveries between 68 and 82%. A new matrix based on 0·5—1% agarose incorporated with 2.5% Sephadex G-200 superfine and 3% Pharmalyte has also been described;[389] it combines the good electrofocusing properties of Sephadex with the more simple handling and gel uniformity of agarose. Preparative i.e.f has been applied to a variety of proteins including erythropoietin,[390] thyroid proteins,[391] and aspartate aminotransferase isoenzymes.[392]

Two-dimensional Electrophoresis. The widespread use of two-dimensional electrophoresis for the separation of isoenzymes and other closely related families of protein in clinical studies is illustrated in a special issue of *Clinical Chemistry*, introduced by Young and Anderson, devoted to applications of the technique.[393]

Two-dimensional electrophoresis was applied particularly to the study of blood and brain proteins and also to the investigation of changes in protein pattern related to a number of disease conditions, notably cancers and inborn errors of metabolism. Several papers in the issue describe the use of computer analysis of two-dimensional gels. Programs for the automatic identification and measuring of proteins in two-dimensional autoradiograms have been described[394] together with semi-automatic programs to follow the synthesis of an individual polypeptide through a series of gels. As an alternative to radioactive marker proteins, computerized densitometry measurements on protein spots visualized by Coomassie Blue Stain have been used for the analysis of bacteriophage T_4 coat proteins.[395]

The use of i.e.f in the first dimension with SDS-p.a.g.e. in the second, introduced by O'Farrell,[396] has been applied both in its original form, for acidic proteins (pI < 7.0), and using the modified version for more basic proteins,[397] in which non-equilibrium pH-gradient electrophoresis forms the first dimension. The technique has been used to study the heterogeneity of collagen,[398-400] non-histone chromatin proteins,[401] myofibrillar proteins,[402] complement proteins,[403] α-1-antitrypsin,[404] and fibrinogen.[405]

[388] C. Chapuis-Cellier and P. Arnaud, *Anal. Biochem.*, 1981, **113**, 325.
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The heterogeneity of human-leukocyte interferon has been eliminated by chemical oxidation with sodium periodate prior to purification by chromatography and two-dimensional electrophoresis.[406] Two-dimensional electrophoresis using formic acid in the first dimension was used for separation and analysis of polio-virus proteins,[407,408] and a novel two-dimensional system on layers of cellulose acetate membrane has been described.[409]

Immunoelectrophoresis. A new review[410] describes the development and modern application of crossed-immunoelectrophoresis (x-i.e.p.) and serves as a useful reference source, including 1981, for cell antigens analysed by x-i.e.p. The coupling of SDS-p.a.g.e. with x-i.e.p. has yielded a hybrid technique with a high degree of resolution and specificity, and a procedure for transfer of polypeptide from the first dimension (polyacrylamide gel) to the second dimension (agarose/antibody gel) without the use of an intermediate layer of non-ionic detergent has been described.[411] The procedure allows more precise alignment of previously fixed and stained polypeptide bands, with the immunoprecipitates. P.a.g.e. in 7M urea followed by x-i.e.p. has been used for the determination of specificity of antibodies raised against serum lipo- and apolipo-proteins.[412] The method has advantages over the use of cellulose acetate in the first dimension, which tends to bind apolipoprotein E and is very limited in protein capacity.

I.e.f. in agarose, for ease of protein recovery, has also been combined with immunoelectrophoresis to analyse and assay human-serum transferrin, and it may be applicable to many other proteins.[413]

Immunoelectrophoresis methods in general are described in one revised and one new book on immunological techniques,[414,415] including some of the difficulties and artifacts arising in immunoelectrophoresis and quantitation of antigens in gels by densitometric scanning. Determination of relative antigen–antibody affinities has been attempted as a function of rocket area *versus* antigen or antibody concentration in quantitative immunoelectrophoresis (q.i.e.p.)[416] and reverse q.i.e.p.[417]

Affinity Electrophoresis. The term affinity electrophoresis denotes a technique where movement of a charged molecule within an electric field is altered by interaction with a second molecule within the medium to form a complex. In the strict sense this would include immunoelectrophoresis, where antigen and antibody interact, but this particular case has been dealt with separately in the previous section. There are basically two approaches to affinity electrophoresis. The first is closely related to immunoelectrophoretic methods in that ligand-

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binding proteins, such as lectins, are used essentially as analogues of antibodies incorporated into agarose gels. Glycoproteins capable of interaction with the lectins are retarded or even precipitated when electrophoresed in such gels. In a recent publication, cell surface proteins of mouse lymphocytes have been analysed on the basis of concanavalin-A binding using such a system.[418] The second approach is based on electrophoresis in polyacrylamide gels containing immobilized ligands and is more closely related in principle to affinity chromatography. Ligands may be attached *via* spacer arms and include sugars, nucleotides, and the reactive triazine dyes becoming popular for affinity chromatography. Gels containing hydrophobic substituents (*N*-alkyl-substituted acrylamides) have been used to study a number of proteins, including bovine serum albumin and β-lactoglobulin A.[419]

The preparation of affinity gels and their present and future qualitative and quantitative application have been reviewed.[420] The review also draws attention to the problems of applying affinity electrophoresis theory to actual experimental conditions. An evaluation of the effects of protein multi-valency and of the heterogeneity and microdistribution of immobilized ligand has been attempted.[421] Equations are presented that relate electrophoretic mobility of multi-valent proteins to the strength of the protein–ligand interaction in a quantitative manner. At low concentrations of immobilized ligand (Cim) fairly exact K and L/n values can be obtained (L = intrinsic dissociation constant of the protein–immobilized ligand complex; n = the number of independent but identical ligand-binding sites on the protein; K = intrinsic dissociation constant of the protein–mobile ligand complex). The conclusions are that (i) there are no simple means of estimating n by affinity electrophoresis, (ii) for a bivalent protein with two independent non-identical binding sites apparent K and L values are obtained that lie between true K_1 and K_2 or L_1 and L_2 values characterizing the two sites, (iii) effective Cim values can be obtained by following the dependence of the mobility of a protein in affinity gels, containing a fixed Cim, on the protein concentration, and (iv) the effects of non-homogeneity of immobilized ligand molecules can be quantitatively evaluated.

Affinity i.e.f. has also been described[422] for the qualitative identification of particular ligand-binding proteins, including lactate dehydrogenase on a Blue Dextran 2000 gel.

Isotachophoresis. There are some signs that in the early 1980s isotachophoresis is finally finding a practical place amongst more standard electrophoretic techniques for the separation and analysis of proteins. To most researchers it is perhaps the least well understood of the modern electrophoretic methods, although the theory dates back to the last century, and the familiar stacking gel of the Ornstein–Davis p.a.g.e. system, used to concentrate the protein zone prior to separation, in fact employs the principle of isotachophoresis.[423] In the isotachophoretic 'steady

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state', ions with different electrophoretic net mobility, but carrying the same sign of charge, are brought to migrate with equal velocity in separate zones by an electric field. The boundaries between the zones are only traversed by the common counter-ion. A unifying model for the ionic composition of diversely described 'steady-state' electrophoresis systems can be defined.[424]

In aqueous media, H^+ and OH^- ions for cationic or anionic separations, respectively, must not contribute significantly to the conduction of electrical current. For cationic isotachophoresis the 'safe region' is above pH 5, but separation of weak bases requires pH values below 5.0. Under these conditions the migration of H^+ must be controlled by selection of a suitable terminating cation. The migration behaviour of H^+ during cationic isotachophoresis has been mathematically defined and the model applied to the separation of some amino-acids and dipeptides with different counter-ions.[425,426]

Capillary isotachophoresis has found increased use for the analysis of amino-acids and peptides[427-429] and has been used as an analytical monitor to follow the synthesis of the *C*-terminal pentapeptide of bombinin.[430] The latter application follows the course of reaction more accurately than conventional techniques such as t.l.c. and could help in the development of peptide synthesis methodology. Capillary isotachophoresis has also been used for the separation of proteins, both analytically[431] and preparatively for erythropoietin[432] and lipoproteins HDL_2 and HDL_3.[433]

Chromatofocusing.—Chromatofocusing takes advantage of the buffering action of the charged group of an ion exchanger to produce a pH gradient. If a buffer initially adjusted to one particular pH is run through an ion-exchange column and buffered at a second pH, then a gradient is formed and can be used to elute proteins previously bound to the ion exchanger in order of their isoelectric points.[434,435] A number of papers have appeared since the middle of 1981, showing that the technique is now finding practical application. Chromatofocusing was used to study variants of human haemoglobulin,[436-438] lysosomal hydrolases,[439] glutathione *S*-transferases,[440] carbamylphosphate synthetases,[441] and yeast hexokinase isoenzymes.[442]

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The properties of a new DEAE-agarose anion exchanger for chromatofocusing of proteins have been described,[443] as has an agarose-polyethylenediamine ion exchanger,[444] which overcomes problems of pH maxima within the gradient caused by slow pH equilibration between DEAE-agarose beads and the surrounding buffer. Chromatofocusing in 6 M urea has been used to separate the four subunits of the lens protein, α-crystallin, on a preparative scale.[445]

Molecular-weight Determination.—An updated table of molecular weights and isoelectric points of proteins has appeared with more than 900 references and entries.[446]

High-performance gel-permeation chromatography (h.p.g.p.c.) using TSK gels SW2000 and 3000 under denaturing conditions (sodium dodecyl sulphate or guanidine hydrochloride) has been used to determine the molecular weight of virus polypeptides.[447] A new matrix for h.p.g.p.c., Toyopearl HW55F, has also been assessed for molecular-weight determinations in the range 10^4—10^6 dalton.[216] Gram quantities of protein can be separated in minutes with better than 86% recovery of material on a preparative scale.

Determination of molecular weight and Stokes radii of non-denatured proteins using polyacrylamide gradient gel electrophoresis has been demonstrated for six standard marker proteins and applied to plant isoenzymes.[448,449] SDS-pore gradient electrophoresis of ovalbumin, avidin, and fetuin in Tris–glycine and Tris–borate buffer systems was used to assess the reliability of this method for molecular-weight estimation of glycoproteins.[450]

PART IB: Primary Structures, 1980 and 1981 *By J. Gagnon and D. L. Christie*

This section comprises comprehensive lists of the amino-acid sequences reported during 1980 (Table 1) and 1981 (Table 2). The proteins and peptides in each list are given in alphabetical order, in thirteen different categories: blood-clotting proteins, complement proteins, electron-transport proteins, enzymes, globins, histocompatibility antigens and antibodies, histones, hormones, ribosomal proteins, structural proteins, toxins, viral proteins, and miscellaneous proteins. The primary structures cited are those derived from automated and manual methods of protein-sequence determination. Amino-acid sequences deduced from nucleotide sequences, but not directly determined, have not been included.

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Table 1 *Amino-acid sequences reported during* 1980

Protein	*Origin*	*Comments*	*Ref.*
Blood-clotting Proteins			
Factor VII	Human	*N*-Terminal sequence, 10 residues	1
Factor XI	Bovine	Complete sequence of light chain, 140 residues	2
Factor XI	Bovine	*N*-Terminal sequence of whole molecule, heavy and light chains, 16 residues each	3
Protein Z	Bovine	*N*-Terminal sequence, 14 residues	4
Complement Proteins			
Anaphylotoxin	Porcine	*N*-Terminal and *C*-terminal sequence, 19 and 12 residues, respectively, identification with des-Arg-C5a	5
Factor B	Human	*N*-Terminal sequence of whole molecule and Ba, 36 residues; *N*-terminal sequence of Bb, 18 residues	6
Factor B	Human	Partial sequence, 100 residues, homology with serine proteinases	7
Factor B	Human	Partial sequence of Bb fragment, 210 residues, active-site homology with serine proteinases	8
Factor D	Human	Partial *N*-terminal sequence, 48 of 57 residues	9
Factor D	Human	*N*-Terminal sequence of CNBr peptide containing serine of active site, 25 residues	10
Factor D	Human	*N*-Terminal sequence of major CNBr fragments, 60 residues	11

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Pro-C4	Guinea pig	Partial *N*-terminal sequence, 17 of 22 residues, same sequence as β-chain	12
C4	Mouse	Partial *N*-terminal sequence of α-, β-, and γ-chains, 9—12 residues, and pro-C4, 5 of 7 residues	13
C5a anaphylotoxin	Porcine	Complete sequence, 74 residues	14
Properdin	Human	*N*-Terminal and *C*-terminal sequence, 10 and 5 residues, respectively	15
Electron-transport Proteins			
Cytochrome *b*5	Rabbit	Sequence of membranous segment, 38 residues	16
Cytochrome *c*	Rice	Complete sequence, 103 residues, *N*-terminal acetylated, 2 lysine residues methylated	17
Cytochrome C1	Bovine	Complete sequence, 241 residues	18
Cytochrome C3	*Desulfovibrio vulgaris*	Complete sequence, 107 residues	19
Cytochrome C557	*Crithidia oncopelti*	*N*-Terminal dimethyl proline residue identified by n.m.r.	20
Ferredoxin	*Halobacterium*	Complete sequence, 128 residues, one lysine residue acetylated	21
Ferredoxin	*Phytolacca esculenta*	Complete sequence of ferredoxins I and II, 96 and 98 residues, respectively	22
Haemocyanin chain e	Spider	Sequence of *N*-terminal and *C*-terminal peptides, 28 and 41 residues, respectively	23

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Enzymes			
Acrosin	Boar sperm	*N*-Terminal sequence, 52 residues	24
Acylphosphatase	Horse muscle	Complete sequence, 98 residues	25
D-Alanine carboxypeptidase	*Bacillus stearothermophilus*	*N*-Terminal sequence, 40 residues, penicillin acetylates Ser-36	26
Alcohol dehydrogenase	*Drosophila melanogaster*	Sequence of 3 alloenzymes, 254 residues each, location of mutant substitutions	27
D-Amino-acid oxydase	Porcine kidney	Sequence of active-site tyrosine containing hexapeptide	28
Cyclic AMP-dependent protein kinase II	Bovine heart	Sequence of hinge region of regulatory subunit, 26 residues including site of autophosphorylation	29
L-Asparaginase	*E. coli*	Complete sequence, 321 residues	30
Aspartate amino-transferase	Porcine heart mitochondria	Sequence of 39 tryptic peptides, 318 of 401 residues	31
Aspartate amino-transferase	Porcine	Sequence of 12 CNBr peptides, 367 of 401 residues	32
Aspartate amino-transferase	Porcine	Complete sequence, 401 residues	33
Ca-ATPase	Rabbit	Sequence of soluble tryptic peptides, 586 residues	34
Ca-ATPase	Rabbit	Sequence of chymotryptic peptides, 629 residues	35
Ca-ATPase	Rabbit	Sequence of some peptic, thermolytic, tryptic, and staphylococcal protease peptides, 397 residues	36

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Ca-ATPase	Rabbit	Sequence of CNBr peptides, 562 residues, construction of 3 long stretches of sequences representing half of the protein	37
γ-Carboxymuconolactone decarboxylases	*Acinetobacter calcoaceticus* and *Pseudomonas putida*	*N*-Terminal sequence of enzymes from 2 sources, 36 and 49 residues, respectively	38
Citrate synthase	Porcine heart	*N*-Terminal sequence of 2 subtilisin fragments, 15 and 8 residues, one fragment is *N*-terminal	39
Collagenase	Fiddler crab	Complete sequence, 226 residues	40
Cytochrome *b*5 reductase	Porcine liver	*N*-Terminal sequence of lysosome solubilized fragment, 20 residues	41
Cytochrome C peroxidase	Yeast	Partial sequence of 28 tryptic and 47 chymotryptic peptides, about 90% of polypeptide sequence	42
Cytochrome C peroxidase	Yeast	Complete sequence, 293 residues	43
Dihydrofolate reductase	Chicken	Complete sequence, 189 residues	44
Diphosphoglycerate mutase	Human	Sequence of an active-site fragment, 32 residues	45
Exopenicillinase (β-lactamase)	*Bacillus licheniformis*	*N*-Terminal sequence of exo-large and exo-small subunits, 14 and 4 residues, respectively	46
Fungal laccase B ('Blue' oxidase)	*Polyporus versicolor*	Sequence of peptides containing single cysteine and cystine, 23 residues	47
β-Glucosidase A3	*Aspergillus wentii*	Sequence of active site containing glycopeptide, 63 residues	48

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Glutamate oxaloacetic transaminase	Rat, mitochondria	Complete sequence, 401 residues	49
D-Glyceraldehyde-3-phosphate dehydrogenase	*Bacillus stearothermophilus*	Complete sequence, 333 residues	50
D-Glyceraldehyde-3-phosphate dehydrogenase	*Thermus aquaticus*	Complete sequence, 332 residues	51
L-3-Hydroxyacyl CoA dehydrogenase	Porcine heart	Complete sequence, 307 residues, a second form in equal amount is missing the first 5 residues	52
p-Hydroxybenzoate hydroxylase	*Pseudomonas fluorescens*	Partial sequence, 149 of 391 residues	53
p-Hydroxybenzoate hydroxylase	*Pseudomonas fluorescens*	*N*-Terminal sequence, 110 residues	54
Inorganic pyrophosphatase	Yeast	Identification of residue-binding inorganic pyrophosphate, Arg-77	55
β-Ketoadipate enol-lactone hydrolase	*Pseudomonas putida*	*N*-Terminal sequence, 54 residues	56
β-Ketoadipate enol-lactone hydrolase I	*Acinetobacter calcoaceticus*	*N*-Terminal sequence, 30 residues	57
2-Keto-3-deoxy-6-phosphogluconate	*Pseudomonas putida*	Complete sequence, 225 residues	58
β-Lactamase	*Pseudomonas*	Sequence of active-site serine peptide, 3 residues	59
Lactate dehydrogenase	Mouse testis	Partial sequence, 298 of 332 residues	60
Lactose permease	*E. coli*	*N*-Terminal sequence of *in vitro* and *in vivo* products, 42 residues	61
Leucine aminopeptidase	Bovine lens	Sequence of *N*-terminal CNBr peptide, 171 residues	62
Lysozyme	Australian black swan	Complete sequence, 185 residues	63

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
NAD-specific glutamate dehydrogenase	*Neurospora crassa*	Sequence of CNBr peptides from *N*-terminal portion, 2 segments of 43 and 313 residues	64
Pancreatic ribonuclease	Impala, Thompson's gazelle, nilgai, and water buffalo	Complete sequence of protein from 4 bovidae	65
Pancreatic ribonuclease	Hippopotamus and two- and three-toed sloth	Complete sequence, 124 and 128 residues, respectively	66
Penicillin-sensitive D-alanine carboxypeptidase	*Bacillus subtilis*	Sequence of active-site peptides, *N*-terminal sequence, 64 residues	67
Pepsinogen A	Human	Sequence of activation peptide, 47 residues	68
Pepsinogen	Japanese monkey	Sequence of activation peptide, 47 residues	69
Pepsinogens	Crab-eating monkey	*N*-Terminal sequence of pepsinogens I-a, III-1, III-2, III-3, and C, 14 residues each	70
Peroxidase 7	Turnip	Sequence of 25 tryptic peptides, 296 residues	71
Peroxidase 7	Turnip	Sequence of CNBr peptides and complete sequence, 296 residues	72
Phenylalanine hydroxylase	Rat liver	Sequence of phosphorylated peptide, 10 residues	73
Phosphofructokinase	Sheep	Sequence of allosteric site, 9 residues	74
Phosphofructokinase	*Bacillus stearothermophilus*	Complete sequence, 316 residues	75
Phosphoglycerate kinase	Human	Sequence of 46 tryptic peptides	76
Phosphoglycerate kinase	Human	Sequence of 14 CNBr peptides, complete sequence, 417 residues	77

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Phosphoglycerate kinase	Human	Amino-acid substitution in variant PGK München, Asp-268→Asn	78
Phosphoglycerate mutase	Rabbit muscle	Sequence of 2 histidine-containing peptides, 8 and 6 residues	79
Phospholipase	Siamese cobra	Sequence of 2 enzymes CM-II and CM-III, 119 amino-acids each	80
Phospholipase A2	Cobra venom	*N*-Terminal sequence, 35 of 40 residues	81
Phosphorylase	*E. coli* and potato	*N*-Terminal sequence, 19 and 15 residues, respectively	82
Phosphorylase	Potato	Sequence of 10 cysteine-containing peptides, 173 residues	83
Phosphorylase	Potato	*N*-Terminal sequence, 104 residues	84
Proelastase	Human pancreas	*N*-Terminal sequence, 16 residues	85
Proteolipid subunit of ATPase	Spinach	Complete sequence, 81 residues	86
Proteolipid subunit of ATPase	Thermophilic bacterium PS-3	Complete sequence, 72 residues	87
Proteolipid subunit of ATPase	*E. coli*	Sequence of 6 mutants, 79 residues, point mutation at position 28	88
Proteolipid subunit of ATPase	*E. coli*	Location of amino-acid replacement in 2 mutants DG7/10 and DC1	89
Pyruvate dehydrogenase multi-enzyme complex	*E. coli*	Sequence around 2 lipoic acid residues of lipoate acetyltransferase component	90
Pyruvate kinase, type L	Porcine liver	Sequence of a phospho-peptide, 33 residues	91

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Pyruvate phosphate dikinase	*Bacillus symbiosus*	Sequence of active-site histidine peptide, 12 residues	92
Ribulose-1,5-bisphosphate carboxylase/oxygenase	*Rhodospirillium rubrum*	Sequence of pyridoxal 5′-phosphate active-site peptide, 15 residues	93
Saccharopine dehydrogenase	Yeast	Sequence of peptide containing essential cysteine, 11 residues	94
Superoxide dismutase Fe/2Mn	7 micro-organisms	*N*-Terminal sequence of 7 enzymes, 40 to 58 residues	95
Superoxide dismutase	Human erythrocyte	Complete sequence, 153 residues	96
Superoxide dismutase Cu/Zn	Human	Complete sequence, 153 residues	97
Superoxide dismutase Cu/Zn	Baker's yeast	Complete sequence, 153 residues	98
Superoxide dismutase Mn	*Bacillus stearothermophilus*	Complete sequence, 203 residues	99
Triosephosphate isomerase	*Bacillus stearothermophilus*	Sequence of 249 residues	100
Trypsin 1 and 2	Starfish	*N*-Terminal sequence of both enzymes, 17 residues each	101
Trypsin and chymotrypsin	Frog	N-Terminal sequence 30 and 20 residues, respectively	102
Tryptophan synthetase	*E. coli*	*N*-Terminal sequence of $\beta 2$ subunit, 99 residues	103
Tyrosine-sensitive 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase	*E. coli*	*N*-Terminal sequence, 30 residues	104
Globins			
Haemoglobin, α-chain	Baboon	Complete sequence, 141 residues	105

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Haemoglobin, (Hb Bari) α-chain	Human	Sequence of tryptic peptide of σ-chain variant, 16 residues	106
Haemoglobin, α-chain of component III	Tadpole of bullfrog	Complete sequence, 141 residues	107
Haemoglobin, α- and β-chains	Horse	Revision of both sequences, 141 and 146 residues, respectively	108
Haemoglobin, α- and β-chains	Barheaded goose and ostrich	Complete sequence of α- and β-chains, 141 and 146 residues, respectively	109
Haemoglobin, α- and β-chains	Goldfish	Complete sequence, 142 and 147 residues, respectively, *N*-terminus of α-chain is acetylated	110
Haemoglobin, chain of component III	Tadpole of bullfrog	Complete sequence, 146 residues	111
Haemoglobin, βA- and βB-chains	Carp	Complete sequence, 147 residues each	112
Haemoglobin, foetal, γ-chain	Rhesus monkey	Complete sequence, 146 residues	113
Haemoglobin, embryonic	Porcine	*N*-terminal sequence, 30 residues, homologous to ε-chain	114
Haemoglobin CTT1	*Chironomus thummi thummi*	Complete sequence, 144 residues	115
Haemoglobin CTT VII A	*Chironomus thummi thummi*	Complete sequence, 145 residues	116
α-Globin, embryo	Chicken	Complete sequence of π and π^1 chains, 141 residues, both chains identical except for Ala/Glu at position 124	117
Leghaemoglobin	Pea	Complete sequence of component I, 147 residues	118
Myoglobin	Yellow-fin tuna	Complete sequence, 146 residues, *N*-terminus acetylated	119

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Myoglobin	Hubb's beaked whale	Complete sequence of major component, 153 residues	120
Myoglobin	Alligator	Complete sequence, 154 residues	121
Myoglobin	Two old-world monkeys	Complete sequence inferred from homology of tryptic peptides	122
Myoglobin	Goose-beaked whale	Complete sequence, 153 residues	123
Histocompatibility Antigens and Antibodies			
Antiazophenyl arsonate antibodies, H- and L-chains	Hybridoma mouse A/J spleenocytes and myeloma	*N*-Terminal sequence, 39 and 47 residues, respectively	124
Antidextran antibodies, H-chains	Myeloma and mouse hybridoma proteins	Complete sequence of V-domain of 10 homogeneous hybridoma and 2 myeloma proteins	125
Antigalactan antibodies, L-chains	Myeloma J-539, S-10, and T-191	Sequence of V-domain, 108 residues each	126
Antiphosphorylcholine antibody, H-chain	Mouse (C57/BL)	Complete sequence of V-domains, 115 residues	127
Antiphosphorylcholine antibodies, H- and L-chains	Mouse (Balb/c)	*N*-Terminal sequence of V-domain from 5 PC-specific antibodies, 30—40 residues	128
Antiphosphorylcholine antibody, L-chain	Mouse (Balb/c) (myeloma protein M511)	Sequence of V-domain, 108 residues	129
Bence-Jones protein Wes, κ-type, L-chain	Human	Sequence from tryptic peptides aligned by homology, 214 residues	130
Bence-Jones protein Wh, λ-subgroup	Human	Complete sequence of V-domain, 111 residues	131

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Disease protein BOT, μ-chain	Human	Sequence of CH_2, CH_3, and CH_4 domains, 353 residues	132
Disease protein EST, γ1 H-chain	Human	*N*-Terminal sequence, 16 residues, protein lacks VH and CH_1 domains	133
Disease protein Wis, λ3 H-chain	Human	Complete sequence, 289 residues	134
H-2D	Mouse	*N*-Terminal sequence of 5 CNBr peptides, 146 of 280 residues	135
$H\text{-}2K^b$	Mouse	*N*-Terminal sequence, 173 residues	136
$H\text{-}2K^b$	Mouse	Sequence of CNBr fragment Ia, 90 residues	137
$H\text{-}2K^b$	Mouse	Sequence of CNBr fragment Ic, 55 residues, completes sequence of *N*-terminal portion external to cell membrane, 284 residues	138
$H\text{-}2K^k$	Mouse	Partial sequence of 6 CNBr fragments, 87 residues	139
H-2L	Mouse	Partial sequence of CNBr peptide, alignment by homology	140
HLA-A, -B, and -C	Human	Sequence of 80% of molecule despite heterogeneity, 273 residues	141
GPLA-B	Guinea pig	Partial *N*-terminal sequence of 3 GPLA-B antigens	142

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Ia antigen	Rat	Partial *N*-terminal sequence of α and β components of H-1^a, H-1^1 and H-1^a, H-1^1 and H^{1n} haplotypes, 7—10 residues	143
IgA, H-chain	Mouse (MOPC 511)	Sequence of H(α)-chain, 432 residues	144
IgD, myeloma protein N1G-65	Human	Sequence of 3 glycopeptides from Fc region, 9, 21, and 15 residues	145
IgG, b5 L-chain	Rabbit	Partial sequence of C-domain, 90 residues	146
IgG, b9 L-chain	Rabbit	*N*-Terminal sequence of a fragment from C-domain	147
IgG, b5 and b6 L-chains	Rabbit	Sequence of tryptophan containing peptides, 5—33 residues	148
IgG, γ2, H-chain (Til protein)	Human	Complete sequence, 440 residues	149
IgG, γ2b—γ2a hybrid, H-chain	Mouse (MPC11), myeloma variant ICR 11.19.3	*N*-Terminal sequence of Fc, 4 Fc CNBr fragments, Fab and 3 Fab CNBr fragments, 149 residues	150
IgG, κ proteins	Human	*N*-Terminal sequence of γ-chain and κ-chain, 27 and 33 residues each	151
IgM, μ H-chain	Human	Complete sequence and 33 residues each	151
IgM, μ H-chain	Human	Complete sequence of V-domain, 136 residues	152
β2-Microglobulin	Guinea pig	Complete sequence, 99 residues	153
Myeloma protein C3H	Mouse, myeloma 5563	Partial sequence of precursors of H- and L-chains synthesized *in vitro*, 18 of 36 and 14 of 30 residues, respectively	154
Myeloma protein M149	Mouse	Sequence of k-chain V-domain, 108 residues	155

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Myeloma protein MOT	Human	Sequence of λL-chain, 216 residues	156
Histones			
H1	Sea urchin	*N*-Terminal sequence, 84 residues	157
H1	Maize	Partial sequence, some 60 residues	158
H1	Rat liver	Identification of ADP-ribosylation site, Glu-2, Glu-14, and a Lys at *C*-terminal end	159
H1	Sea urchin	Complete sequence, 248 residues	160
$H1^0$	Bovine	*N*-Terminal sequence of a CNBr peptide, 17 residues	161
H2A	Human	Comparison of tryptic peptides of H2A (1) and H2A (2) variants	162
H2A	Sea urchin	Complete sequence, 125 residues	163
H2B	Starfish	Complete sequence, 120 residues, blocked *N*-terminus	164
H2B	Rat	Identification of ADP-ribosylation site, Glu-2	165
H3, H4, H2A, and H2B	Yeast	Partial sequence of 4 histones, about 30% of each	166
H5	Chicken	Complete sequence, 189 residues	167
H5, H2A, H2B, and dimer H2A–H2B	Rat	Identification of phosphorylation site by cAMP-dependent protein kinase	168

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Hormones			
Adrenal polypeptides	Bovine	Complete sequence of peptide F and partial sequence of peptide I, 34 and 31 residues, respectively	169
Big gastrin	Human	Complete sequence, 34 residues	170
Chorionic gonadotropin	Human	Assignment of disulphide bonds, 7–31, 10–32, 28–60, 59–87, 82–84	171
Corticotropin/β-lipotropin precursor	Porcine	*N*-Terminal sequence of Trp-peptide I, 35 residues	172
Corticotropin/β-lipotropin precursor	Rat	Partial *N*-terminal sequence, signal peptide of 26 residues	173
Egg-laying peptides	*Aplysia californica*	Complete sequence of neuropeptides A and B, 34 residues each, B differs from A in 4 positions	174
Endorphin II	Salmon	Complete sequence, 30 residues	175
Gastric proventricular peptide	Chicken	Complete sequence, 27 residues	176
Insulin	Porcupine	Complete sequence of A- and B-chains	177
β-Lipotropin and corticotropin	Turkey	*C*-Terminal sequence of β-LPH, residues 41—91, complete sequence of ACTH, 39 residues	178
β-Melanotropin	Salmon	Complete sequence, 17 residues	179
β-Melanotropin	Salmon	Sequence of new form of MSH, 15 residues	180

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Pituitary peptide containing —MSH sequence	Human	Sequence of γ-MSH by homology of tryptic peptides with bovine γ-MSH, 15 residues	181
Polypeptide hormones PH1 and PYY	Porcine	*N*-Terminal sequence of PH1 and PYY, 7 and 9 residues, respectively; *C*-terminal sequence, 2 residues	182
Pro-opiomelanocortin	Human	*N*-Terminal sequence of *N*-terminal peptide, 40 of 103 residues	183
Pro-opiomelanocortin	Pig	Sequence of *N*-terminal peptide, 103 residues, determination of 2 glycosylation sites	184
Pro-somatostatin	Pig	Complete sequence, 28 residues	185
Secretin	Chicken	Complete sequence, 27 residues, *C*-terminal Met is amidated	186
Somatostatin I	Catfish	Complete sequence, 22 residues	187
Somatostatin 28	Pig	Complete sequence, 28 residues	188
Somatomedin-C	Human	Partial sequence, some 40 residues	189
Urotensin II	*Gillichthys mirabilis*	Complete sequence, 12 residues	190
Vasoactive peptides	Human	Sequence of 3 peptides derived from plasmin digest of fibrinogen, 5, 5, and 11 residues	191
Ribosomal Proteins			
Acid protein B-L9	*Bacillus subtilis*	Complete sequence, 122 residues	192

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
'A' protein	Methanobacterium	*N*-Terminal sequence, 48 residues	193
BL-17	*Bacillus stearothermophilus*	Complete sequence, 147 residues	194
L4	*E. coli*	Complete sequence, 201 residues	195
L11	*E. coli*	Complete sequence, 141 residues, contains *N*-terminal *N*-trimethyl alanine and 2 N^{ε}-trimethyl lysine	196
L22	*E. coli*	Complete sequence, 110 residues	197
S10	*E. coli*	Complete sequence, 103 residues	198
S11	*E. coli*	Complete sequence, 128 residues	199
YP A1	Yeast	Complete sequence, 110 residues	200
Structural Proteins			
Collagen peptide α1 (III)-CB5	Human	Complete sequence, 327 residues	201
Link protein	Bovine cartilage	*N*-Terminal sequence of a tryptic and 2 CNBr fragments (D and C), 32, 8, and 41 residues, respectively	202
Membrane collagen, type IV	Mouse	*N*-Terminal sequence of peptic peptide and 2 CNBr peptides derived from it (CB-7 and CB-5), 18, 13, and 30 residues, respectively	203
Procollagen, Pro 1 (I)	Chicken	*N*-Terminal sequence of medium propeptide and a tryptic fragment, 29 and 16 residues	204

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Toxins			
Antitumour toxin ricin D	Small beans	*N*-Terminal sequence of CNBr peptides from A- and B-chains, 75 and 73 residues, respectively	205
CM-10 and CM-9a	*Naja nivea* and *Naja naja Kaou-thia*	Complete sequence of 2 toxins, 65 and 64 residues, respectively	206
C10 S2 C2 and C13 S1 C1	*Dendroaspis angusticeps* (green mamba)	Complete sequence, 60 and 58 residues, respectively	207
C13 S1 C3 and C13 S2 C3	*Dendroaspis angusticeps*	Complete sequence, 57 and 59 residues, respectively	208
Viroisin	*Amanita virosa*	Sequence of cyclic hexapeptide	209
Viral Proteins			
Adenovirus type-2 protein	Adenovirus	Partial *N*-terminal sequence of 4 proteins: hexon, fiber, component IX, and early protein E1B-15K	210
Core proteins	Semliki forest virus and Sindbis virus	Partial sequence, 67 and 88% of SF and SIN proteins	211
Gene 32 DNA-binding protein	Bacteriophage T4	Partial sequence, 281 of 333 residues	212
Haemagglutinin	A/Hong Kong· influenza virus (H3 N2)	Changes in sequence of molecules from 9 strains, 18 in 262 residues of HA1 and 2 in 129 residues of HA2	213
Haemagglutinin	Hong Kong influenza virus	Assignment of disulphide bonds, 6 of 8 bonds	214
Structural protein	Semliki forest virus	*C*-Terminal sequence of 4 proteins	215
Structural proteins E1 and E2	Semliki forest virus	*N*-Terminal sequence, 19 and 20 residues, respectively	216
Tumour antigen	Simian virus 40	Partial *N*-terminal sequence of 5 fragments from limited proteolysis	217

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Miscellaneous Peptides and Proteins			
Acetyl choline receptor	Electric ray	*N*-Terminal sequence of 4 subunits, 54 residues each	218
Albumin	Bovine	*N*-Terminal sequence of tryptic peptide, 32 residues; this completes the sequence	219
Allergen Ra3	Ragweed	Complete sequence, 101 residues	220
Amyloid fibril AA proteins	Mink	Sequence of 2 proteins, 53 and 64 residues each	221
Amyloid fibril AA protein	Human	Complete sequence, 83 residues	222
Asclepain	Milkweed	*N*-Terminal sequence of 2 cysteinyl proteases, 21 residues each, homology to papain	223
Bacterio-opsin	*Halobacterium halobium*	*N*-Terminal sequence of a higher mol. wt. species, elongation at *N*-terminus	224
Blood-group antigens	Human	*N*-Terminal sequence of Ss and MN glycoproteins, 35 and 30 residues, respectively, 13 residues peptide from component D	225
Bromelain	Pineapple	Partial sequence, *C*-terminal 110 residues, homology to papain	226
Calmodulin	Bovine	Complete sequence, 148 residues, *N*-terminal alanine is acetylated	227
Ceruloplasmin, histidine-rich fragment CpF5	Human	*N*-Terminal sequence, 18 residues, sequence of 9 CNBr peptides	228
Ceruloplasmin, histidine-rich fragment CpF5	Human	Sequence of 19 tryptic peptides, complete sequence of fragment, 159 residues	229

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
DNA-binding proteins HU-1 and HU-2	*E. coli*	Complete sequence of both proteins, 90 residues each	230
Elastase-cathepsin G inhibitor	Leech	Complete sequence, 70 residues	231
Elongation factor Tu	*E. coli*	Complete sequence, 393 residues	232
Elongation factor Tu	*E. coli*	Complete sequence, 393 residues, *N*-terminal serine is acetylated, Lys-56 is partially methylated	233
Glycophorin	Porcine	Complete sequence, 133 residues, 2 *N*-linked and 10 *O*-linked carbohydrate sites	234
Haptoglobin	Human	Complete sequence of α- and β-chains, 83 and 245 residues, respectively, some homology to serine proteases	235
Lactotransferrin	Human	*N*-Terminal sequence of 2 glycopeptides, 13 and 36 residues, assignment of carbohydrate attachment site	236
LATH lectin α- and β-chains	*Lathyrus odoratus*	*N*-Terminal sequence of both chains, 25 residues each	237
Lipoproteins HDL	Human	*N*-Terminal sequence of 3 different apo-HDL, 15 residues each	238
Lipoprotein VLDL-II	Cockerel	Sequence of signal peptide, 23 residues	239
M-Protein	*Streptococcus* type 5, strain B788	*N*-Terminal sequence, 29 residues	240
M-Protein	*Streptococcus* type 24	Sequence of CNBr peptides, 35 residues each	241

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Protein	*Origin*	*Comments*	*Ref.*
α2-Macroglobulin	Human	Sequence of a tryptic peptide, 56 residues, containing the alkylamine reactive site	242
α2-Macroglobulin	Human	*N*-Terminal and *C*-terminal sequence of 2 peptides resulting from cleavage of temperature-sensitive bond	243
α2-Macroglobulin	Human	Localization of reactive thiol-ester	244
Major secretory protein	Rat seminal vesicle	Complete sequence, 90 residues	245
Myosin	Rabbit	*N*-Terminal sequence of short and long subfragment-2, 17 residues; both have identical sequence	246
Myosin, L-1 light-chain	Chicken	Complete sequence, 192 residues	247
Myosin	Chicken	Complete sequence, 166 residues, alignment of tryptic peptides by homology to rabbit myosin L-2 light chain	248
Non-histone chromosomal protein HMG-1 and -2	Calf	Complete sequence, 259 and 256 residues, respectively	249
Non-histone chromosomal protein HMG-17	Chicken	Complete sequence, 89 residues	250
Non-histone chromosomal proteins, HMG-like	Trout and chicken	*N*-Terminal sequence of HMG-14 (chicken), 52 residues, and protein C and D (trout), 27 and 18 residues, respectively	251
Interferon, fibroblast	Human	*N*-Terminal sequence, 13 residues	252
Interferon, C-10, fibroblast	Human	*N*-Terminal sequence, 13 residues	253

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Table 1 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Interferons lymphoblastoid	Human	Partial sequence of at least 2 types of IFN-αA, 140 residues, 3 types of IFN-αB, 161 residues	254
Interferon leucocyte	Human	*N*-Terminal sequence, 22 residues	255
Interferon, major component, lymphoblastoid	Human	*N*-Terminal sequence, 20 residues	256
Interferons, types A, B, and C	Mouse	*N*-Terminal sequence, 24, 24, and 20 residues, respectively	257
Outer-membrane protein II	*E. coli*	Complete sequence, 325 residues	258
P2 protein	Rabbit sciatic nerve	*N*-Terminal sequence, 64 residues, *N*-terminal serine is acetylated	259
P2 protein	Bovine peripheral nerve	Complete sequence, 131 residues	260
Phosphatidylcholine-exchange protein	Bovine liver	Sequence of *N*-terminal half of protein, 122 residues	261
Phospholipase A2 homologue	*Notechis scutatus scutatus* (Australian tiger snake)	Complete sequence, 119 residues, non-neurotoxic and non-enzymic protein	262
PO glycoprotein	Human peripheral nerve	*N*-Terminal sequence of protein and a glycopeptide, 17 and 27 residues, respectively	263
Prolamins	Barley (hordein) and wheat (gliadin)	*N*-Terminal sequence of each, 27 and 28 residues, respectively	264
Proline-rich peptide, P-C	Human saliva	Complete sequence, 44 residues, is *C*-terminal portion of proline-rich phosphoprotein	265

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Protein	*Origin*	*Comments*	*Ref.*
Proline-rich phosphoprotein (protein C)	Human saliva	Complete sequence, 150 residues, *N*-terminal, 106 residues, is protein A	266
Protein A	*Dendroaspis polylepis polylepis* (black mamba)	Complete sequence, 81 residues	267
Proteins A, B, and C	*Bacillus megaterium* spores	Sequence around sites cleared by spore-specific protease	268
Protein B	*Bacillus megaterium* spores	Complete sequence, 96 residues, internal duplication	269
Protein C	*Bacillus megaterium*	Complete sequence, 71 residues	270
Proteinase inhibitors	Silktree	Sequence of reactive site of A-II and B-II, 21 residues each, homology to soya-bean trypsin inhibitor	271
Purothionin homologue	Barley	Complete sequence, 45 residues	272
Sulphate-binding protein	*Salmonella typhi-murium* LT2	Complete sequence, 310 residues	273
Tropomyosin	Rabbit heart	Complete sequence, 284 residues	274
β-Tropomyosin	Rabbit	Complete sequence, 284 residues	275
Vitamin K-dependent protein	Human bone	Complete sequence, 49 residues, containing 2 γ-carboxyglutamic acid residues	276

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Table 2 *Amino-acid sequences reported during* 1981

Protein	*Origin*	*Comments*	*Ref.*
Blood-clotting Proteins			
Fibrinogen	Human	Localization of fibrin polymerization site, residues 373—410 of γ-chain	277
Fibrinogen, γ-chain variant	Human	*C*-Terminal sequence	278
Fibronectin	Human	*N*-Terminal sequence of 4 fragments, 4—8 residues	279
Fibronectin	Bovine	Sequence of the factor VIIIa acceptor site	280
Plasminogen	Porcine	Partial sequence of CNBr fragments	281
Plasminogen	Human, bovine, porcine	Sequence of *N*-terminal CNBr fragments of 3 species	282
Complement Proteins			
C_{1s}	Human	*N*-Terminal sequence of light chain, 52 residues	283
C_3	Guinea pig	Partial *N*-terminal sequence of pro-C_3	284
C_{3b} inactivator	Human	*N*-Terminal sequence of heavy and light chains, 30 and 25 residues, respectively	285
C_{4a}, anaphylatoxin	Human	Complete sequence, 77 residues	286 287
C_4	Human	*N*-Terminal sequence of a′-chain and of 3 inactivation fragments	288
C_4	Human	*N*-Terminal sequence of fragment C_{4d}, 90 residues, localization of thiol and reactive acyl groups	289 290

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
C_4	Human	Sequence around the thio-ester site, 44 residues	291
C_4 binding protein	Human	*N*-Terminal sequence, 13 residues	292
Cobra-venom factor	Cobra	*N*-Terminal sequence of α-, β-, and γ-chains, 25, 23, and 12 residues, respectively	293
Properdin	Human	Partial sequence, 225 of 480 residues	294
Electron-transport Proteins			
Allophycocyanin	*Anabaena variabilis*	Complete sequence of the β-subunit, 161 residues	295
Allophycocyanin	*Mastigocladus laminosus*	Complete sequence of the α- and β-subunits, 160 and 161 residues	296
Allophycocyanin	*Synechococcus* 6310	*N*-Terminal sequence of the α- and β-subunits of AP-B and the α-subunit of AP, 12, 11, and 9 residues, respectively	297
Cytochrome *c*	Yeast	Complete sequence, 103 residues	298
Cytochrome *c'* and *c*-556	Bacteria	Complete sequence of the proteins from 14 different species	299
Cytochrome c_3	*Desulfovibrio desulforicans*	Complete sequence, 118 residues	300
Cytochrome *c*-553	Brown alga	Complete sequence, 85 residues	301
Cytochrome P-450	Porcine	*N*-Terminal sequence, 16 residues	302
Cytochrome P-450	Rabbit	*N*-Terminal sequence of 3 forms, 5 residues each	303
Cytochrome P-450	Rabbit	*N*-Terminal sequence of 2 forms	304
Ferredoxin	Parsley	Complete sequence, 96 residues	305

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Ferredoxin	*Thermus thermophilus* HB8	Complete sequence, 78 residues	306
Haemocyanin	Horseshoe crab	Sequence of *N*-terminal CNBr fragments of α-chain, 87 residues	307
Phycocyanin	*Cyanidium caldarium*	Complete sequence of β-subunit, 172 residues	308
Phycocyanin	*Cyanidium caldarium*	Complete sequence of α-subunit, 162 residues	309
Thioredoxin	*Corynebacterium nephridii*	Complete sequence, 105 residues	310
Thioredoxins	Spinach	Partial *N*- and *C*-terminal sequence of thioredoxins f, mf, and mc	311
Enzymes			
Aconitase	Porcine	Sequence of tryptic peptide containing an essential Cys residue, 13 residues	312
D-Alanine carboxy-peptidase	*Bacillus stearothermophilus* and *Bacillus subtilis*	Sequence of *C*-terminal membraneous segments, 30 residues each	313
D-Alanyl-D-alanine peptidase	*Streptomyces*, strain R61	*N*-Terminal sequence, 28 residues	314
Alcohol dehydrogenase	Human	Sequence of tryptic peptide of active site of β_1 and β_2 subunits, 42 residues	315
Alkaline phosphatase	*E. coli*	Complete sequence, 449 residues, localization of 2 disulphide bonds	316
cAMP-dependent protein kinase	Bovine	Complete sequence of catalytic subunit, 349 residues	317
cAMP-dependent protein kinase	Porcine	Sequence of 2 Cys-containing peptides of catalytic subunit, 8 and 17 residues	318

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
cAMP-dependent protein kinase	Porcine	Sequence of peptide labelled with fluorosulphonyl benzoyl adenosine, 27 residues	319
α-Amylase	Porcine	Sequence of isoenzyme I, 496 residues	320
α-Amylase	Porcine	Sequence of major part of the molecule, from residue 35 to 410	321
α-Amylase	*Bacillus amyloliquefaciens*	Sequence of CNBr peptide containing essential Tyr residue, 73 residues	322
α-Amylase	*Bacillus amyloliquefaciens*	Sequence of CNBr fragments D and E, 88 and 56 residues, respectively	323
Arginine kinase	Lobster	Partial sequence, alignment of CNBr fragments	324
Aspartokinase I-homoserine dehydrogenase	*E. coli*	Identification of hinge between the 2 regions of bifunctional protein	325
ATPase	Bovine	*N*-Terminal and *C*-terminal sequence of an active cleavage product	326
DD-Carboxypeptidase-transpeptidase	*Actinomadura*	Sequence of peptide containing the active Ser residue, 7 residues	327
Cathepsin D	Porcine	Partial *N*-terminal sequence of 2 biosynthetic forms	328
Citrate synthetase	Porcine	Complete sequence, 437 residues	329
Copper–zinc superoxide dismutase	Horse	Complete sequence, 153 residues	330
Crotalase	Eastern diamond-back rattlesnake	Partial sequence, 98 residues	331
Cytochrome oxidase, subunit VI	Bovine	Complete sequence, 85 residues	332

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
3-Deoxy-D-arabinoheptulosonate-7-phosphate synthetase	*E. coli*	*N*-Terminal sequence of 3 isoenzymes, 38—40 residues	333
Endopeptidase III	Hornet	Sequence of 2 peptides containing the active Ser and His residues, 39 and 30 residues, respectively	334
Enolase	Yeast	Complete sequence, 436 residues	335
Extracellular serine proteinases	*Bacillus thuringiensis* and *Thermoactinomyces vulgaris*	*N*-Terminal sequence of 2 microbial proteinases, 14 residues each	336
Fatty-acid synthetase	Goose	Sequence of peptide containing the active Ser residue of the thioesterase domain, 8 residues	337
Fructose 1,6-bisphosphatase	Rabbit	Sequence of nonapeptide from active site	338
Fructose 1,6-bisphosphatase	Rabbit	Sequence of peptide from *C*-terminus, 22 residues	339
Fructose 1,6-bisphosphatase	Porcine	Sequence of peptide containing phosphorylated Ser residue, 63 residues	340
Fructose 1,6-bisphosphatase	Porcine	Sequence of *N*-terminal peptide, 60 residues	341
Glutamic oxaloacetic transaminase	Rat	Complete sequence, 401 residues	342
Glutamine synthetase	*Anabaena* 7120	*N*-Terminal sequence, 27 residues	343
Glutathione reductase	Human	Sequence of FAD-binding domain, 159 residues	344
Glyceraldehyde 3-phosphate dehydrogenase	Human	Complete sequence, 334 residues	345

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Glyceraldehyde 3-phosphate dehydrogenase	Rat	Sequence of 2 tryptic peptides	346
L-Histidinol dehydrogenase	*Salmonella typhimurium*	Sequence of complete set of tryptic peptides (36), alignment from nucleotide sequence	347
β-Lactamase	*Staphylococcus aureus*	Partial sequence of leader peptide	348
L-Lactate dehydrogenase	*Lactobacillus xylosus, curvatus*, and *stearothermophilus*	Sequence of dodecapeptide from the substrate-binding region of 3 bacterial enzymes	349
Lipase	Human	*N*-Terminal sequence, 25 residues	350
Lipase	Porcine	Complete sequence, 449 residues	351
Low-molecular-weight proteinase	Crayfish	*N*-Terminal sequence, 19 residues	352
Luciferase	Firefly	Sequence of hexapeptide from nucleotide-binding site	353
Malate dehydrogenase	*E. coli*	*N*-Terminal sequence, 36 residues	354
MoFe-protein (nitrogenase)	*Azotobacter vinelandii*	Sequence of 15 Cys-containing peptides of α- and β-subunits, 221 residues	355
MoFe-protein (nitrogenase)	*Clostridium pasteurianum*	*N*-Terminal sequence of larger subunit, 179 residues	356
Myeloperoxidase	Human	*N*-Terminal sequence, 8 residues	357
Pentulokinases	*Klebsiella aerogenes*	*N*-Terminal sequence of D-ribulokinase and D-xylulokinase, 15 and 24 residues	358
3-Phosphoglycerate kinase	Horse	Complete sequence, 416 residues	359 360

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
3-Phosphoglycerate kinase	Yeast	Partial sequence, alignment of CNBr peptides	361
Phospholipase A	*Elapidae*	Complete sequence, 118 residues	362
Phospholipase A_2	Habu snake	*N*-Terminal sequence, 9 residues	363
Phospholipase A_2 (DE-I)	Hime-habu snake	Complete sequence, 123 residues	364
Phosphorylase kinase	Rabbit	Complete sequence of δ-subunit, 148 residues	365
Prenyltransferase	Chicken	Sequence of photoaffinity-labelled CNBr peptide, 30 residues	366
Procarboxy peptidase A	Bovine	Sequence around an active site of subunit III, 20 residues	367
Propanediol dehydrastase	*Klebsiella pneumoniae*	Partial *N*-terminal sequence of the 60 K subunit, 34 residues	368
Proteinase A	Mouse	*N*-Terminal sequence, 40 residues	369
Protocatechuate 3,4-dioxygenase	*Pseudomonas aeruginosa*	Complete sequence of β-subunit, 228 residues	370
Ribulose bisphosphate carboxylase	Spinach	*N*-Terminal sequence of peptide bearing the activator carbon dioxide, 17 residues	371
Ribulose bisphosphate carboxylase	*Rhodospirillum rubrum*	Sequence of 5 Cys-containing peptides, 94 residues	372
RNA polymerase	*E. coli*	Partial sequence of β-subunit, 930 of 1372 residues	373
D-Serine dehydratase	*E. coli*	Sequence of 444 residues	374
Superoxide dismutase	*Bacillus stearothermophilus*	Partial sequence by mass spectrometry	375

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Tonin	Rat	Partial sequence, 95 residues	376
Triosephosphate isomerase	Human	Tentative sequence by homology with the rabbit enzyme	377
Urine diphosphate glucose dehydrogenase	Bovine	Sequence of peptide containing the catalytic site thiol group, 14 residues	378
Globins			
Erythrocruorin CTT IIIa	Diptera	Complete sequence, 151 residues	379
Erythrocruorin CTT IV	Diptera	Complete sequence, 136 residues	380
Erythrocruorin CTT VI	Diptera	Complete sequence, 147 residues	381
Erythrocruorin CTT VIII	Diptera	Complete sequence, 151 residues	382
Erythrocruorin CTT IX	Diptera	Complete sequence, 145 residues	383
Haemoglobins	Caiman, Nile crocodile, and Mississippi crocodile	Complete sequence of α- and β-subunits of the 3 crocodile species	384
Haemoglobin	European mole	Complete sequence of α- and β-subunits, 141 and 146 residues	385
Haemoglobin	Greylag goose	Complete sequence of α- and β-subunits, 141 and 146 residues	386
Haemoglobin	Musk shrew	Complete sequence of α, β^{I}, and β^{II} subunits, 141, 146, and 146 residues	387
Haemoglobin	Yak	Complete sequence of β^{I} and β^{II} subunits, 146 residues each	388
Haemoglobin	Armadillo	Complete sequence of major β-subunit, 146 residues	389

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Haemoglobin (embryonic)	Human	Complete sequence of ζ-chain, 141 residues	390 391
Myoglobin	Gastropodic mollusc	Complete sequence, 144 residues	392
Myoglobin	Lace monitor lizard	Complete sequence, 153 residues	393
Myoglobin	Map turtle	Complete sequence, 153 residues	394
ρ-Globin (embryonic)	Chicken	Complete sequence, 146 residues	395
Histocompatibility Antigens and Antibodies			
Anti-azobenzenearsonate	Guinea pig	Sequence of second hypervariable region of different isotypes	396
Anti-azophenylarsonate monoclonal Ab	Mouse (A/J)	Partial sequence of H- and L-chains from 7 idiotype negative and positive molecules	397
Anti-*p*-azophenylarsonate monoclonal Ab	Mouse	Complete sequence of 5 L-chain variable regions, 108 residues each	398
Anti-γ-globulin, Sie and Wol (IgM)	Human	Complete sequence of variable regions of L- and H-chains of proteins Sie and Wol, 109 and 124 residues, respectively	399 400
Anti-GAT and anti-GA monoclonal AB	Mouse (DBA/2)	*N*-Terminal sequence of 5 IgM and 5 IgG H-chains	401
Anti-(4-hydroxy-3-nitrophenyl) acetyl monoclonal Ab (IgD)	Cell line B1-881	Sequence of *C*-terminal portion of secreted H-chain	402
Anti-inulin myelome proteins	Mouse	Sequence of variable region of H-chains of proteins AMPC1 and T957, 113 residues each	403

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Anti-micrococcus Ab	Rabbit	Partial sequence of H-chain of three a100 allotypes, 35 to 50 residues	404
Anti-phosphorylcholine myelome Ab M603 (IgA)	Mouse	Sequence of variable region of L-chain, 110 residues	405
Anti-phosphorylcholine Ab	Mouse	*N*-Terminal sequence of variable region of L- and H-chains from 16 hybridoma proteins	406
Bence-Jones protein Den	Human	Complete sequence, 214 residues	407
Bence-Jones protein NIG-58	Human	Complete sequence, 213 residues	408
Cryoglobulin McE (monoclonal IgM)	Human	Sequence of variable region of H-chain, 125 residues	409
Disease protein CHA	Human	*N*-Terminal sequence, 39 residues	410
H-2D^b histocompatibility antigen	Mouse	*N*-Terminal sequence, 98 residues, by radiosequencing	411
H-2D^d histocompatibility antigen	Mouse	Sequence of CNBr peptide CN–C, 75 residues, by radiosequencing	412
H-2K^d histology compatibility antigen	Mouse	*N*-Terminal sequence, 98 residues, by radiosequencing	413
H-2K^b histocompatibility antigen	Mouse	Complete sequence, 346 residues, by radiosequencing	414—417
HLA, DW2,2; DR2,2 histocompatibility antigen	Human	*N*-Terminal sequence of β-chain, 198 residues	418

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
I-A and I-E, Dr-like molecules	Marmoset (cell line GM 3158)	Partial *N*-terminal sequence of α- and β-chains of each molecule	419
Immunoglobulins, λ-chains	Rabbit	Partial sequence of variable and constant regions from different sources	420
Immunoglobulins, λ-chain	Rabbit	Sequence of constant region, 106 residues	421
Immunoglobulin, L-chain	Rabbit–mouse hybridomas	Sequence of constant region of λ_3-chain, 95 of 110 residues	422
Immunoglobulin, L-chain	Rabbit–mouse hybridomas	Partial sequence of L-chains from cell lines 12F2 and 1D4P5	423
Immunoglobulin, allotype Iglb	Mouse	Complete sequence of γ2a H-chain of CBPC-101 Fc region, 216 residues	424
Immunoglobulin Smλ	Human	Complete sequence of L-chain, 135 residues, carbohydrate moiety at Ser-21 and Asn-25	425
Immunoglobulin Sac	Human	*N*-Terminal sequence of H-chain, 20 residues	426
β_2-Microglobulin	Mouse	Complete sequence, 99 residues	427
Myeloma protein Goe	Human	Partial sequence of Fc region, 118 residues	428
Myeloma protein N1G-65 (IgD, δ-chain)	Human	Complete sequence of Fc region, 266 residues	429
Myeloma protein WAH (IgD)	Human	Complete sequence of Fc region, 226 residues, sequence of first constant and hinge regions of δ H-chain, 207 residues	430 431
Myeloma C.C58 L-chains	Mouse	*N*-Terminal sequence of 3 L-chains, M75, F171, and F67, 49 residues each	432

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Histones			
H1	Bovine	*N*-Terminal sequence of subfraction CTL-1, 104 residues	433
$H1^0$	Bovine	Sequence of a *N*-bromosuccinimide cleavage peptide, 37 residues	434
H3	Human	Complete sequence, 135 residues	435
H3 and H4	Chicken	*N*-Terminal sequence of limit peptides of H3 and H4	436
H3 and H4	Calf	Localization of the sites of *in vitro* phosphorylation	437
H5	Chicken	Localization of 5 phosphorylation sites by cAMP-dependent protein kinase	438
Histone-like protein (HTa)	*Thermoplasma acidophilum*	Complete sequence, 89 residues	439 440
Hormones			
Angiotensinogen	Rat	*N*-Terminal sequence, 17 residues	441
Angiotensinogen	Human	*N*-Terminal sequence, 25 residues	442
Chorionic gonadotropin	Human	Assignment of the 6 disulphide bonds of the β-subunit	443
Choriogonadotropin	Human	Sequence of leader peptide of α- and β-subunits, 24 and 20 residues, respectively	444
Corticotropin-releasing factor	Sheep	Complete sequence, 141 residues	445 446
Dermorphin	Frog	Sequence of opiate-like heptapeptide	447

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Dynorphin	Porcine	Sequence of heptadecapeptide	448
β-Endorphin	Horse	Complete sequence, 31 residues	449
β-Endorphin	Ostrich	Complete sequence, 31 residues	450
Enkaphalin-containing peptides	Bovine	Sequence of 2 opioid polypeptides, 30 and 39 residues	451
Follitropin	Ovine	Sequence of α-subunit, 96 residues, based on homology with lutropin	452
Follitropin	Ovine	Complete sequence of β-subunit, 111 residues	453
Growth hormone (20 000-molecular-weight variant)	Human	*N*-Terminal sequence, 38 residues	454
Hypothalamic peptide	Frog	Sequence of the decapeptide HS2D4	455
β-Lipotropin	Human	Reinvestigation of *N*-terminal sequence, 60 residues	456
β-Lipotropin	Human	Complete sequence, 89 residues	457
β-Lipotropin	Ostrich	Complete sequence, 79 residues	458
Melanocyte-stimulating hormone	Dogfish	Sequence of γ-MSH, 12 residues	459
α-Neo-endorphin	Porcine	Complete sequence, 10 residues	460
β-Neo-endorphin	Porcine	Complete sequence, 9 residues	461

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Nerve-growth factor	Mouse	Complete sequence of γ-subunit, 233 residues, assignment of 5 disulphide bonds and one *N*-linked carbohydrate moiety	462
Neuropeptide	Human, bovine, and rat	Sequence of an undecapeptide, identical in all species	463
Neurophysin VLDV	Horse	Complete sequence, 93 residues	464
Neurophysins MSEL and VLDV	Rat	Complete sequence of both, 93 residues each	465
Neurophysins	Rat	*N*-Terminal sequence of VP-RNP, OT-RNP, and OT-RNP', 44, 40, and 69 residues, respectively	466
Neurophysins	Human, ovine, and guinea pig	Complete sequence of hNP-I, 93 residues, *N*-terminal sequence of hNP-II, oNP-I and -II, and gNP-I and -II	467
Preproinsulin	Anglerfish	Sequence of leader peptide, 24 residues	468
Preproinsulin	Atlantic hagfish	Partial sequence of leader peptide, 25 residues	469
Pro-adrenocorticotropin/endorphin	Mouse	Sequence of *N*-terminal of 16 K fragments, 29 residues, and γ-lipotropin, 38 residues	470
Pro-opiocortin	Salmon	Complete sequence, 76 residues, assignment of 2 disulphide bonds	471
Pro-γ-melanotropin	Porcine	Sequence of a novel fragment, 30 residues	472
Pro-opiomelanocortin	Human	Complete sequence of maturation product, 76 residues, assignment of 2 disulphide bonds	473—475

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Pro-opiomelanocortin	Mouse	Partial sequence of leader peptide	476
Pro-opiomelanocortin	Porcine	*N*-Terminal sequence, 80 residues	477
Pro-opiomelanocortin	Rat	Partial sequence of various forms	478
Relaxin	Sand-tiger shark	Complete sequence of A-chain, 24 residues, partial sequence of B-chain, 18 residues	479
Secretin	Bovine	Complete sequence, 27 residues	480
Secretory protein I (parathyroid)	Bovine	Partial *N*-terminal sequence, 20 residues	481
Somatostatin-like peptide	Ovine	Sequence of somatostatin, 28 residues, and of a major component of somatostatin fraction, 30 residues	482
Somatostatin-14	Catfish	Complete sequence, 14 residues	483
Somatostatin	Human and porcine	*N*-Terminal sequence, 20 residues each	484
Sperm-activating peptides	Sea urchin	Sequences of 2 different peptides, 9 and 10 residues	485
Tremerogen A-10 and a-13	*Tremella mesenterica*	Sequence of both peptide sex hormones, 12 and 13 residues, respectively	486
Ribosomal Proteins			
Acidic ribosomal protein MA1	*Micrococcus lysodeikticus*	Complete sequence, 118 residues	487
Acidic ribosomal protein YPA1	Yeast	Complete sequence, 110 residues	488
Ribosomal protein BL10	*Bacillus stearothermophilus*	Complete sequence, 177 residues	489

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Ribosomal protein S2	*E. coli*	Complete sequence, 240 residues	490
Structural Proteins			
Collagen, type III	Human	Sequence of CNBr fragment α1 (III)-CB9, 236 residues, complete sequence of human type-III collagen, 1023 residues	491
Collagen, type IV	Human (placental basement membrane)	*N*-Terminal sequence of several peptic peptides	492
Preprocollagen, type I	Chicken	Partial *N*-terminal sequence of pro-α1(I) and pro-α2	493
Tropoelastin b	Chicken	Sequence of leader peptide, 24 residues	494
Tropoelastin	Chicken	*N*-Terminal sequence of a tryptic peptide, 73 residues	495
Toxins			
Cholera toxin	*Vibrio cholerae*	*N*-Terminal sequence of precursor of A-subunit, 8 residues	496
Cholera toxin	*Vibrio cholerae*	Complete sequence of γ-chain of A-subunit, 46 residues	497
Heat-stable enterotoxin	*E. coli*	Complete sequence, 18 residues	498
Neurotoxin	Common-death adder	Complete sequence of the short and long chains, 62 and 73 residues, respectively	499 500
Peptide toxins	Marine snail	Sequence of 3 toxic peptides, GI, GIA, and GII, 13, 15, and 13 residues, respectively	501
Toxins B-II and B-IV	Heteronemertine	Complete sequence of B-II, 55 residues, structure revision of B-IV	502

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Toxin D	Indian cobra	Complete sequence, 71 residues	503
Viral Proteins			
Capsid proteins C and D	Phage M13	Partial *N*-terminal sequence	504
Core proteins	Semliki forest virus and sindbis virus	Complete sequence, 267 and 264 residues, respectively	505
DNA-helix-destabilizing protein (32p)	Bacteriophage T4	Complete sequence, 301 residues	506
Gag gene proteins p10, p12, p15, and p30	Baboon and cat type-c viruses	*N*-Terminal and *C*-terminal sequence	507
Gag gene protein p15	Avian myeloblastosis	Complete sequence, 124 residues	508
Gene 0.3 protein	Bacteriophage T4	Complete sequence, 116 residues	509
Glycoprotein ERA-G	Rabies virus	Short *N*- and *C*-terminal sequence	510
Haemagglutinin	Influenza virus [A/Japan 305/57 (H2N1)]	Assignment of 5 disulphide bonds	511
Haemagglutinin	Influenza virus [A/duck/Ukraine/1/63 (Hav 7)]	Sequence of HA1-chain, 328 residues, partial sequence of HA2-chain, 192 residues	512
Haemagglutinin	Influenza virus [A/Aichi/2/68 (X-31)]	Sequence of HA1-chain, 328 residues, partial sequence of HA2-chain, 188 residues	513
Hepatitis B protein	Hepatitis virus	*N*-Terminal sequence of p-25 and gp-30, 30 residues each	514
Hexon protein	Adenovirus	Complete sequence, 966 residues	515—518

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Low-m.w. nucleic acid-binding proteins	Leukaemia viruses	Complete sequence of Rausher strain p10, 56 residues, partial sequence of 6 other MuLV p10s	519
Major coat proteins	Filamentous bacterial virus, If1 and Ike	Complete sequence of protein from If1 and Ike, 51 and 53 residues, respectively	520
MoMuLV envelope polypeptide	Moloney murine leukaemia virus	Partial *N*-terminal sequence of PR15E	521
Poliovirus replication proteins	Poliovirus	Partial *N*-terminal sequence of polypeptides P3-1b, P3-2, P3-7c, and P3-4b	522
Poliovirus P-2 products	Poliovirus	Partial sequence of P2-3b, P2-5b, and P2-X	523
SBMV coat protein	Southern bean mosaic virus	Sequence of *N*- and *C*-terminal peptides, 3 and 9 residues, respectively	524
SFV protein ns86	Semliki forest virus	Partial *N*-terminal sequence of ns86	525
SFV protein P62 and E3	Semliki forest virus	Partial *N*-terminal sequence	526
STNV coat protein	Satellite tobacco necrosis virus	Near-complete sequence, identical to nucleotide sequence	527
SV40 agnogene product	Simian virus 40	Partial *N*-terminal sequence	528
Miscellaneous Peptides and Proteins			
α_1-Acid glycoprotein	Rat	*N*-Terminal sequence of protein from serum and intracellular precursor form, 14 residues each	529
Amelogenin polypeptide	Bovine	Complete sequence of TRAP and LRAP, 45 and 46 residues, respectively	530

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
cAMP receptor protein	*E. coli*	*N*-Terminal sequence of 2 large chymotryptic peptides	531
α-Amylase inhibitor (Hoe-467A)	*Streptomyces tendae*	Complete sequence, 74 residues, assignment of 2 disulphide bonds	532
Amyloid-fibril protein AR	Human	Complete sequence, 154 residues	533
Amyloid protein L1N	Human	*N*-Terminal sequence, 20 residues	534
Anionic proline-rich proteins	Human and monkey	Sequence of active tryptic peptides of 4 human and 1 monkey proteins	535
α_1-Antitrypsin	Human	Assignment of 3 carbohydrate-moiety attachment sites	536
α_1-Antitrypsin	Rat	Partial sequence of leader peptide	537
Apoferritin	Horse	Complete sequence, 174 residues	538
Apolipoprotein A-I (HDL)	Rat	*N*-Terminal sequence, 7 residues	539
Apoprotein E	Human	Cys/Arg interchanges in 3 Apo-E isoforms	540
Bacillomycin D	*Bacillus subtilis*	Sequence of cyclic heptapeptide	541
Calcium-binding protein	Bovine	Complete sequence, 75 residues	542
Calmodulin	Human	Sequence of a half-molecule fragment, 77 residues	543
Calmodulin	Scallop	Complete sequence, 148 residues	544
Calmodulin	*Tetrahymena pyriformis*	Complete sequence, 147 residues	545

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Carboxypeptidase inhibitor	Tomato	Complete sequence, 37 residues	546
Cecropin A and B	Cecropia moth	Complete sequence of both proteins, 37 residues each	547
Ceruloplasmin	Human	Sequence of fragment CpF4, 405 residues	548
Colipase	Chicken	*N*-Terminal sequence, 39 residues	549
Colipase B	Horse	Complete sequence, 96 residues	550
Crambin	*Crambe abyssinica*	Complete sequence, 46 residues	551
α-Crystallin	Aardvark and manatee	Complete sequence of both proteins, 173 residues	552
Desmin and vimentin	Chicken and pig	Sequence of *C*-terminal fragment of chicken desmin, pig desmin, and virmentin, 140, 138, and 139 residues, respectively	553
Elongation factor G	*E. coli*	Sequence of fragment T5, 228 residues	554
Elongation factor Tu	*E. coli*	Complete sequence, 393 residues	555 556
Elongation factor Tu	Rabbit	*N*-Terminal sequence of fragment eEF-Tu^1, 12 residues	557
Ferric pseudobactin	*Pseudomonas* B10	Sequence of hexapeptide and *X*-ray structure	558
Fimbrial protein (K88 antigen)	*E. coli*	Complete sequence, 264 residues	559
Fulvocin C	*Myxococcus fulvus*	Complete sequence, 45 residues	560
D-Galactose-binding protein	*E. coli*	Complete sequence, 309 residues	561

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Gap-junction protein	Rat	*N*-Terminal sequence, 52 residues	562
Glial fibrillary acidic protein	Calf	*N*-Terminal sequence of a CNBr peptide, 28 residues	563
α_{2u}-Globulin	Rat	Partial *N*-terminal sequence, 28 residues	564
α_{2u}-Globulin	Rat	Partial sequence of leader peptide	565
Glycinin	Soybean	Partial sequence of 5 acidic peptides	566
Glycinin	Soybean	Identification of acidic and basic subunit complexes	567
Glycoprotein (alveolar)	Human	Partial sequence of several peptides, 74 residues	568
G-Substrate (for cGMP-dependent protein kinase)	Rabbit	Sequence around 2 phosphorylation sites, 31 and 29 residues	569
High-mobility-group protein (HMG-T)	Trout	Partial sequence, 174 residues	570
High-mobility-group protein (HMG)	Mouse	*N*-Terminal sequence, 9 residues	571
High-mobility-group protein (HMG)	Duck	Identification of the acetylation sites in HMG-14 and -17	572
High-potential iron–sulphur proteins	*Thiocapsa roseopersicina* and *Chromatium gracile*	Sequence of both proteins, 85 and 82 residues, respectively	573
Histidine-binding protein	*Salmonella typhimurium*	Complete sequence, 238 residues	574
Interferon (fibroblast)	Human	Partial *N*- and *C*-terminal sequence	575

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Interferon (leukocyte)	Human	Sequence of interferon species α_2 and β_1, 155 residues	576
Interferon A (leukocyte) (IFN-αA)	Human	Assignment of 2 disulphide bonds	577
Intestinal peptide PH1	Porcine	Complete sequence, 27 residues	578
Lactotransferrin	Human	Partial sequence, alignment of CNBr fragments	579
Legumin	Pea	*N*-Terminal sequence of α^M-subunit, 25 residues	580
Lentil lectin	Lentil	Complete sequence of the β-chain, 159 residues	581
Light harvesting protein	*Rhodospirillum rubrum*	Complete sequence, 52 residues	582
α_2-Macroglobulin	Human	Sequence around the putative transglutaminase crosslinking site and the 'bait' region	583—585
Metallothionein-II	Mouse	Complete sequence, 61 residues	586
Metallothionein	Crab	Complete sequence, 58 residues	587
Membrane receptor for glycoprotein	Chicken	Complete sequence, 207 residues	588
α_1-Microglobulin	Human	Complete sequence, 167 residues, assignment of 3 carbohydrate-moiety attachment sites	589
Mithochondrial ADP, ADP carrier protein	Bovine	Sequence of *C*-terminal region, 94 residues	590
Mitochondrial ATPase inhibitor	Yeast	Complete sequence, 63 residues	591

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Mitogenic lectin	Plant	Complete sequence of α-subunit, 52 residues	592
Morphogenic peptides	*Anthopleura elegantissima* and *Hydra attenuata*	Sequence of peptide from both sources, 11 residues each	593
Multiplication-stimulating activity peptide	Rat (liver-cell culture)	Complete sequence, 67 residues	594
Myosin, L-chain	Chicken	Complete sequence of L-4, 150 residues	595
Myosin, low-m.w. L-chain	Chicken	Complete sequence, 171 residues	596
Myosin, L-chain	Chicken	Complete sequence of L-1, 190 residues	597
Myosin, L-chains	Chicken	Complete sequence of cardiac L-2A, L-2B, and gizzard 17K L-chains, 163 163, and 150 residues, respectively	598
Nucleolar protein B23	Novikoff hepatoma ascites cells	Sequence of peptide containing phosphorylation site, 5 residues	599
Ovalbumin	Birds	Sequence of 16 phosphoserine peptides from 8 species	600
Ovalbumin	Chicken	Complete sequence, 385 residues, carbohydrate moiety at Asn-292, Ser-68 and 344 are phosphorylated	601
Osteocalcin	Chicken	Complete sequence by gas chromatography–mass spectrometry, 50 residues	602
P2 myelin basic protein	Bovine	Sequence of *C*-terminal segment, 18 residues	603
Pancreatic polypeptide	Dog	Sequence of an icosapeptide	604
Peptidoglycan-associated lipoprotein	*Proteus microbilis*	*N*-Terminal sequence, 13 residues	605

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Phage F22 repressor protein	*Salmonella*	Complete sequence, 216 residues	606
Phosphatidylcholine transfer protein	Bovine	Complete sequence, 212 residues	607
Pilin	*Caulobacter crescentus*	*N*-Terminal sequence, 32 residues	608
Post γ-globulin	Human, dog	Partial sequence of protein from both sources	609
Protamine Z3	Dogfish	Complete sequence, 31 residues	610
Protein B of colicin E3	*E. coli*	Complete sequence, 84 residues	611
Protein HC (complex forming)	Human	Complete sequence, 181 residues, assignment of a disulphide bond	612
Proteinase inhibitor	Acacia tree	*N*-Terminal sequence of a- and b-chains, 11 and 16 residues	613
Proteinase inhibitor (DE-1)	Weeping wattle	Complete sequence, 162 residues	614
Proteinase inhibitor (DE-3)	Broad-leaved erythrina	*N*-Terminal sequence, 20 residues	615
Proteinase inhibitors I-A and I-A′	Adzuki beans	Sequence of I-A and I-A′, 78 and 72 residues, respectively	616
Proteoglycan	Human, porcine, and shark	Sequence of peptides from various proteoglycans	617
Pseudobactin A	*Pseudomonas* B10	Structure by comparison with ferric pseudobactin	618
Retinoid-binding protein	Bovine	*N*-Terminal sequence of 3 proteins, 29 residues each	619
Retinoid-binding protein	Rat	*N*-Terminal sequence, 33 residues	620
Rhodopsin	Bovine	Partial sequence of a hydrophobic region, 50 residues	621

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Table 2 (*cont.*)

Protein	*Origin*	*Comments*	*Ref.*
Rhodopsin	Bovine	Sequence of *C*-terminal third of the protein, 109 residues, localization of retinal-binding and phosphorylation sites	622
S-100a protein	Bovine	Complete sequence of α-subunit, 93 residues	623
Sauvagine	Frog	Complete sequence, 40 residues	624
Steroid-binding protein (component C3)	Rat	Complete sequence, 77 residues, carbohydrate moiety at Asn-17	625
Thy-1 glycoprotein	Rat	Complete sequence, 111 residues, assignment of 2 disulphide bonds and 3 *N*-linked carbohydrate attachment sites	626
Thymopoietins I, II, and III	Bovine	Complete sequence of TP-I, TP-II (revision), and TP-III, 49 residues each	627
Thymosin β4	Bovine	Complete sequence, 43 residues	628
Trypsin inhibitor III	Squash	Complete sequence, 28 residues	629
Trypsin inhibitor B	Black mamba	Complete sequence, 57 residues	630
Trypsin inhibitor HI-30	Human	Complete sequence, 143 residues, assignment of 2 carbohydrate attachment sites	631
Trypsin inhibitor (low m.w.)	Human	*N*-Terminal sequence, 8 residues	632
α-Tubulin	Porcine	Complete sequence, 450 residues	633
β-Tubulin	Porcine	Complete sequence, 445 residues, positions 37, 48, 57—59, and 275 are heterogeneous	634

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PART IC: Chemical Modification of Proteins *By R. Cassels and R. A. G. Smith*

1 Introduction

A summary of modified proteins is given in the Table. As in previous reports, this list is intended to be comprehensive, but simple radiolabelling experiments, prosthetic-group modifications, and routine ELISA coupling techniques have been excluded.

Table *Chemical modification of proteins*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Acetate kinase	*E. coli*	2,3-Butanedione	Arg	Evidence for essential Arg residue	1
Acetylcholine receptor (membrane bound)	*Torpedo californica*	Various thiol/disulphide reagents	Cys	Disulphide reduction alters Na^+ permeability	2
Acetylcholine receptor	*Torpedo californica*	5-(Iodoacetamido)salicylic acid	—SH	Fluorescence probe	3
Acetylcholine receptor	*Electrophorus electricus*	Dimethyl suberimidate	(Lys)	Determination of mol. wt. by PAGE	4
	Torpedo marmorata	Glutaraldehyde/EDC	(Lys)		
Acetylcholine receptor	Cat skeletal muscle	Bromo[^{3}H]acetylcholine		Receptors from different sources compared	5
Acetylcholine receptor	*Torpedo marmorata*	5-Azido[^{3}H]trimethisoquin		Labelling of δ- and α-subunits	6
Acetylcholinesterase	*Electrophorus electricus*	Methyl(acetoxymethyl)nitrosamine		Photosuicide alkylation (enzyme participation)	7
Acetylcholinesterase	*Torpedo californica, Electrophorus electricus*	*N*-Dansylaziridine	Cys	Groups exposed by Zn^{2+} or Cd^{2+}	8
Acetylcholinesterase	*Torpedo californica*	(Pyrenebutyl)methylphosphonyl fluoride	Ser	Fluorescence probe of binding to peripheral sites	9
Acetylcholinesterase	Human erythrocytes	DCCI, CMC		Irreversible inhibition	10
N-Acetyl transferase	Rat pineal gland	Cystamine	Disulphide bonds	Disulphide interchange inactivation	11

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Acid phosphatase	Bovine liver	Iodoacetate, Rose Bengal/light		Essential Cys	12
Actin, myosin subfragment-1	Rabbit muscle	4-(*N*-Maleimido)benzyltrimethyl-ammonium chloride	Cys-373	G-Actin modified	13
F-Actin	Rabbit skeletal muscle	*N*-(1-Pyrenyl)iodoacetamide	Cys	Fluorescence probe of actin–meromyosin interaction	14
F-Actin	Rabbit skeletal muscle	Phenylenebismaleimide	Cys	Characterization of actin dimer	15
Actin		$[^3H]CH_2O + NaBH_3CN$	Lys	Reactivity change on polymerization	16
Actinidin, papain	*Actinida chinensis, Carica papaya*	n-Propyl 2-pyridyl disulphide, 4-(*N*-aminoethyl)-2′-pyridyl disulphide, 7-nitrobenzo-2-oxa-1,3-diazole, 4-chloro-7-nitrobenzofurazan	Cys	Active-site reactivity probes	17
Acyl carrier protein	*E. coli* K-12	*N*-Acylimidazole	Cys	Specific Cys modification allows chemical alternative to enzymatic synthesis of acyl carrier proteins	18
Adenine nucleotide carrier	Beef-heart mitochondria	2-Hydroxy-5-nitrobenzyl bromide 2,3-Butanedione, phenylglyoxal	Trp Arg	Atractyloside- and bongkrekic acid-binding sites modified	19
5-Adenosyl homocysteine hydrolase	Bovine liver	Various nucleoside analogues		Irreversible inhibition by different mechanisms	20
5-Adenosyl homocysteine hydrolase	Human erythrocytes	5′-Deoxy-5′-methylthioadenosine		Enzyme inactivation by unknown mechanism	21
Adenylate cyclase	Rabbit heart	*p*-Aminophenyl arsenoxide, *p*-aminophenyl dichloroarsine		Essential SH	22
Adenylate kinase	Pig muscle	3-(4-Azido-2-nitrobenzoylseleno) propionic acid	Cys-25, $-NH_2$	New photo-activatable cross-linking reagent	23

ADP/ATP carrier protein	Rat-heart mitochondria	*N*-Ethylmaleimide	—SH	Investigation of relationship between binding sites	24
		Butanedione	Arg		
		N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline	$-CO_2H$		
		2-Hydroxy-5-nitrobenzyl bromide	Trp		
ADP-glucose synthetase	*E. coli*	TNBS	Lys	Effect on allosteric properties	25
ADP-ribosylated histone H1	Ehrlich ascites tumour	*N*-Bromosuccinimide	Trp	Location of ribosylated residues	26
β-Adrenergic receptor	Duck erythrocyte membrane	(±)-1-(Indol-4-yloxy)-3-[1-(*p*-azido-*m*-iodophenyl)-2-isobutylamino]-2-propanol		Identification of receptor protein	27
D-Ala-D-Ala peptidases	*Streptomyces* R61, *Actinomadura* R39	Various β-lactams		Short- and long-lived intermediates	28, 29
Alanine dehydrogenase	*Bacillus natto*, KMD 1126	TNBS	$-NH_2$	Modification of $-NH_2$ in the presence of —SH	30

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Alanine racemase	*E. coli* B	ββ-Difluoroalanine, βββ-trifluoroalanine		Suicide substrates	31
Alanine tRNA synthetase	*E. coli*	Dimethyl suberimidate	Lys	α_4-Tetrameric structure	32
Albumin	Human serum	5′-*O*-[Methyl-(4-phenylimidate)]uridine		Uridine conjugates	33
Albumin	Bovine serum	Hydrogen peroxide	Cys, Met	Gliclazide-binding site	34
		Iodacetic acid	Cys, Met, His		
		Iodoacetamide	Cys, Met, His		
		2-Hydroxy-5-nitrobenzyl chloride	Trp		
Albumin	Bovine serum	2-Imino-2-methoxyethyl-1-thioglycopyranoside		Preparation of neoglycoprotein	35
Albumin	Human serum	(±)-2-Methylpenem-3-carboxylic acid	Lys-195	Destruction of β-lactam ring by albumin drug-binding site	36
Albumin	Bovine serum	*N*-(3-Pyrene)maleimide	Cys	Lacks specificity	37
Albumin	Bovine serum	Ranitidine, thioether analogue	Lys	Preparation of immunogen for radioimmunoassay	38
Albumin	Bovine serum	*p*-Chloromercuribenzoic acid	Cys	Effects on tolbutamide binding	39
		p-Aminophenylmercuric acetate	Cys		
		p-Hydroxynitrobenzyl bromide	Trp		
Albumin	Bovine	2-Nitro-4-azidophenylsulphenyl-ACTH		Identification of corticotropin-binding domain	40
Albumin	Bovine serum	6β-Hydroxycortisol 21-hemisuccinate/isobutylchlorocarbonate	(Lys)	Immunoassay conjugates	41
Albumin	Bovine	5-*N*-Succinylamino-aspirin/EDC		Aspirin-hypersensitivity study	42
Albumin	Bovine serum	Amanitinylazobenzoyl-*N*-glycylglycine/carbodi-imide		Conjugation increases cytotoxicity	43

Albumin	Bovine serum	Glutaraldehyde		Polymerized albumin entraps drugs	44
Albumin	Bovine	*S*-Acetylmercaptosuccinic anhydride		Use in coupling to derivatized daunomycin	45
Alcohol dehydrogenase	Horse liver	(*R,S*)-Bromo-3-(5-imidazolyl) propionic acid, iodoacetic acid	Cys-46	Affinity labelling of Zn-binding site	46
Alcohol dehydrogenase	Horse liver	Bromoimidazolyl propionate, haloacetate, halopropionate	Cys-46	Selective alkylation	47
Alcohol dehydrogenase	Horse liver	3-Chloroacetylpyridine-adenine dinucleotide		Alkylates active-site residue	48
Alcohol dehydrogenase	Horse liver	Haloacetate, halopropionate, halobutyrate	Cys-46	Effect of imidazole on affinity alkylation	49
Aldehyde dehydrogenase	Sheep liver	Disulphiram		Glutathione does not protect	50

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Aldehyde reductase	Pig liver	Phenylglyoxal	Arg	Specific modification suggests anion-binding sites	51
		p-Carboxyphenylglyoxal	Arg		
Aldolase	Rabbit muscle, human erythrocyte	Di-isoethionyl-3,3′-dithiobis-propionimidate		New membrane-impermeable cleavable crosslinking reagent	52
Aldolase	*Saccharomyces cerevisiae*	Diethylpyrocarbonate	His	N.m.r. monitoring of His modification	53
Aldolase	Rabbit muscle	Methyl acetimidate	Lys	Reaction conditions investigated	54
Aldolase	Rabbit liver	1-Chloro-2,6-dinitrobenzene			55
	Rabbit muscle	1-Chloro-2,4-dinitrobenzene, 4-hydroxymercuribenzoate, pyridoxal phosphate			
Alkaline phosphatase	Human placenta	Periodate-oxidized AMP/$NaBH_4$	Lys	1.5 moles incorporated per mole enzyme	56
Alkaline phosphatase	*E. coli*	Ethoxyformic anhydride	His	3 His residues implicated in Zn^{2+} binding	57
Alkaline phosphatase	Human placenta	Butanedione, phenylglyoxal, methylglyoxal	Arg	Phosphate-binding site labelled	58
Allergen Ra3	Ragweed	Mercaptoethanol/iodoacetic acid		Role of thiol and disulphide studies	59
Alveolysin	*Bacillus alvei*	DTNB, TLCK	(Cys)	One essential thiol	60
D-Amino-acid oxidase	Pig kidney	1,2-Cyclohexanedione	Arg	Role of arginine in substrate binding	61
Aminoacyl tRNA synthetases	*E. coli*	Periodate-oxidized ATP, GTP, and uridine		Partial reaction inhibited	62
4-Aminobutyrate aminotransferase	Rabbit brain	Ethanolamine *o*-sulphate	(Lys)	Suicide inhibition *via* β-elimination	63
4-Aminobutyrate aminotransferase	Pig brain	*o*-Phthalaldehyde	Lys	Specific inactivation	64
γ-Aminobutyric acid–α-ketoglutaric acid aminotransferase	Pig brain	(*S*)-4-Amino-5-halopentanoic acids		Every turnover gives inactivation	65

γ-Aminobutyric acid–α-ketoglutarate transaminase	Mouse	4-Amino-5-fluoropentanoic acid		Inactivation *in vivo*	66
5-Aminolevulinic acid dehydratase	Bovine liver	5-Halolevulinic acids	Cys	Role of active-site —SH in catalysis	67
5-Aminolevulinic acid dehydratase	Bovine liver	DTNB	Cys	Mechanistic role of Cys residues	68
Aminopeptidase	Japanese apricot (*Prunus mune sieb.*)	*p*-Chloromercuribenzoate, diethyl pyrocarbonate	Cys, His	Characterization of activity	69
AMP deaminase	Rat muscle	Tetranitromethane	Cys, Tyr	Partial and complete inactivation	70
AMP receptor proteins	HeLa cells	8-Azido[^{32}P] cyclic AMP		Protein characterization	71

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
AMP receptor protein	*Coprinus macrorhizus*	8-Azidoadenosine-3′,5′-monophosphate		Identification and characterization of receptor	72
Anion transporter (band 3)	Human erythrocytes	4-Benzamido-4′-isothiocyanostilbene-2,2′-disulphonate, 2[*N*-(4-azido-2-nitrophenyl)amino] ethanesulphonate		Fluorescence study of labelled-site relationship	73
Anion transporter (band 3)	Human erythrocytes	4-Benzamido-4′-isothiocyanostilbene-2,2′-disulphonate		Fluorescent label	74
Anion transporter	Human erythrocytes	1,2-Cyclohexanedione	Arg	Inhibition of transport	75
Anion transporter (band 3)	Human erythrocytes	*N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide	Cys	E.s.r. probe of conformational changes	76
Anisotropic inhibitor binding protein	Rat-liver mitochondria	Ethidium monoazide		Receptor location	77
Anthranilate synthase component I	*Serratia marcescens*	Various reagents	Arg, His, Cys	Location of essential residues	78
Anti-*p*-azobenzenearsonate antibodies	Guinea pig	*N*-(Bromoacetyl)-β-[(*p*-arsonophenyl)azo]-L-tyrosine, *N*-(bromoacetyl)-*p*-arsanilic acid	Tyr, Lys	Both chains labelled	79
Antibodies	Various	Horseradish peroxidase/periodate/$NaBH_4$		Histochemical applications of conjugates	80
Anti-DNP IgG	Rabbit	Bis-(α-bromoacetyl-ε-DNP-Lys-Pro) ethylenediamine		Preparation of affinity crosslinked IgG dimers	81
Anti-β2-microglobulin	Human lymphoblasts, human urine	4-(6-Formyl-3-azidophenoxy) butyrimidate	Lys	Stepwise heterobifunctional chemical/photochemical crosslinker	82
Anti-pilin antibody ferritin	*Caulobacter crescentus*	Glutaraldehyde		Ferritin-labelled antibody for histology	83
Apo-E high-density lipoproteins	Canine plasma	Cyclohexanedione	Lys, Arg	Investigation of receptor binding	84
Apolipoprotein E	Rat serum	Iodine monochloride		Radiolabelling to produce lipoprotein separation probe	85

Arginine decarboxylase	*E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*	DL-α-(Difluoromethyl)arginine		Suicide inhibitor	86
Aromatase	Human placenta	Various allenic and acetylenic sterols		Suicide inhibition	87
Asparaginase, glutaminase	*E. coli, Erwinia carotovora, Achromobacter*	Ethyl acetimidate, *O*-methyl isourea, succinic anhydride, formaldehyde/borohydride	$-NH_2$	Improvement of therapeutic persistence in plasma	88
Aspartase	*E. coli*	Dimethyl suberimidate	$-NH_2$	Subunit arrangement	89
Aspartate aminotransferase	Porcine heart	Pyridoxal 5-phosphate	Lys-258	Coenzyme role in stability	90
Aspartate aminotransferase	Rat hepatocytes	L-Cycloserine		Mitochondrial and cytosolic enzymes both inactivated	91

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Aspartate transcarbamoylase	*E. coli*	*p*-Hydroxymercuribenzoate	Cys	Subunit dissociation	92
Aspartate transcarbamoylase	*E. coli*	Tartryl diazide	$-NH_2$	Allosteric role of catalytic–regulatory subunit reactions	93
Aspartate transcarbamoylase	*E. coli*	Tetranitromethane	Tyr	Investigations of co-operativity	94
Aspartate transcarbamoylase	*E. coli*	Tartryl diazide, *p*-hydroxy-mercuribenzoate	$-NH_2$, Cys	Co-operativity of crosslinked subunits	95
Aspartyl proteinases	*Penicillium roqueforti* and others	Butane-2,3-dione + light	Trp, Tyr	Photosensitized modification	96
Aspergillopepsin A	*Aspergillus awamori*	*p*-Nitrophenyldiazonium chloride	Tyr-75, Tyr-189	Pepstatin protects one Tyr	97
Aspirinase	Guinea-pig liver	Bis-(4-nitrophenyl)hydrogen phosphate, various thiol reagents	Ser	Thiol-dependent carboxylesterase	98
ATPase	Beef-heart mitochondria	Phenylglyoxal 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole	Arg Tyr	Role of Tyr in ATP hydrolysis	99
ATPase	Sarcoplasmic reticulum	Maleimide spin label	Cys	E.s.r. study of protein–lipid interactions	100
ATPase	Sarcoplasmic reticulum	Various maleimide spin labels	Cys or (Lys)	E.s.r. probe for conformational studies	101
ATPase	Canine renal medulla	*N*-Ethylmaleimide	Cys	Activity studies	102
ATPase	*Saccharomyces cerevisiae* mitochondrion	Dithiobis(succinimidyl)propionate	Lys	Probe of subunit structure	103
ATPase	*E. coli*	Arylazido-adenine nucleotides		Differential subunit photolabelling	104
ATPase	Rabbit sarcoplasmic reticulum	*N*-(1-Anilinonaphthyl-4)maleimide	Cys	Fluorescence probe of phosphorylation	105

Ca^{2+}-ATPase	Rabbit muscle	*N*-[2-(16-doxylstearoyl)ethyl]-maleimide	Cys	Spin label indicates oligomeric structure	106
(Ca^{2+}, Mg^{2+})-ATPase	Sarcoplasmic reticulum	*N*-Ethylmaleimide	Cys	Labelling of 10—11 Cys	107
(Ca^{2+}, Mg^{2+})-ATPase	*E. coli*	2′,3′-Dialdehyde ADP and ATP		Some non-specific labelling	108
Ca^{2+}-ATPase	Rabbit sarcoplasmic reticulum	Fluorescein isothiocyanate	Lys	Phosphorylated and non-phosphorylated intermediates	109
Ca^{2+}-ATPase	Rabbit sarcoplasmic reticulum	Fluorescein isothiocyanate	Lys	Affinity probe	110
(Na^+, K^+)-ATPase	Porcine kidney	Various ATP analogues, chloromethylphosphonic acid		Inactivation of ATP- and P_i-binding sites	111
(Na^+, K^+)-ATPase, oligomycin-sensitive ATPase, cytochrome C oxidase	Porcine kidney, bovine mitochondria	Various arylazido phospholipids		Photolabelling in reconstituted membranes	112

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
$(Na^+ + K^+)$-ATPase	Canine kidney	Chromium arylazido-β-alanyl ATP		ATP-binding sites	113
H^+-ATPase ($F_0.F_1$)	*Thermophilic bacterium* PS3	Iodine	Tyr	Involvement of tyrosine in H^+ transport	114
F_1-ATPase	Mitochondria	TNBS	Lys	Essential amino group	115
TF_1-ATPase	*Thermophilic bacterium* PS3	DCCI	Glu	Essential Glu residue	116
F_1-ATPase	Bovine mitochondrion	FDNB	Tyr	Inactivation reversed by dithiothreitol	117
F_1-ATPase	Beef-heart mitochondrion	Phenylglyoxal, butanedione	Arg	Active-site modification	118
		7-Chloro-4-nitrobenzene-2-oxa-1,3-diazole	Tyr		
F_1-ATPase	Bovine mitochondrion	[^{14}C]DCCI	Glu	Single modified Glu residue	119
		4-Chloro-7-nitrobenzofurazan	Tyr		
F_1-ATPase	Yeast	Citreoviridin		Fluorescence studies	120
		Aurovertin			
ATPase (IF_1)	Beef-heart mitochondria	Meth-4-azido[^{14}C]benzimidate		Photoattachment to βf_1	121
ATPase component protein	Beef-heart mitochondrion	DCCI			122
ATP synthetase complex	Bovine-/porcine-heart mitochondrion	*p*-Chloromercuribenzoate	Cys	pCMB reaches thiols	123
		Carboxypyridine disulphide, *N*-ethylmaleimide	Cys	unreactive to NEM	
Bacteriorhodopsin	*Halobacterium halobium*	Fluorescamine	Lys	Study of light-to-dark adaptation	124
Bacteriorhodopsin	*Halobacterium halobium*	[^{14}C]DCCI		Light-induced reaction	125
Bacteriorhodopsin	*Halobacterium halobium*	Dansyl chloride, pyrene-1-sulphonyl chloride		Fluorescence probes	126
Bacteriohodopsin	*Halobacterium halobium*	Various	Lys	Fluorescamine alters H^+ pumping	127

Bacteriorhodopsin	*Halobacterium halobium*	Tetranitromethane	Tyr-26, -64, -131, -133	Role of Tyr in chromophore function	128
Benzodiazepine receptor protein	Rat-brain membrane	Diethyl pyrocarbonate	His	Modification affects [^{3}H]-diazepam binding	129
Benzodiazepine receptor	Calf brain	[^{3}H]Flunitrazepam		Direct photolabelling	130
Benzodiazepine receptor	Rat brain	[^{3}H]Flunitrazepam		Localization of receptors	131
Benzodiazepine receptor	Calf cerebral cortex	Tetranitromethane	Tyr	Involvement of Tyr residues in drug binding	132
Biliverdin reductase	Rat-liver cytosol	Various reagents	Cys	Characterization of enzyme activity	133

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
α-Bungarotoxin/erabutoxin *b*	*Bungarus multicinctus*/ *Laticauda semifasciata*	*N*-Succinimidyl [^{3}H]propionate	—NH_2		134
α-Bungarotoxin	*Bungarus multicinctus*	*N*-Succinimidyl [2,3-^{3}H]propionate		Production of probe for receptor-binding studies	135
Calmodulin	Bovine brain	*S*-({[(Iodoacetyl)amino]ethyl}-amino)-1-naphthalenesulphonate	Cys	Fluorescence probe	136
Calmodulin	Bovine brain	*S*-Adenosyl methionine/protein carboxymethylase		Non-specific methylation	137
Calmodulin		[^{3}H]Chloropromazine, [^{3}H]trifluorperazine		Direct photoaffinity labelling	138
cAMP receptor proteins	Human lymphocyte	8-Azidoadenosine-3′,5′-monophosphate		Location of binding sites	139
Carbonic anhydrase B	Bovine	$Br_2^{\cdot-}$, $(SCN)_2^{\cdot-}$	(Trp, His, Tyr)		140
Carbonic anhydrase B	Bovine	$OH^{\cdot-}$ $(SCN)_2^{\cdot-}$ $Br_2^{\cdot-}$	Trp His Tyr	Essential Trp residues	141
DD-Carboxypeptidase-transpeptidase	*Actinomadura* R39	Benzylpenicillin	Ser	Acyl enzyme formed	142
DD-Carboxypeptidase	*Streptomyces* R61	Various	Ser, Arg	Penicillin-binding properties	143
Carotid body	Cat	Various sulphydryl reagents		Modification increases hypoxia response	144
Cathepsin B, C, proteinase, clostripain	Bovine-spleen streptococcus	Peptidyl diazomethyl ketones	Cys	Selective inactivators	145
Cathepsin D	Chicken liver	*N*-Diazoacetyl-*N*′-(2,4-dinitrophenyl)ethylenediamine, 1,2-epoxy-3-(4-acetylphenoxy) propane		Labels on heavy chain	146
Cathepsin L	Rat liver	CBZ-Phe-Phe diazomethyl ketone CBZ-Phe-Ala diazomethyl ketone		Selective inactivation	147

Cells	Various	Methyl *p*-hydroxybenzimidate, 1,3,5-trichlorotriazine		Hapten coupling	148
Cell-surface receptor (cAMP)	*Dictyostelium discoideum*	8-Azido-[^{32}P]cAMP		Membrane cAMP receptor identified	149
cGMP-dependent protein kinase	Bovine lung	[^{32}P]n8cIMP (8-azidoinosine-3′,5′-[^{32}P]monophosphate)		Identification of protein in cell extracts	150
cGMP-dependent protein kinase	Bovine lung	N^{α}-Tosyl-L-lysine-chloromethyl-ketone			151
Chloramphenicol acetyltransferase	*E. coli*	Methyl acetimidate, citraconic anhydride	Lys	Buried lysine residues	152
Chloroplast CF_1	Lettuce	Fluorescein isothiocyanate	Lys	ATP protects against labelling	153
Chloroplast membranes	*Pisum sativum*	4-Azidoatrazine		Labelling of triazine-binding site	154

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Cholera toxin and its A and B promoters	*Vibrio cholerae*	2-Nitrophenylsulphenyl chloride 2,4-Dinitrophenylsulphenyl chloride	Trp Trp	Structure–function studies	155
Cholera toxin subunits	*Vibrio cholerae*	12-(4-Azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine		Glycolipid labels for membrane penetration study	156
Cholesterol 7-α-hydroxylase (cytochrome P450)	Rabbit and rat liver	Various thiol reagents		Reversible inactivation	157
Choline acetyltransferase	Squid-head ganglia	Butanedione, phenylglyoxal, camphor-quinone-10-sulphonic acid	Arg	Arg in coenzyme A-binding site	158
Choriogonadotrophin	Human	4-Azidobenzoylglycine, *N*-hydroxysuccinimide ester		Heterobifunctional photocrosslinking reagent for protein–receptor interactions	159
Chorionic gonadotropin and receptor	Rat-testis membrane	Disuccinimidyl suberate Dithiobis(succinimidyl propionate)	(Lys) (Lys)	Characterization of receptor	160
Chromosomal proteins (non-histone)	Rat organs	*S*-Adenosyl-L-[methyl-^{3}H]methionine/carboxy-methyltransferase	Carboxyl	Possible regulatory role of methylation	161
α-Chymotrypsin		*N*-Nitrosovalerolactam *N*-Nitroso-1,4-dihydro-6,7-dimethoxy-3-(2H)-isoquinolinone		Carbonium-ion generation during hydrolysis	162
α-Chymotrypsin	Bovine	Fluorocinnamoylimidazoles	Ser	^{19}F n.m.r. study	163
α-Chymotrypsin, trypsin inhibitor	Bovine	Fluoresceinisothiocyanate	(—NH_2)	Method for isolating fluorescein-labelled proteins	164

Chymotrypsin	Bovine	4-*trans*-Benzylidene-2-phenyl-oxaloline-5-one, various acyl imidazoles	Ser	Active-site acylation	165
Chymotrypsin	Bovine pancreas	Dansyl chloride Fluorescein isothiocyanate	$-NH_2$	α-Macroglobulin–chymotrypsin interaction	166
C-1 inhibitor	Human plasma	Various	Tyr, Lys, Arg	Essential Lys	167
Citrate synthase	Pig heart	Diethylpyrocarbonate Acetic anhydride Methyl mercuric chloride	His $-NH_2$ Cys	Evidence against two separate catalytic sites/monomer	168
Coat protein	PfI virus	Methyl-3-mercaptopropion-imidate	$-NH_2$	Heavy-atom site for *X*-ray analysis	169
CoA transferase	Pig heart	2-Nitro-5-(thiocyanato)benzoate	Cys	Two pathways for reaction	170
Colipase	Pig, horse	Diethylpyrocarbonate	His	Hydrophobic interactions unaffected	171
Collagenase	*Clostridium histolyticum*	Various	Various	Essential carboxyl, Tyr, and Lys	172

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Collagenase	Pig synovium	Various mercurials and thiols	Cys	Effect on collagenase activation	173
Complement C3	Human serum	Methylamine	Internal thioester bond	Conformational changes on modification	174
Complement C3b-like glycoprotein, monoclonal antibody	Cobra venom	*N*-Succinimidyl-3-(2-pyridyl-dithio)propionate	Lys, Cys	Immunocytolytic complex	175
Complement C4	Human	[^{14}C]Methylamine [^{14}C]Iodoacetamide	Asp or Glu Cys	Internal thiol ester linkage	176
Complement C4	Human	*N*-(7-Dimethylamino-4-methyl-coumarinyl)maleimide	Cys	Localization of single Cys residue	177
Concanavalin A	Jackbean	3-[(4-Azidophenyl)dithio]-propionic acid, oxysuccinimide ester		Cleavable reagent used to crosslink subunits	178
Concanavalin A	Jackbean	Succinic anhydride, acetic anhydride	(Lys)	Modified lectin still activates complement	179
Coupling factor I	Spinach chloroplasts	Woodward's reagent K, dicyclohexylcarbodi-imide	—CO_2H	Essential carboxylate groups	180
Coupling factor I	Spinach chloroplasts	3′-*O*-[3-(*N*-4-Azido-2-nitro-phenyl)amino]propionyl-ATP		Labelling of β-subunit	181
Creatine kinase	Equine skeletal muscle	Various —SH reagents	Cys	Two Cys residues essential for activity	182
Creatine kinase	Rabbit muscle	γ-(*p*-Azidoanilide)-ATP		Two moles analogue/mole enzyme	183
Crotalase	*Crotalus adamanteus*	Peptide chloromethyl ketones	(His)	Best inhibitor also inhibits kallikrein	184
γ-Cystathionase	Rat liver	γ-Fluorinated amino-acids		Suicide inhibitors	185
Cytochrome *c*		Acetic anhydride, succinic anhydride	Lys	Effect on reactions with superoxide and P450 reductase	186

Cytochrome *c*	Various microbial and mammalian sources	*O*-Methylisourea	Lys	Lys converted to homoArg	187
Cytochrome *c*	Horse heart	Trifluoroacetyl, trifluoromethylphenyl-carbamyl	Lys	$Fe(CN)_6^{3-}$ interaction	188
Cytochrome *c*	Horse heart	Trifluoroacetyl Trifluoromethylphenylcarbamyl	Lys Lys	Lys in adrenoxin interaction	189
Cytochrome *c*	Horse heart	Maleic anhydride	Lys	Stepwise charge modification	190
Cytochrome *c*	Various	Iodoacetic acid	Met-65	N.m.r. study	191
Cytochrome *c*	Horse heart	4-Chloro-3,5-dinitrobenzoic acid	Lys-13, -72	N.m.r. and e.s.r. studies	192
Cytochrome *c* peroxidase	Yeast	Arylazidocytochrome *c*	Lys-13	Delineation of protein–protein binding site	193

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Cytochrome P450	Rat liver	Chloramphenicol	Lys	Suicide inactivation	194
Cytochrome P450	Rat	Various		Suicide substrates	195
Dehydrogenases (various)	Various species	Diazonium salt derivatives of ADP	Cys, Lys, His, Tyr	Affinity labelling	196
Dihydrofolate reductase	*Streptococcus faecium*	TNBS	Lys	One essential Lys	197
Dihydrofolate reductase	Chicken liver	3-Chloro-4-(4-{2-chloro-4-[4,6-diamino-2,2-dimethyl-*S*-triazin-1-(2H)-yl]phenyl}-butyl)benzenesulphonyl chloride	Tyr-31	Characterization of affinity labelling	198
Dihydropteridine reductase	Rat liver	Thiol reagents		Cys role in tertiary structure	199
Diol dehydratase	*Klebsiella pneumoniae*	Pyridoxal 5'-phosphate/$NaBH_4$	Lys	Lys essential to component interactions	200
Dipeptidyl peptidase IV	*Streptococcus mitis*	DFP	Ser	DFP- and chelator-sensitive enzyme	201
Diphtheria toxin fragment A	*Corynebacterium diphtheriae*	*N*-Succinimidyl-3-(2-pyridyldithio)propionate	Lys, Cys	Toxic asialofetuin–diphtheria toxin conjugate	202
Diphtheria toxin A-chain	*Corynebacterium diphtheriae*	*NN'-O*-Phenylenemaleimide		Crosslinking to immunoglobulin	203
dnaB protein	*E. coli* YS1	Dimethyl-3,3'-(tetramethylene dioxy)dipropionimidate		Probe of subunit structure	204
Elongation-factor G 70-S ribosome	*E. coli*	4-(6-Formyl-3-azidophenoxy)-butyrimidate	NH_2	Identification of ribosomal proteins at EF-G-binding site	205
Elongation-factor Tu	*E. coli*	N^{ε}-Bromoacetyl-Lys-tRNA	His	Essential His near tRNA-binding site	206
Emulsin β-D-glucosidase	Sweet almond	*N*-Acetylimidazole	Tyr	Catalytic role of Tyr	207
Enkephalin receptor	Rat-brain membrane	Tyr-D-Ala-Gly-Phe-Leu-chloromethyl ketone			208

Enoyl-CoA hydratase	Bovine liver	Diethyl pyrocarbonate	His, Lys		209
2-Enoyl-CoA reductase	*Mycobacterium smegmatis*	Diacetyl, phenylglyoxal	Arg	Inactivation by Arg-specific reagents	210
Erythrocruorin	*Chironomus thummi thummi*	Cyanate	α-NH_2	Primary structure investigation	211
		1,2-Cyclohexanedione	Arg		
		Maleic anhydride	Lys		
		Diethyl pyrocarbonate	His		
Erythrocyte membranes	Human	3-(Trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine		Labelling of band-3 protein	212
Erythrocyte proteins	Human	Ozone	Cys	Disulphide crosslinking of membrane proteins	213
17β-Estradiol dehydrogenase	Human placenta	5'-[*p*-(Fluorosulphonyl)benzoyl]-adenosine		Both enzyme activities at single active site	214

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
17β-Estradiol dehydrogenase	Chicken liver	Dimethyl suberimidate	Lys	Subunit structure	215
17β-Estradiol dehydrogenase	Human placenta	16α-Bromoacetoxyprogesterone	His	Dual activities at single site	216
Fab′ antibody fragment	Rabbit, goat	Pyridyldithio derivatives of phosphatidylethanolamine	Lys	Immunodirected liposome targetting	217
Factor Xa	Bovine	Dansyl-Glu-Gly-Arg chloromethyl ketone	His-96	Fluorescence probe for interactions with other prothrombinase components	218
Fatty-acid synthetase	Yeast	1,3-Dibromo-2-propanone	Cys, pantatheine	Subunit crosslinking	219
Ferredoxin-$NADP^+$ oxidoreductase	Spinach chloroplasts	*N*-Ethyl-5-phenylisoxazolium-3′-sulphonate	$—CO_2H$	Single CO_2H in nucleotide-binding site	220
α-Fetoprotein	Rat	Diethylpyrocarbonate	His	His involved in oestrogen binding	221
Fetuin and others	Calf mucosa	2-Iminobiotin		Membrane-protein affinity probe	222
Fibrinoligase (Factor XIIIa)	Human	Fluorescent thiocholine esters	Cys	Fluorescent acyl enzyme formation	223
Flavoenzyme alcohol oxidase	*Candida buidinii*	*p*-Chloromercuribenzoate [1-^{14}C]Diethylpyrocarbonate	Cys His	Inactivation specific to PCMB	224
Formate dehydrogenase	*Achromobacter parvulus I*	2,3-Butanedione	Arg	Role of Arg residues	225
Formyl peptide chemotactic receptor	Human neutrophil membrane	^{125}I-*N*-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys Ethylene glycol bis(succinimidyl succinate)	Cys (Lys)	Strategy for receptor isolation	226
Fucosidase	Pig thyroid	CMC *p*-Mercuribenzoate, *N*-ethylmaleimide, iodoacetate	$—CO_2H$ Cys	Characterization of activity	227

β-Fucosidase	*Achatina balteata*	Iodine, *N*-acetylimidazole, tetranitromethane	Tyr	Essential Tyr	228
Fumarase	Pig heart	EDC	Asp, Glu	Difference labelling	229
Fumarase	Rat-liver mitochondrion and cytosol	Glutaraldehyde		Determination of subunit structure	230
4-Fumarylaceto-acetate fumarylhydrolase	Rat liver	*p*-Hydroxymercuribenzoate DTNB	Cys Cys	Characterization of enzyme structure and activity	231
L-Galactonolactone oxidase	Yeast	4,4'-Dipyridyldisulphide and other —SH reagents	Cys	Single inessential Cys modified	232
Galactose 1-phosphate uridylyltransferase	Human erythrocytes	Various		Cys and His in active centre	233
Galactose-specific lectin	*Mormordica charantia*	Various reagents	Lys, Trp, Tyr, Arg, Asp, Glu	Residues involved in carbohydrate binding	234

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
β-Galactosidase	*E. coli*	Cortisol derivatives		*N*-Succinimidyl ester coupling for immunoassay	235
β-Galactosidase		Glycyrrhetylamine acids	(Lys)	Immunoassay conjugate	236
β-Galactosidase	*E. coli*	*N*-Hydroxysuccinimide-biotin, *m*-maleimidobenzoyl-DNP-lysine		Doubly labelled enzyme immunoassay	237
Galactosyl transferase, α-lactalbumin	Human, bovine	Dimethyl pimelimidate	(Lys)	Studies of lactase synthetase complex	238
GAP junction	Chick lens	Cu^{2+}/1,10-phenanthroline	Thiol	Crosslinking to determine GAP structure	239
Gelonin anti-thy 1.1 antibody	*Gelonium multiflorum*	SPDP	Lys, Cys	Cytotoxic conjugate	240
Glucose oxidase	*Aspergillus niger*	Glutaraldehyde, dimethyl suberimidate, dimethyl adipimidate	Lys	Crosslinking on succinoylated lectin matrix	241
Glucose 6-phosphate	Rat liver	Various *N*-alkylmaleimides		Two essential thiols in non-polar environments	242
Glucose phosphate isomerase	Human and rabbit muscle, yeast	Various reagents		Identification of essential catalytic residues	243
Glucose phosphate isomerase	Porcine muscle, human placenta	2-Nitro-5-thiocyanobenzoic acid	Cys	Cleavage and sequencing	244
Na^+/D-glucose transport component	Rabbit small intestine	4-Azidophlorizin		Identification of transport-protein component	245
D-Glucose transporter	Rabbit intestine	6-*O*-[*N*-(2-Nitro-4-azidophenyl)-β-alanyl]phlorizin		Flash photolysis necessary for specific labelling	246
α-1,4-Glucosidase/albumin/IgE	Yeast/rat/rabbit	Glutaraldehyde		Antibody targetting of copolymer	247

Glutamate decarboxylase	Mouse brain	[α-^{3}H]Acetylenic γ-aminobutyric acid		Mechanism-based enzyme inactivation	248
Glutamate dehydrogenase	*E. coli*	α-(Fluoromethyl)glutamic acid		Mechanism-based inactivation	249
Glutamine synthetase	*E. coli*	5′-*p*-Fluorosulphonyl benzoyladenosine			250
γ-Glutamyl transferase	Rat kidney	6-Diazo-5-oxo-L-norleucyl-glycine		Inactive precursor of affinity labelling reagent	251
γ-Glutamyl transpeptidase	Rat kidney	L-(α*S*,5*S*)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid		Glutamine antagonist labels specific subunits	252
γ-Glutamyl transpeptidase	Human-foetal lung fibroblasts	6-Diazo-5-oxo-L-norleucine, 5-iodoacetamidofluorescein		Modification of light subunit	253
Glutathione reductase	Yeast	*N*-Alkylmaleimides	Cys	Role of Cys residues	254
Glutathione reductase	Yeast	Iodacetamide	Cys	Monoalkylated derivative produced	255

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
D-Glyceraldehyde-3-phosphate dehydrogenase	Rat muscle	[^{14}C]Butanedione	Arg	Essential Arg located	256
Glyceraldehyde-3-phosphate dehydrogenase	Yeast, rabbit muscle	DTNB	Cys-149	Specific reagent	257
Glyceraldehyde-3-phosphate dehydrogenase	Pig muscle	Fluorescein isothiocyanate		Investigation of G3PDH interaction with 3-PGK	258
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	[^{15}N]-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide	Cys-149	Introduction of ^{15}N and ^{2}H for e.s.r. studies	259
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	*N*-([^{15}N]-1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide	Lys (?)	E.s.r. study of G3PDH binding to erythrocyte membrane	260
Glyceraldehyde-3-phosphate dehydrogenase	Yeast	Arylazido-β-alanyl derivatives of NAD^+ and $NADP^+$		Reduction of covalent label *in situ*	261
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	β-(2-Furyl)acryloyl phosphate	Cys	Chromophoric acyl enzyme	262
DL-Glycerol-1-phosphate	*Duriella salina*	*p*-Hydroxymercuribenzoate	Cys	Demonstration of active-site SH	263
Glycine receptor	Rat spinal cord	[^{3}H]Strychnine/*hv*		Direct photoaffinity labelling	264
Glycogen phosphorylase	Rabbit muscle	Phenylglyoxal	Arg	One essential Arg	265
Glyoxalase II	Rat erythrocytes	Various	Various	Active-site Arg binds substrate	266
Gonadotropin-releasing hormone receptors	Rat pituitary	(D-Lys^{b}-N^{ε}-azidobenzoyl)GnRH		Localization of receptors	267

Growth-factor membrane protein	Rabbit cervical ganglion	Hydroxysuccinimidyl *p*-azidobenzoate		Receptor identification	268
Growth hormone	Bovine	*N*-Acetylimidazole	Tyr, Lys	Tyr not important to activity	269
Growth hormone	Bovine	TNBS	*N*-Terminal amino	Lys-69 reacts after *N*-terminal blocking	270
		Potassium isocyanate	Lys		
		Iodoacetamide	Met		
GTPase	Bovine	8-Azido-GTP		Tryptic map located GTP-binding component	271
Guanylate cyclase	Rat liver	Various thiol reagents	Cys	Reversible inactivation *via* disulphide reduction	272
Guanylate cyclase	Rat liver	Various oxidizing and thiol-specific agents	Cys	Two closely juxtaposed SH groups	273

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Haemagglutinin	Asian influenza virus	[^{14}C]Iodoacetamide	Cys	Disulphide bond location	274
Haemoglobin	Human	4-Isothiocyanatobenzene sulphonic acid	*N*-Terminus	Changed O_2 affinity, *etc.*	275
Haemoglobin A	Human	Potassium isocyanate	α-NH_2	Allosteric effects of carbamylation	276
Haemoglobin A and S	Human	Various bis-salicyl esters (diaspirins)	—NH_2	Variation of crosslink length and nature of aspirin substituents	277
Haemoglobin	Human	1,5-Iodoacetylaminoethyl-5-naphthylamine-1-sulphonate	Cys-β93, Cys-β112	Fluorescence studies of subunit dynamics	278
Haemoglobin	Rat	[*NN'*-Diethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine] sulphoxide	Thiol	Single thiol modified	279
Haemoglobin	Human	2,2,2-Trifluoroethanol/DTNB	Cys-β93	Potential ^{19}F n.m.r. probe	280
Haemoglobin/erythrocyte membrane proteins	Human	Dimethyl suberimidate		Membrane-permeable crosslinker	281
Haemoglobin S	Human	Methyl acetimidate		Anti-sickling effect	282
Haptenated serum albumin	Rabbit	Succinic anhydride/EDCI	Carboxyl	Coupling to sheep erythrocytes	283
Haptoglobin/haemoglobin	Human pleural fluid	1,5-Difluoro-2,4-dinitrobenzene	Lys, Tyr	β-Chains and H-chains in close proximity in Hb.Hp complex	284
Herbicide receptor protein	*Pisum sativum*, *Amaranthus hybridus*	2-Azido-4-ethylamino-6-isopropylamino-*S*-triazine		Identification of receptor protein	285
Hexokinase	Yeast	ATP-4-(*N*-2-chloroethyl-*N*-methylamino)benzylamide		Investigation of ATP-binding-site differences between monomeric and dimeric enzyme	286

Hexokinase	Yeast	Procion Green H-4G		Mg^{2+} protects	287
Histidine decarboxylase	*Lactobacillus* 30a	Glutaraldehyde	Lys	Subunit structure E.M.	288
Histidine decarboxylase	*Micrococcus* sp. N	*N*-Bromosuccinimide	Trp	Essential Trp	289
Histidine-rich glycoprotein	Rabbit serum	Diethylpyrocarbonate	His	Modification of Zn^{2+} binding	290
L-Histidinol : NAD^+ oxidoreductase (histidinol dehydrogenase)	*Salmonella typhimurium*	Pyridoxal 5-phosphate	Lys	Evidence for active-site lysine	291
Histone H3	Rat liver	*N*-Pyrenemaleimide		Fluorescence study of chromatin accessibility	292
Histone H4	Calf thymus	Dipotassium phosphoramidate	His	^{31}P n.m.r. study of chemical phosphorylation	293

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
HLA-A2 antigen	Lymphoblastoid cells	*N*-{[(Iodoacetyl)amino]ethyl}-*S*-naphthylamine-1-sulphonic acid		Fluorescent probe of cell–protein interactions	294
Hyaluronidase	Bovine testis	Butane-2,3-dione	Arg	Investigation of role of Arg in catalytic mechanism	295
D-(−)-β-Hydroxybutyrate dehydrogenase	Beef-heart mitochondria	(i) NEM, *p*-(chloromercuri)-phenylsulphonate	Thiols	Essential thiol and disulphide	296
		(ii) Phenylarsine oxide, diamide	Vicinal thiols		
		(iii) Sulphite, sulphide, cyanide	Disulphides		
D-(−)-β-Hydroxybutyrate dehydrogenase	Beef-heart mitochondria	(i) 2,3-Butanedione	Arg	Essential Arg and His	297
		(ii) Phenylglyoxal	Arg		
		(iii) Diethyl pyrocarbonate	His		
D-β-Hydroxybutyrate dehydrogenase	*Zoogloea ramigera* I-16-M	*p*-Chloromercuribenzoic acid, DTNB	Cys	Inhibition of activity	298
Δ^5-3β-Hydroxysteroid dehydrogenase	Various mammals	4,5-Allenic 3β-hydroxy-5,10-secosteroids		Suicide inactivation *via* ketones	299
IgE receptor	Mast cells	5-Iodo-1-azidonaphthalene		Hydrophobic photolabel	300
IgG	Rabbit	EDLP		Coupling to liposomes	301
IgG	Human	6-(7-β-Galactosylcoumarin-3-carboxamido)hexylamine/dimethyl suberimidate	Lys	Homogeneous substrate-labelled fluorescent immunoassay	302
IgG	Rabbit	Carboxymethyldextran/trypsin inhibitor		Co-immobilization on polymer	303
IgG (anti-thy 1.2 antigen)		α-Amanitinylazobenzoylglycyl-glycine/carbodi-imide		*T. lymphoma* toxin targetting	304
IgG	Mouse	Neocarzinostatin apoprotein/SPDP	Cys	Toxin targetting	305
IgG	Goat	Citraconic anhydride/CMC		Coupling to phosphatidyl ethanolamine liposomes	306

IgG (anti-human)	Sheep	SPDP		Crosslinking to peroxidase	307
IgG 2a	Mouse	Phosphatidylethanolamine liposomes modified with SPDP		Targetting of methotrexate	308
Inhibitor I-1	Ragi	TNBS	Lys	Bifunctional inhibitor	309
		Cyclohexane-1,2-dione	Arg	with two active sites	
Initiation complex	Ribosomes	Diepoxybutane, methyl-*p*-azidobenzoylaminoacetimidate		Crosslinking in quaternary complex	310
Initiation Factor 3	*E. coli* MRE 600	Pyridoxal 5-phosphate/sodium borohydride	Lys	Active-site lysine	311
Initiation Factor 3	*E. coli* MRE 600	1-Dimethylaminonaphthalene-5-sulphonyl chloride	Lys-73, -80, -91, -96, -99, -112	Fluorescence probe for protein–ribosome interaction	312
Inorganic pyrophosphatase	*Streptococcus faecalis* ATCC 8043	TNBS	Lys	Essential Lys and Cys	313
		Various —SH reagents	Cys		

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Insulin	Bovine	*NN'*-Carbonyl-bis-(L-methionine *p*-nitrophenyl ester)	Gly-A1, Lys-B29	Crosslinking reduces sensitivity to pepsin degradation	314
Insulin	Porcine	Carbonyl-bis-(L-methionine *p*-nitrophenyl ester)	Gly, Lys	Receptor-binding sites	315
		t-Butyloxycarbonyl-L-[^{125}I]iodotyrosine *N*-hydroxysuccinimide ester	Val		
Insulin	Porcine	N^{α}-Boc-N^{γ}-(4-azidophenylacetyl)-D-α,γ-diaminobutyric acid 4-nitrophenyl ester	Gly-A1	Photolabelled insulin derivative iodinated and then crosslinked to membrane proteins	316
Insulin	Bovine	*NN'*-Carbonyl-bis-(L-methionine *p*-nitrophenyl ester)	Gly-A1, Lys-B29	Trypsin used to resynthesize Arg-B22–Gly-B23 bond in crosslinked insulin	317
Insulin	Porcine	1-Fluoro-2,4-dinitrobenzene	$-NH_2$, His, Tyr	pH dependence of group reactivity	318
Insulin		Bis(azoisobutyrimidate)	Gly-A1, Lys-B29	Retention of biological activity	319
Insulin B-chain	Pig	Cyclohexane-1,2-dione	Arg-22	Activity retained	320
Insulin B-chain	Bovine	(i) Trypsin/phenylhydrazine, pH 6.5	(i) Carboxyl terminal Arg	Activation of carboxyl *via* phenyldi-imide	321
		(ii) BNPS-skatole	(ii) A-Chain *N*-terminus		
Insulin/ricin B-toxin	*Ricinus communis*	SPDP, insulin SPDP, ricin	Lys-B29 Cys	Conjugate with altered receptor-binding properties	322
Insulin-like growth factors	Human	Disuccinimidyl suberate	(Lys)	Two types of rat-liver-membrane growth-factor receptors	323
Insulin receptor	Rat liver	N^{B29}-[(4-Azido-2-nitrophenyl)-acetyl]insulin		Hydrodynamic characterization of labelled product	324

Insulin receptor	Rat adipocyte	Disuccinimidyl suberate	Lys		325
Insulin receptor Insulin	Pig	Disuccinimidyl suberate	(Lys)	Crosslinking of insulin to its receptor	326
Insulin receptor	Rat adipocyte	Disuccinimidyl suberate Disuccinimidyl adipate Disuccinimidyl succinate	(Lys)	Affinity labelling of adipocyte membranes	327
Insulin receptor	Human placenta	Disuccinimidyl suberate	(Lys)	Insulin crosslinked to receptor	328
Isocitrate dehydrogenase (TPN-dependent)	Pig heart	4-Iodoacetamidosalicylic acid	Cys, Lys	Affinity label not very specific	329
Isocitrate dehydrogenase	Pig heart	(*RS*)-3-Bromo-2-ketogluterate	Thiol?	Substrate and inactivator	330
Kallikrein	Human urine	Chloro-(N^{α}-*p*-tosyl-lysyl)methane	His	Active-site His modified	331
2-Keto-4-hydroxy-glutarate aldolase	*E. coli* K-12	Dimethyl suberimidate	Lys	Subunit structure	332

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Δ^5-3-Ketosteroid isomerase	*Pseudomonas testosteroni*	Pregnyne, estryne		Acetylenic suicide substrates give allenic ketones	333
Δ^5-3-Ketosteroid isomerase	*Pseudomonas testosteroni*	Estryne	Arg-57	Arg forms enol imidate with enzymically generated allene	334
Lac repressor	*E. coli*	*N*-(Iodoacetylaminoethyl)-5-naphthylamino-1-sulphonate		Fluorescence study of local conformational mobility	335
α-Lactalbumin	Bovine	[^{3}H]Acetic anhydride/dimethyl 3,3′-dithiobispropionimidate	Lys	Identification of crosslinking sites in protein complex	336
β-Lactamase	*E. coli*	Phenoxymethyl penicillanic acid sulphone, methicillin sulphone, quinacillin sulphone	Ser-70	Mechanism-based inhibitors forming acyl-enzymes	337
β-Lactamase	*E. coli*	Olivanic acid derivatives	Ser and others	Mechanism-based inhibitors	338
β-Lactamase	*E. coli*	Clavulanic acid, 9-deoxyclavulanic acid	Ser and others	Inactivation following acyl-enzyme formation	339
β-Lactamase	*E. coli*	Deuterio derivatives of penicillanic acid sulphone	Ser and others	Stereospecific hydrogen abstraction precedes inactivation	340
β-Lactamase	*Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae*	Clavulanic acid		Transient and suicide inhibition	341
Lactate dehydrogenase		Dichlorotriazinyl dyes		Active-site-directed affinity labels	342
Lactate dehydrogenase	Pig heart	Methyl methanethiosulphonate Diethylpyrocarbonate	Cys-165 His-195	Differential protection by cofactors	343
Lactate dehydrogenase	Pig muscle	Glutaraldehyde		Kinetics of reassociation studies by crosslinking	344
L-Lactate mono-oxygenase	*Mycobacterium phlei*	2,3-Butanedione, phenylglyoxal	Arg	Arg involved in lactate binding	345

Lactogen	Human placenta	(2-Nitrophenylsulphonyl)-3-methyl-3′-bromoindolenine	Trp	Role of Trp	346
β-Lactoglobulin	Bovine	Citraconic anhydride	Lys	Disulphide-mediated polymerization on modification of Lys	347
Lactose receptor	*E. coli* CSH 46	Tetranitromethane *N*-Ethylmaleimide	Tyr Cys	NEM protects Cys from modification by tetranitromethane	348
Lectin	*Solanum tuberosum*	Various	Various	Systematic study of effects on haemagglutinating activity	349
Lipase	Pig pancreatic	Diethyl *p*-nitrophenyl phosphate	Ser	Determination of active-site sequence	350
Lipoamide dehydrogenase	Pig heart	Various imidoesters, pyridoxal 5′-phosphate	Lys	Essential Lys and His	351
Lipoamide dehydrogenase	Pig heart	Iodoacetamide	Cys	Monoalkyl derivative	352

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Lipoamide dehydrogenase	Pig heart	2-Mercaptoethanol/iodoacetate	Disulphide	Stable monomeric enzyme	353
Lipoprotein (low density)	Human	1,2-Cyclohexanedione Diketene Diazotized arsanilic acid	Arg Lys Tyr, His	Arg and Lys in platelet interaction	354
Lipoprotein (very low density)	Rat liver	1,2-Cyclohexanedione	Arg	Effects of modification uptake by liver	355
Luciferase	*Beneckea harvey* 1	2,4-Dinitrofluorobenzene	Amino groups	Subunit modification	356
Luciferase	Firefly	*p*-Fluorosulphonylbenzoyl-*S'*-adenosine	Lys	Nucleotide-binding-site peptide located	357
Lutropin/thyrotropin	Bovine anterior pituitaries	Iodoacetic acid	Met	Activity studies	358
Lutropin/thyrotropin	Bovine	Dithioerythritol	Disulphide	Location of disulphides	359
Lysozyme	Hen egg-white	2,4-Dinitrobenzene sulphonic acid	Lys-33, Lys-96	Lys-33 modified in absence, and Lys-96 in presence, of maleic anhydride	360
Lysozyme	Hen	*N*-Bromosuccinimide	Trp-62	*X*-Ray and n.m.r. study of Trp function	361
Lysozyme	Hen egg	EDC/amine nucleophile	Asp-101	Specific labelling	362
Lysozyme	Hen egg	Osmium tetroxide-pyridine, oxo-osmium(VI)-pyridine	Various	Crosslinking and peptide-bond cleavage	363
Lysozyme		Methanol/HCl	Carboxyl	Immunological study of lysozyme methyl ester	364
Lysozyme/albumin	Hen egg-white, bovine serum	SPDP	Lys, Cys	Preparation of immunoadsorbents by coupling to Sepharose-gelatin	365
α_2-Macroglobulin	Human plasma	Methylamine	Cys-Glx	Forms γ-glutamyl, methylamide internal ester	366

α_2-Macroglobulin	Human plasma	Methylamine Iodoacetic acid	Glu Cys	Cys, thiolester intermediate	367
Malate dehydrogenase	Bovine	Quercetin + *hv*		Pseudo-photolabelling	368
Malate dehydrogenase	Porcine-heart mitochondrion	*N*-Ethylmaleimide		Activity studies	369
Malic enzyme	Pig liver	Various diketones	Arg	Arg residues involved in substrate binding	370
Melanocyte α-MSH receptor	*Zenopus laevis*	*p*-Azidophenylalanine-^{13}α-melanotropin/*hv*		Irreversible stimulation	371
Membrane proteins	Erythrocyte ghosts	4-*NN*-Dimethylaminoazobenzene-4′-isothiocyanate	Lys(?)	Chromophoric hydrophobic probe	372
Membrane protein	Human platelet	5′-*p*-Fluorosulphonylbenzoyl adenosine		ADP receptor labelling	373

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Membrane proteins	Human sickle-cell erythrocyte	Pyridoxal 5-phosphate	$-NH_2$	Reversible protection of amino groups during acetamidination	374
Membrane proteins	Rat-liver mitochondrion, insect flight-muscle mitochondrion	*N*-Polymethylenecarboxymaleimides	Cys	Probes of depth of membrane SH groups	375
Membrane proteins	Human erythrocyte	Maleimidomethyl-3-maleimidopropionate	Cys	New crosslinking reagent	376
Membrane proteins	Human erythrocyte	Iodine monochloride	Tyr (His)	Labelling studies	377
Membrane proteins	*Halobacterium halobium*	DCCI		Effect on light-induced membrane potential	378
Membrane protein/glucagon	Rat liver	Hydroxysuccinimidyl *p*-azidobenzoate	$-NH_2$(?)	Location of glucagon receptor	379
Mesentericopeptidase	Bacillus mesentericus	Dansyl fluoride		Fluorescence study	380
Metallothionein	Rat liver, horse kidney	DTNB	Cys	Metal ions released on modification	381
Methaemoglobin	Opossum	3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	Cys-β93	Conformation probe for e.s.r. studies	382
Methionine γ-lyase	*Pseudomonas ovazis*	L-2-Amino-3-*cis*-pentenoate		Suicide inhibition *via* pyridoxal *p*-quinoid derivative	383
Met-tRNA synthetase	*E. coli*	tRNA-Met modified with (i) propane-1,3-diamine (ii) dithiobis(succinimidyl propionate)	Lys	Cleavable crosslinker	384
Monoamine oxidase	Pig liver	*N*-(1-Methyl)cyclopropylbenzylamine		Suicide inhibitor	385
Monoamine oxidase	Rat liver	[^{3}H]Pargyline	Covalent flavin moiety	Identification of amino-acid sequence at active site	386

M protein mutants	Vesicular stomatitis virus	[^{125}I]Iodonaphthyl azide		Interactions with viral nucleocapsid and envelope studies	387
Multiplication-stimulating activity	Rat liver	Disuccinimidyl suberate Disuccinimidyl succinate Ethylene glycolyl bis(succinimidyl succinate)	(Lys)	Affinity label attachment to membrane receptor	388
Myoglobin	Sperm whale	Potassium cyanate	—NH_2	α-NH_2 and ε-NH_2 groups carbamylated	389
Myoglobin	Map turtle	Citraconic anhydride, 1,2,4-benzenetricarboxylic anhydride	Lys	Blockage of ε-NH_2 groups for sequencing	390
Myosin	Rabbit skeletal muscle	DTNB	Cys	Study of nucleotide binding	391
Myosin	Rabbit muscle	[^{14}C]Nitrothiocyanobenzoic acid	Thiol	Two essential thiols labelled	392
Myosin	Pig heart	*N*-Methyl-2-anilino-6-naphthalene-sulphonyl chloride	Lys	Selectivity of Lys modification achieved	393

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Myosin	Pig heart	*N*-Methyl-2-anilino-6-naphthalene-sulphonyl chloride	Lys	Localization and isolation of modified Lys residues	394
Myosin	Rabbit skeletal muscle	2,4,6-Trinitrobenzenesulphonate	Lys	PPi affects modification	395
Myosin B ATPase	Dog colon	*N*-Ethylmaleimide	Cys		396
Myosin (g_1)	Rabbit skeletal muscle	Tetranitromethane	Tyr	Tyr-52 and -180 modified	397
Myosin, actin	Rabbit muscle	5′-*p*-Fluorosulphonylbenzoyladenosine		Location in membrane	398
Myosin subfragment 1	Rabbit muscle	TNBS	Lys	Reactive Lys on 27k fragment	399
Myosin subfragment 1	Rabbit skeletal muscle	*p-NN′*-Phenylenedimaleimide		Bifunctional reagent traps ADP non-covalently	400
Myosin subfragment 1	Skeletal muscle	*p-NN′*-Phenylenedimaleimide	Cys	Subunit interaction and catalysis	401
Myosin subfragment 1	Rabbit muscle	*N*-[7-(Dimethylamino)-4-methyl-3-coumarinyl]maleimide	Cys	Location of SH groups	402
Myosin subfragment 1	Rabbit muscle	CMC/3-nitrotyrosine ethyl ester	Carboxyl	ATPase activity altered: one essential carboxyl	403
Myosin subfragment 1	Rabbit muscle	*N*-(Iodoacetyl)-*N′*-(5-sulpho-1-naphthyl)ethylene diamine	Cys	Excitation-energy-transfer experiments	404
Myosin subfragment 1	Rabbit muscle	TNBS	Lys	One Lys linked to K^+-ATPase activity	405
NADH dehydrogenase	Ox-heart mitochondrion	Arylazido-β-alanyl NAD^+		Photoaffinity identification of nucleotide-binding site	406
NADPH-cytochrome P450 reductase	Rabbit-liver microsome	*p*-Hydroxychloromercuribenzoate	Cys	Location of functional SH groups	407
NAD(P)H dehydrogenase	Rat liver	3-(α-Acetonyl-*p*-azidobenzyl)-4-hydroxycoumarin		Specific photoinactivation	408
NADPH:photochlorophyllide oxidoreductase	Various plant etioplasts	[^{3}H]-*N*-Phenylmaleimide	Thiols	NADPH and photochlorophyllide protect active centre	409

Neurotoxic esterase	Hen brain	[^{3}H]Di-isopropyl phosphofluoridate	(Ser)	Electrophoretic identification of labelled enzyme	410
Neurotoxin	*Clostridium botulinum*	*N*-Bromosuccinimide Tetranitromethane	Trp Tyr	One essential Trp	411
Neurotoxin II	*Naji naji oxiana*	Phenyl trifluoroacetate	—NH_2	^{19}F n.m.r. investigation of structure	412
Nicotinamide nucleotide transhydrogenase	Mitochondrion	DCCI Diethylpyrocarbonate	(—CO_2H) (His)	Inhibition studies	413
Oligomycin-sensitive ATPase	Beef-heart mitochondrion	Tartryl di(glycylazide), diepoxybutane	—SH	Subunit interactions in oligomycin sensitivity	414
Ornithine decarboxylase	*Physarum polycephalum*	α-Difluoromethylornithine			415

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ornithine decarboxylase	*Pseudomonas aeruginosa*	DL-α-Difluoromethylornithine		No inhibition of *E. coli* enzyme	416
Ornithine transcarbamylase	*E. coli*	Phenylglyoxal	Arg	Catalytic role of Arg	417
Ovoinhibitor	Chicken	Ozone		Anti-elastase activity reduced	418
Ovomucoid	Turkey egg	Dimethylamine borane / Trimethylamine borane + formaldehyde	Lys	Reductive methylation without disulphide reduction	419
Papain	*Carica papaya*	2-Chloromethyl 4-nitrophenyl(*N*-carbobenzyloxy)glycinate	Trp-177	Effect on thiol reactivity	420
Papain	*Carica papaya*	7-(α-Bromoacetyl)-10-methylisoalloxazine	Cys	Flavopapain semisynthetic enzyme	421
Pepsin	Porcine	KI_3	Tyr	Effect of iodination on c.d. spectra	422
Pepsinogen	Porcine	Diethylpyrocarbonate	Lys	Inactivation not reversible	423
Peptidoglycans	*Micrococcus lysodeikticus*	1-Fluoro-2,4-dinitrobenzene		Radiochemical microdetermination	424
Peptidylarginine deiminase	Rat epidermis	Iodoacetate, *p*-chloromercuribenzoate	Cys	Inhibition of activity	425
Peptidyl transferase	*E. coli* 50S ribosomal subunit	*N*-Bromoacetyl puromycin		Location of antibiotic binding site	426
Peroxidase	Rat uterus	*N*-Ethylmaleimide	Cys	Activity increased	427
Phenylalanine	Rat liver	*N*-Ethylmaleimide	Cys	Activity increased 20—30 times (possible conformational trigger)	428
Phenylalanyl tRNA synthetase	*E. coli*	Pyridoxal 5′-phosphate, 2′,3′-dialdehyde ATP	Lys	Inhibition of aminocylation step	429
Phosphodiesterase (Ca^{2+}-sensitive)	Bovine heart muscle	Azidocalmodulin	$-NH_2$	Photoaffinity label for calmodulin-binding proteins	430
6-Phosphogluconate dehydrogenase	*Candida utilis*	Pyridoxal 5′-phosphate	Lys	Studies of P-5′-P binding site	431
Phosphoglucose isomerase	Rabbit muscle	1-Chloro-2-oxohexanol-6-phosphate		Weak non-covalent inhibition	432

Phosphoglycerate kinase	Yeast	DTNB, 2-chloromercuri-4-nitrophenol	Cys	Effects on proteolysis of PGK	433
Phospholipase A	*Bungarus multicinctus*	*p*-Bromophenacyl bromide	His	One of two His modified: enzyme inactivated	434
Phospholipase A_2	Porcine pancreas	Phenylglyoxal monohydrate	Arg-6	Micelle interface recognition site modified	435
		1,2-[1-^{14}C]Cyclohexanedione	Arg-6		
Phospholipase A_2	Bovine pancreas	1-Trimethylamino-2-bromoethyl bromide	Cys	Role of Asp binding Ca^{2+}	436
		EDC, semicarbazide	Asp, Glu		
Phospholipase A_2	Various snake venoms	Glyoxylic acid/Cu^{2+}	α-Amino group	Transamination: some activity retained	437
Phospholipase A_2	*Crotalus atrox* venom	1,2-Di-*O*-hexylglycero-3-(ethyl diazomalonamidoethyl)phosphate		*N*-Terminal region labelled	438

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Phospholipase A_2	Pig pancreas	*N*-Ethyl-*S*-isoxazolium-3-sulphonate	Carboxylate	Single essential carboxylate	439
Phospholipase C	*Bacillus cereus*	Pyridoxal 5′-phosphate/$NaBH_4$	Lys	Essential Lys	440
Phosphorylase kinase	Rabbit muscle	2′,3′-Dialdehyde ATP		Inactivation influenced by protein substrates	441
Phosphorylase kinase	Rabbit muscle	2′,3′-Dialdehyde ATP/$NaBH_4$		Inactivation sensitive to metal-ion effectors	442
Photosystem 1 core complex	Spinach	EDC/[^{14}C]ethylenediamine	Carboxyl	Charge reversal alters light utilization	443
Plasmin	Human	4-Amidinophenyl 4′-methoxy-benzoate	Ser	Acyl-enzyme prodrugs	444
Plasmin	Human	4-Azidobenzoylglycyl-L-lysine		Labelling of lysine-binding site	445
Plastocyanin	Spinach	EDC/[1,2-^{14}C]ethylenediamine	Carboxyl	Interaction with photosystem 1 increased	446
Platelets	Human	Photolabile derivative of thrombin		Labelling of thrombin receptor	447
Porin	*E. coli* B	Acetic anhydride Succinic anhydride Glycinamide	—NH_2 —NH_2 —CO_2H	Investigation of permeability in membrane	448
Porphobilinogen deaminase	*Rhodopseudomonas spheroides*	[^{14}C]Porphobilinogen		Stable enzyme–substrate complex	449
Phenyl transferase	Chicken liver	*O*-Azidophenylethyl pyrophosphate	At least 16 amino-acids, esp. Arg and Ala	Location of photolabelling site	450
Progesterone receptor	Human uterus	17,21-Dimethyl-19-norpregna-4,9-diene-3,20-dione + u.v.		Confirmation of identity with mol. wt. 42 000 protein	451
Prolactin	Sheep pituitary	Fluorescein isothiocyanate	α-NH_2	Study of recombinant complex of peptide fragments	452
Prolyl 4-hydroxylase	Chicken embryo	Hydrogen peroxide	Cys	Induces S—S crosslinking of monomers	453

Protein A	*Staphylcoccus aureus*	SPDP		Crosslinking to peroxidase	454
α-Proteinase inhibitor	Human plasma	Tetranitromethane, *N*-acetyl-imidazole	Tyr	Tyr essential for elastase inhibition	455
α-1-Proteinase inhibitor	Canine plasma	*N*-Ethylmaleimide	Cys	Effects on inhibitor ability	456
Protein kinase (AMP-dependent)	Human erythrocyte	*p*-Chloromercuribenzoate, DTNB, *N*-ethylmaleimide	Cys	Essential Cys residue	457
Protein kinase, types I and II	Chinese-hamster ovary	8-Azidoadenosine-3′:5′-[^{32}P]mono-phosphate			458
Protein kinase (cAMP-dependent)		Various disulphide reagents (*e.g.* DTNB)	Cys	Channelling of reagent to specific site	459
Protein kinase (cAMP-dependent)	Bovine lung	5′-*p*-Fluorosulphonyl benzoyl adenosine			460

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Protein kinase (cAMP-dependent)	Rat adrenal microsome	8-Azidoadenosine-3′:5′-[^{3}H]monophosphate		Determination of cellular location	461
Proteins S3, S6, S8, and S10	*E. coli* ribosome	Various	Mainly Lys	Ribosomal protein mapping	462
Purine-transport-system protein	*Saccharomyces cerevisiae*	8-Azidoadenine		Isolation and characterization of receptor	463
Pyridine nucleotide transhydrogenase	*Azotobacter vinelandii*	*p*-Aminophenylarsenoxide	Cys	Reversible divalent reagent	464
Pyridoxamine-*S*′-phosphate oxidase	Rabbit liver	Phenylglyoxal, 2,3-butanedione, 2,4-pentanedione	Arg	Essential Arg exposed in holoenzyme	465
Pyroglutamyl aminopeptidase	*Bacillus amyloliquefaciens*	N^{α}-Carbobenzoxy-L-pyroglutamyl chloromethyl ketone	Cys	Inactivation by affinity labelling	466
Pyroglutamyl aminopeptidase	*B. amyloliquefaciens*	L-Pyroglutamyl chloromethyl ketone	Cys	Investigation of active site	467
Pyruvate kinase	Rabbit muscle	DTNB	Cys	Study of allostery	468
Pyruvate kinase	Rabbit muscle	5′-[*p*-Fluorosulphonyl)benzoyl]-1-N^6-ethenoadenosine	Cys	Fluorescent label	469
Pyruvate kinase	Rabbit muscle	5′-[*p*-(Fluorosulphonyl)benzoyl]-guanosine	Cys	Two residues modified *via* thiosulphonate	470
Pyruvate transporter	Rat mitochondria	[^{3}H]-*N*-Phenylmaleimide		α-Cyanocinnamate protects	471
Restriction endonucleases	Various	Thiol-reactive agents	Cys	Most enzymes not SH dependent	472
Restriction endonucleases EcoRI	*E. coli* EcoRY13	CMC	—CO_2H (Tyr, Cys)	Water-soluble reagent	473
Rhodopsin	Bovine	Iodoacetamide	Cys	Sequence analysis	474
Rhodopsin	Bovine	1-Azido[^{3}H]pyrene		Hydrophobic photolabelling in retinal rod membrane	475
Ribonuclease A	Bovine pancreas	4-Arsono-2-nitrofluorobenzene	—	Spectrophotometric titration of derivatives	476
Ribonuclease	*Streptomyces erythreus*	Iodoacetate Iodoacetamide	—CO_2H His	Study of active site	477
Ribonuclease T_1	*Aspergillus oryzae*	Iodoacetate, bromoacetate	Glu-58	N.m.r. probe of adjacent His	478

Ribosomes	Rat liver	[^{3}H]Puromycin		Irradiation at 254 nm	479
Ribosomes	*E. coli*	Bromoacetyl pleuromutilin		S18, L2, and L27 subunits labelled	480
Ribosomes	*E. coli*	Dimethylmaleic anhydride		Reversible modification	481
Ribosomal proteins	*E. coli*	Thiolated aminoacyl tRNA		Photoactivation of thiolated RNA	482
Ribosome-bound proteins L7 and L12	*E. coli*	5-(Iodoacetamidoethyl)amino-naphthalene 1-sulphonic acid		Probe of ribosome structure	483
Ribosome-bound proteins L7 and L12	*E. coli*	IAEDANS, FITC		Fluorescence labelling of ribosomes	484
Ribosomal protein L7 and L12	*E. coli*	4-(6-Formyl-3-azidophenoxy) butyrimidate	Lys	Photocrosslinked complex active	485

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Ribosomal proteins	*E. coli*	Diepoxybutane		Cleavable crosslinking to RNA	486
Ribosomal 30S subunits	*E. coli*	4-Azido-2,3,5,6-tetrafluoropyridine, 4-azido-3,5-dichloro-2,6-difluoro-pyridine		Photocrosslinking	487
Ribosomal 30S subunits/IF_3	*E. coli*	*p*-Nitrobenzylmaleimide		Two-stage crosslinking	488
Ribosomal proteins (30S, 50S)	*E. coli* MRE600	2-Iminothiolane, dithiobis-propionimidate	(Lys)	Proteins at subunit interface	489
Ribosomal subunits (30S, 70S)	*E. coli*	2-Iminothiolane	$-NH_2$, —SH	Examination of ribosome structure	490
Ribosomal subunits (40S)	Rat liver	Dimethyl 3,3'-dithiobispropion-imidate	(Lys)	Neighbouring protein pairs	491
Ribosomal subunit (40S)	Rabbit reticulocyte	2-Iminothiolane	$-NH_2$, —SH	Investigation of topography	492
Ribosomal subunits (70S)	*E. coli*	Dimethyl 3,3-dithiobispropion-imidate	Lys	50% crosslinking after 40% Lys modification	493
Ribosome (70S)	*E. coli*	*N*-(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)maleimide	Cys	E.s.r. study of ribosome–tRNA interaction	494
Ribulosebisphosphate carboxylase	Tobacco	2,3-Butanedione, phenylglyoxal	Arg	Arg residues in catalysis	495
Ribulosebisphosphate carboxylase	Spinach	Diethylpyrocarbonate Rose-Bengal-sensitized photo-oxidation	His His	Essential His residues	496
Ribulosebisphosphate carboxylase	Spinach	CO_2 + diazomethane	Lys	Carbamate intermediate trapped	497
Ribulosebisphosphate carboxylase-oxygenase	Potato	DTNB	Cys	Role of Cys in cold-adaptation	498
Ribulosebisphosphate carboxylase-oxygenase	*Rhodospirillum rubrum*, spinach	2-Bromo-1,5-dihydroxy-3-pentanone 1,5-bisphosphate		Irreversible inactivation	499

Ricin A-chain/IgM	*Ricinus sanguineus*	3-(2-Pyridyldithio)propionic acid/carbodi-imide		Immunotoxin	500
Ricin	*Ricinus sanguineus*	*N*-Acetylimidazole	Tyr	Toxicity inhibition	501
Ricin	*Ricinus sanguineus*	SPDP		Crosslinking to monoclonal antibody	502
RNA polymerase	*E. coli* MRE 600	4-Azidophenyl mono- and di-nucleotides		Photoaffinity probe of subunit orientation during RNA synthesis	503
Saccharotropine dehydrogenase (L-lysine forming)	Yeast	2,3-Butanedione	Arg	One essential Arg	504
Sarcoplasmic reticular proteins	Rabbit skeletal muscle	[^{32}P]8-N_3-cAMP		Location and characterization of glycoprotein	505
Sarcosine oxidase	*Corynebacterium* sp. U-96	Iodoacetamide, *p*-chloromercuri-benzoate, *N*-ethylmaleimide	Cys	Activity inhibited	506

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Scrapie agent	Hamster	Diethylpyrocarbonate	His	Reversible abolition of infectivity	507
Scrapie agent 'hydrophobic protein'	Hamster	Diethylpyrocarbonate	(His, Ser, Tyr)	Evidence for protein necessary for infectivity	508
Serine hydrolase	Rat-spleen lymphocyte	Dithiothreitol, *N*-ethylmaleimide	Cys Cys	Essential free SH group	509
Serine proteases	Various	3′,6′-Bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]-spiro{isobenzofuran-1-(3-H)-9′-[9H]xanthen}-3-one (FDE)	Ser	Fluorogenic active-site titrant	510
Serine transhydroxy-methylases	Lamb/rabbit liver	D-Fluoroalanine	Cys	Suicide inhibition	511
Serotonin receptor	Human platelets	2-Nitroimipramine		Apparently irreversible inhibitor	512
Sodium channels	Rat brain	2-Azidoanthraniloyl glycine hydrazide derivatives of oxidized tetrodotoxin		Production of fluorescent labelled channel	513
Sorbitol dehydrogenase	Sheep liver	Sodium iodoacetate	Cys	Similarities to alcohol dehydrogenases	514
Spectrin, glycophorin	Human	MAL-6	Cys	Erythrocyte membrane studies	515
Succinate dehydrogenase	Bovine heart	Diethylpyrocarbonate	His	Essential His residue	516
Succinate dehydrogenase	Rat-liver mitochondria	*N*-Substituted maleimides	Lys	Two classes of essential SH	517
Succinate thiokinase	Pig heart	*S*-Methanosulphonyl-CoA	Cys	Acyl-CoA sites	518
Succinate ubiquinone reductase (complex II)	Mitochondria	(Arylazido)phospholipids		Interaction with photoactivated lipid vesicles	519
Terrilytin	*Aspergillus terricola*	Human-serum albumin		Conjugation reduces acute toxicity	520
Tetanus toxin	*Clostridium tetani*	Glutaraldehyde		Linking to cells	521

Thiolsubtilisin	*Bacillus amyloliquefaciens*	2,2-Dipyridyl disulphide	Cys	Titration of semi-synthetic enzyme	522
Thioltransferase (glutathione : disulphide oxidoreductase	Porcine pituitary	*N*-Ethylmaleimide	Cys	Essential Cys	523
α-Thrombin	Human	Various modifications		Study of fibroblast thrombin receptors	524
α-Thrombin	Human	Pyridoxal 5′-phosphate	Lys	Inhibition of thrombin–platelet interaction	525
α-Thrombin and γ-thrombin	Human	13 spin-labelled phenylsulphonyl fluoride derivatives	Ser-205	E.s.r. mapping of active-site topography	526
α-Thrombin and γ-thrombin	Human	Dansyl hydrazide/periodate/borohydride		Fluorescence labels	527

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Table *(cont.)*

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Thymidylate synthetase	*Lactobacillus casei*	1-(2-Deoxyribosyl)-8-azapurin-2-one 5′-monophosphate		Enzyme-generated covalent transition-state analogue	528
Thymidylate synthetase	*Lactobacillus casei*	Diethylpyrocarbonate	His	Role of histidine in structure	529
Thyroglobulin	Hog	Iodine, iodide–chloramine T, iodine monochloride	Tyr	Hormone synthesis studies	530
Thyrotropine-releasing hormone receptor	Rat	Thyrotropine-releasing hormone diazomethyl ketone and chloromethyl ketone		Antagonists *in vivo*	531
Thyroxine-binding globulin	Human serum	*N*-Bromoacetyl-α-thyroxine	Met, Lys, Tyr, His	Abolition of thyroxine binding	532
Tissue proteins	Human liver	NaB^3H_4/[^{14}C]formaldehyde		Used for precision two-dimensional PAGE	533
Toxin II	*Anemonia sulcata*	Various	Lys, Arg, His, Asp	Structure–function relationships	534
Transferrins (various)	Human, rabbit, bovine, chicken	Diethylpyrocarbonate	His, (Tyr)	Role of His in Fe-binding sites	535
Transhydrogenase	Bovine-heart mitochondrion	DCCI	—CO_2H	Single residue modified at low concentrations, dimers formed at high concentrations	536
Tri-iodothyronin receptor	Rat-liver nucleus	*N*-Bromoalkyl derivatives of thyronin		Specific labelling	537
Troponin	Bovine heart	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	Thiols	SH role in component interactions	538
Troponin C	Rabbit leg muscle	Acetic anhydride	Lys	Structural studies	539
Troponin C	Rabbit skeletal muscle	*N*-[*p*-(2-Benzimidazolyl)phenyl]-maleimide, *N*-(1-anilinonaphthyl-4)maleimide, dansylaziridine	Cys-98	Fluorescence study of Ca^{2+} binding	540
Troponin C, α-tropomyosin, actin	Rabbit skeletal muscle	*N*-(4-Azidobenzoylglycyl)-*S*-(2-thiopyridyl)cysteine	Cys	Water-soluble, stable, heterobifunctional photoaffinity crosslinking reagent	541

Troponin C, troponin I	Rabbit skeletal muscle, bovine cardiac muscle	*N*-(4-Azidobenzoyl[2-^{3}H]glycyl)-*S*-(2-thiopyridyl)cysteine	Cys-98	Study of protein–protein interactions	542
Troponin T	Rabbit leg muscle	Acetic anhydride	Lys	Probe of troponin T structure and function	543
Trypsin	Bovine	Vinylpyrrolidone–acrolein copolymer		Thermal denaturation study	544
Trypsin inhibitor	Bovine pancreas	Methyl iodide	Met-52	Affects structural role of Met-52	545
Trypsin inhibitor	Soybean	Polyvinylpyrrolidone			546
Tryptophanase	*E. coli* B/IE7-A	Tetranitromethane	Tyr	Identification of active-site residues	547
Tryptophan synthase	*E. coli*	SPDP, various bismaleimides	(Cys)	Crosslinking of active centres in tetramer	548
Tryptophanyl-tRNA synthetase	Bovine pancreas	Diethylpyrocarbonate DTNB	His Cys	Identification of Zn-binding residues	549

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Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Tryptophanyl-tRNA synthetase	Beef pancreas	Mesitoyl-AMP		Evaluation of subunit interactions	550
Tryptophanyl-tRNA synthetase	Beef pancreas	Mesitoyl-AMP			551
Tubulin	Pig brain	2,3-Butanedione	Arg	Arg involved in microtubule assembly	552
Tubulin	Bovine brain	Iodoacetamide		SH reactivity as conformational probe	553
		NN'-Ethylenebis(iodoacetamide)	Cys		
Tubulin	Bovine brain	Iodoacetamide		Effects of vinblastine and maytansine on alkylation	554
Tyrosinase	*Neurospora crassa*	Methylene Blue/*hv*	His	Photo-oxidation study	555
UDP-glucose dehydrogenase	Bovine liver	Iodo[2-^{14}C]acetate	Cys	Active-site tryptic peptide isolated	556
		Iodoacetamidofluorescein			
Urate oxidase	*Streptomyces cyanogenus*	Various —SH reagents	Cys	Inhibition of activity	557
Urate oxidases	Pig liver, *Candida utilis*	Polyethylene glycol (activated)	—NH_2	Immune response and plasma half-life	558
Uricase	*Candida utilis*	2,4-Bis-(*O*-methoxypolyethylene glycol)-6-chlorotriazine	Lys	Reduced antigenicity	559
Uricase	*Candida utilis*	Naphthoquinone disulphonic acid	—NH_2	Two —NH_2 per subunit important for activity	560
Uteroglobulin and other steroid-receptor proteins	Rat-liver cytosol, rabbit-uterus cytosol	Progesterone and other steroids		Direct photoaffinity labelling	561
Various	Various	Bis-(1,1-trifluoroacetoxy)iodobenzene	Asn, Gln	Conversion of carboxamide to amine	562
Various	Various	4-(3'-Phenyl-2'-pyrazolin-1'-yl)-benzenesulphonyl chloride		Fluorescence probe	563
Various	Various	*p*-Nitrophenylglyoxal	Arg	Colorimetric determination of Arg	564
Various	Various	Peroxidized phospholipid		Covalent reaction	565

Various	Rat	2,3-[^{14}C]Acrylonitrile	Thiols		566
Various	Various	Various/liposomes		Liposome immobilization techniques reviewed	567
Various polypeptides	Various	Dimethylaminobenzene isothiocyanate		High-sensitivity h.p.l.c. separation	568
Various	Various	Methyl acetimidate/methylamine	(Lys)	Acetimidoyl groups removable by amine	569
Various polypeptides	Various	Hydrazine	Arg and peptide bonds	Deguanidation and peptide-bond cleavage	570
Various	Various	*O*-(4-Diazo-3,5-di[^{125}I]iodo-benzoyl)sucrose		Radiolabelling procedure	571
Various	Various	*O*-Iodosobenzoic acid	Tyr and Trp	Oxidative halogenation *not* due to iodobenzoic acid	572

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[562] L. M. Soby and P. Johnson, *Anal. Biochem.*, 1981, **113**, 149.
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[566] H. Peter and H. M. Bolt, *Xenobiotica*, 1981, **11**, 51.
[567] V. P. Torchilin and A. L. Klibanov, *Enzyme Microb. Technol.*, 1981, **3**, 297.
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[572] A. Fontana, D. Dalzoppo, C. Grandi, and M. Zambonin, *Biochemistry*, 1981, **20**, 6997.

Table (*cont.*)

Protein	*Source*	*Reagent*	*Residue*	*Comments*	*Ref.*
Various	Various	*O*-Iodosobenzoic acid	Tyr	Modification by *O*-iodoxybenzoic acid impurity	573
Various oligomeric enzymes	Various	L-Glutamyl chloromethyl ketone, iodoacetic acid, 5-(iodoacetamidoethyl)aminonaphthalene-1-sulphonate		'Half of sites' reactivity does not necessarily imply assymmetry	574
Various proteins and membrane proteins	Sheep erythrocyte	SPDP	Lys, Cys	Preparation of protein-labelled cells	575
Various proteins	SV40-infected African Green monkey kidney cells	Diazophenylthioether paper		Immunological detection of proteins	576
Various proteins	Various	Periodate	Cys (vicinal)	Phosphate analogue	577
Vitamin D-binding protein	Human plasma	Various	Various	Modifications of Lys, Arg, and Tyr affect activity	578

* Abbreviations used in this Table: BNPS 2-nitrophenylsulphenyl bromide, CMC 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide metho-*p*-toluenesulphonate, DFP diisopropyl phosphofluoridate, EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide, FDNB 1-fluoro-2,4-dinitrobenzene, FITC fluorescein isothiocyanate, IAEDANS *N*-iodoacetyl-*N'*-(1-naphthyl-5-sulphonic acid) ethylene diamine, NEM *N*-ethylmaleimide, MAL-6 *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl) maleimide, SPDP *N*-succinimidyl 3-(2-pyridyldithio)propionate, TLCK N^{α}-(*p*-toluenesulphonyl)lysine chloromethyl ketone, TNBS 2,4,6-trinitrobenzenesulphonic acid.

573 W. C. Mahoney, P. K. Smith, and M. A. Hermodson, *Biochemistry*, 1981, **20**, 443.
574 J. Herzfield, T. Ichiye, and D. Jung, *Biochemistry*, 1981, **20**, 4936.
575 Y. H. Jou and R. B. Bankert, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 2493.
576 J. Reiser and J. Wardale, *Eur. J. Biochem.*, 1981, **114**, 569.
577 M. Rippa, T. Bellini, M. Signorini, and F. Dallochio, *J. Biol. Chem.*, 1981, **256**, 451.
578 M. Kawakami and D. S. Goodman, *Biochemistry*, 1981, **20**, 5881.

2 Investigations of Known and Novel Reagents and Reactions

Butane-2,3-dione.—Butane-2,3-dione inactivates various aspartyl proteinases not by modifying Arg but by photosensitized modification of Tyr and Trp.[579] Although the modification has not been chemically characterized, oxindole is unlikely to be the major initial product from Trp. Thus butane-2,3-dione remains a specific reagent for Arg in the dark, at pH 8, and in the presence of borate, but as a photo-oxidant it is limited by its lack of specificity.

Diethyl Pyrocarbonate.—The inactivation of thymidylate synthetase (*L. casei*) by diethylpyrocarbonate is associated (spectroscopically) with modification of a large proportion of His residues (and negligible Tyr or Cys), but the modification is not reversed by hydroxylamine.[580] In the case of transferrin it is suggested that this may be due either to conformational changes or to *NN'*-dicarbethoxylation.[581]

Iodoacetate and Iodoacetamide.—In liver alcohol dehydrogenase, zwitterionic buffers like MES, PIPES, and EPPS protect against affinity labelling by iodoacetate to a lesser degree than small ionic buffers like phosphate, particularly below pH 9, although CMES had an anomalously high affinity.[582] Buffer-dependent dissociation constants and first-order rate constants have been determined as functions of pH. In the presence of imidazole, iodoacetate, iodoacetamide, and similar halo compounds alkylate Cys-46 as a metal–thiol complex with increased nucleophilicity, owing to an inner-sphere ligand-field effect.[583] (See also the section 'Affinity Labelling'.)

***o*-Iodosobenzoic Acid.**—Cleavage at and modification of Tyr by this reagent have been stated[584] to result from *o*-iodoxybenzoic acid, an impurity arising from disproportionation and which is removed by pre-incubating with *p*-cresol, allowing selective cleavage at Trp in various proteins.

In cytochrome C (horse heart)[585] cleavage occurs at Tyr and Trp in the presence of 4M guanidinium hydrochloride but at Trp alone in the presence of *p*-cresol. However, here it is concluded that Tyr cleavage is due to the *o*-iodosobenzoic acid–guanidine-HCl–acetate mixture, rather than *o*-iodoxybenzoic acid, as it only occurs in the presence of halides.

Methyl Acetimidate.—The rate of hydrolysis of methyl acetimidate and its rate of amidination of denatured aldolase have been reinvestigated as functions of temperature, pH, and ionic strength (although not for native aldolase) and varied strongly and to a similar extent between 0 and 40 °C. As the mechanism is believed to involve attack of the unprotonated Lys ε-NH_2 on protonated methyl acetimidate, the pH optimum lies between the two pK_as (approximately 7.5 and 10.5, respectively), probably close to 8.8.

[579] J.-C. Eripon and T. Hofmann, *Biochem. J.*, 1981, **193**, 55.
[580] H. H. Daron and J. L. Aull, *Biochim. Biophys. Acta*, 1981, **658**, 132.
[581] J. Mazurier, D. Leger, V. Tordera, J. Montreuil, and G. Spik, *Eur. J. Biochem.*, 1981, **119**, 537.
[582] C. Syvertsen and J. S. McKinley-McKee, *Eur. J. Biochem.*, 1981, **117**, 165.
[583] K. H. Dahl and J. S. McKinley-McKee, *Eur. J. Biochem.*, 1981, **120**, 451.
[584] W. C. Mahoney, P. K. Smith, and M. A. Hermodson, *Biochemistry*, 1981, **20**, 443.
[585] A. Fontana, D. Dalzoppo, C. Grandi, and M. Zambonin, *Biochemistry*, 1981, **20**, 6997.
[586] A. J. Makoff and A. D. B. Malcolm, *Biochem. J.*, 1981, **193**, 245.

A new method for the rapid and quantitative removal of acetimidoyl groups from proteins or peptides modified by alkyl acetimidates has been described.[587] Incubation with 3.44M methylamine·HCl buffer at pH 11.5 and 25 °C for 4 hours gives 95% removal (half-life approximately 26 minutes, 29-fold less than using NH_3), with no deleterious effects on primary structure. The method was successfully applied to the sequencing of a mouse immunoglobulin heavy-chain peptide, and it is a valuable alternative to citraconylation and maleylation.

***N*-(3-Pyrenyl)maleimide.**—The usefulness of this compound as a fluorescent probe was found to be limited by its lack of specificity, as several spectroscopically distinct products (involving covalently bound adsorbed or hydrolysed probe) contribute to the emission of labelled bovine serum albumin.[588]

Tetranitromethane.—Two studies demonstrate that nitration of tyrosine by tetranitromethane produces no gross changes in molecular size, conformation, or subunit structure. Native and nitro-α-proteinase inhibitor monomer have the same elution volume on Sephadex G100 and the same catabolic rate when injected into rats. No modification of Met or Trp was observed at pH 8.0. The approximate 12% yield of polymeric nitro-α_1PI is presumably due to the formation of biphenyl bonds.[589]

When tryptophanase was modified by tetranitromethane, no dityrosine was detected by amino-acid analysis, although there were low levels (<0.5 mole per mole enzyme) of methionine sulphoxide formation.[590]

Triazine Dyes.—Some preparative and analytical applications of triazine dyes have been reviewed.[591]

Procion Green H-4G (a monochlorotriazinyl Cu-phthalocyanine dye) was found to be better than Cibacron Blue F3G-A for isolating yeast hexokinase, as it displays an apparently unique interaction with the enzyme. The binding of hexokinase to agarose-Procion Green H-4G is promoted by Mg^{2+}, and elution is with ATP or *d*-glucose. The reactivity of the chlorotriazine group with nucleophiles, the high molar extinction coefficient, and the high stability and solubility make reactive triazine dyes ideal affinity labels for general use with nucleotide-dependent enzymes.[592] In free solution, Procion Green H-4G is an active-site-directed irreversible inhibitor and alkylates hexokinase at Cys-243.

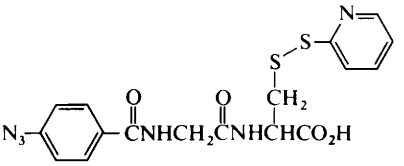
Crosslinking Reagents.—This has been a very active area, with several new reagents being introduced and the scope of application of existing ones increasing.

A wide range of reagents has been used for studying protein subunit structure or topology and protein–protein interactions. Simple reagents like dimethyl suberimidate [593] and tartryl diazide [594] continue to be used to crosslink protein subunits. With *E. coli* aspartase, SDS-PAGE densitometric patterns were compared with

[587] G. C. Dubois, E. A. Robinson, J. K. Inman, R. N. Perham, and E. Appella, *Biochem. J.*, 1981, **199**, 335.
[588] B. Lux and D. Gerard, *J. Biol. Chem.*, 1981, **256**, 1767.
[589] A. Feste and J. C. Gan, *J. Biol. Chem.*, 1981, **256**, 6374.
[590] T. Nihara, T. Toraya, and S. Fukui, *Eur. J. Biochem.*, 1981, **119**, 273.
[591] C. R. Lowe, D. A. P. Small, and A. Atkinson, *Int. J. Biochem.*, 1981, **13**, 33.
[592] Y. D. Clonis, M. J. Goldfinch, and C. R. Lowe, *Biochem. J.*, 1981, **197**, 203.
[593] S. D. Putney, R. T. Sauer, and P. R. Schimmel, *J. Biol. Chem.*, 1981, **256**, 198.
[594] W. W. C. Chan and C. A. Enns, *Can. J. Biochem.*, 1981, **59**, 461.

theoretical distributions and with electron micrography, thus providing a useful means of investigating quaternary structure in the absence of good crystals.[595]

Several new reagents have been described for investigating protein–protein interactions and isolating or characterizing receptors, the most versatile of which is the photoactivatable heterobifunctional reagent *N*-(4-azidobenzoyl glycyl)-*S*-(2-thiopyridyl)cysteine (AGTC) (1).[596]



(1)

Synthesized by coupling of the *N*-hydroxysuccinimide ester of azidobenzoyl glycine to *S*-(2-thiopyridyl)-Cys, AGTC dissolves readily in aqueous buffer at pH 7.5, is stable at room temperature at pH 3—9, and resists degradation after exposure to laboratory fluorescent lighting for one week. The disulphide bond is stable to the conditions for arylazido activation. After 2 h of reaction, 90% of AGTC had reacted with troponin C, α-tropomyosin, or actin through the S—S moiety (monitored *via* pyridine-2-thione release at 343 nm). After treating AGC-tropomyosin with 6M urea and 0.1M dithiothreitol for 16 h, 92% of the photoactivatable probe was removed from the tropomyosin SH, which could be relabelled with AGTC, indicating the reversibility of SH attachment.

Using the troponin C–troponin I (TnC–TnI) interaction as a test system, AGTC reacted quantitatively at Cys-98 of TnC;[597] photolysis of an AGC-TnC–carboxymethylated TnI mixture at 350 nm gave a covalent 1 : 1 TnC–TnI complex. The transfer of labelled AGC from TnC Cys-98 to TnI was completed using dithiothreitol. The maximum crosslinking range of AGTC is approximately 14 Å.

Another photoactivatable heterobifunctional crosslinking reagent, 4-(6-formyl-3-azidophenoxy)butyrimidate, has been used to identify elongation factor G binding proteins in 70S ribosomes.[598] 6.5 moles of crosslinker were incorporated per mole of EFG, and the identification of neighbouring proteins was based on the transfer of radioactivity from EFG, the crosslinked complexes being cleaved by aminolysis. The same reagent has also been used to crosslink polypeptide chains in the L7/L12 dimer[599] (the imidate reacting with Lys-51 of L7/L12, and using reductive alkylation rather than photoactivation) and to identify the lymphoblastoid cell receptor for the Sindbis virus. Again, reductive methylation with NaB^3H_4 was used, subsequent aminolysis leaving ^3H attached to the receptor.[600]

Two more new heterobifunctional reagents, 4-azido-2,3,5,6-tetrafluoropyridine and 4-azido-3,5-dichloro-2,6-difluoropyridine, crosslinked vicinal RNA to protein

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[596] P. C. S. Chong and R. S. Hodges, *J. Biol. Chem.*, 1981, **256**, 5064.
[597] P. C. S. Chong and R. S. Hodges, *J. Biol. Chem.*, 1981, **256**, 5071.
[598] J. A. Maassen and W. Möller, *Eur. J. Biochem.*, 1981, **115**, 279.
[599] J. A. Maassen, E. N. Schop, and W. Möller, *Biochemistry*, 1981, **20**, 1020.
[600] J. A. Maassen, and C. Terhorst, *Eur. J. Biochem.*, 1981, **115**, 153.

amino groups in 30S ribosome subunits. When reacted at a mole ratio of 1000:1 for 12 minutes at 37 °C, then irradiated at 302 nm for 10 minutes, these reagents gave a maximum crosslinking yield of only 3.5%.[601]

p-Nitrobenzylmaleimide (PNBM), designed to react with the unique Cys-66 of initiation factor 3, is another two-stage crosslinker, involving electrophilic and photochemical modification.[602] IF3 and 30S ribosome subunits were crosslinked by two procedures: (i) irradiating a complex between PNBM·IF3 and the 30S subunit and (ii) irradiating the IF3·30S complex in the presence of PNBM.

The use of 2-iminothiolane to crosslink ribosomal proteins extends that of dimethylsuberimidate, by linking additional protein pairs. 2-Iminothiolane has been used to identify proteins at the S1 binding site in 70S and 30S particles [603] (by incubating a 30S–70S mixture with 5 mM reagent for 2.5 h at 0 °C) and to investigate the topography of the 40S subunit.[604] In both cases, crosslinked complexes were identified by two-dimensional PAGE. Reaction with 2-iminothiolane gives amidine-linked sulphydryl derivatives of protein amino groups; mild oxidation by H_2O_2 and addition of iodoacetamide follow.

Identification of the rat-liver membrane receptor for glucagon [605] and of the superior cervical ganglia receptor for nerve growth factor [606] using *N*-hydroxysuccinimidyl 4-azidobenzoate has been reported, and a similar reagent, *N*-hydroxysuccinimidyl 4-azidobenzoylglycine, has been used to label choriogonadotropin receptor polypeptides by binding the labelled hormone to the granulosa cell surface, followed by photolysis.[607]

Potentially useful for membrane-receptor studies is *N*-hydroxysuccinimidyl 3-[(4-azidophenyl)dithio]propionate, up to 5.5 moles of which were attached to Con A tetramer.[608]

Therapeutic protein conjugates have been reported in increasing numbers; crosslinking is used to synthesize either controlled-release systems for drugs or targetted toxins, often coupled to monoclonal antibodies.

A highly immunogenic preparation of tetanus toxin was obtained by crosslinking to leukaemic or lymphocyte cells,[609] and crosslinking ricin A-chain to anti-$thy_{1.2}$ IgM using 3-(2-pyridyldithio)propionic acid–DCCI gave a conjugate that was highly toxic towards tumour cells.[610] SPDP has been used extensively, *e.g.* for crosslinking ricin A-chain,[611] methotrexate-bearing liposomes,[612] neocarzino-

[601] R. Millon, J.-P. Ebel, F. Le Goffric, and B. Ehresman, *Biochem. Biophys. Res. Commun.*, 1981, **101**, 784.
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[603] G. Boileau, A. Sommer, and R. R. Traut, *J. Biol. Chem.*, 1981, **256**, 8222.
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[605] G. L. Johnson, V. L. MacAndrew, and P. F. Pilch, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 875.
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[609] E. H. Relyveld and S. Ben-Efraim, *J. Immunol. Methods*, 1981, **40**, 209.
[610] H. E. Blythman, P. Casellas, O. Gros, P. Gros, F. K. Jansen, F. Paolucci, B. Pau, and H. Vidal, *Nature (London)*, 1981, **290**, 145.
[611] I. S. Trowbridge and D. L. Domingo, *Nature (London)*, 1981, **294**, 171.
[612] L. D. Leserman, P. Machy, and J. Barbet, *Nature (London)*, 1981, **293**, 226.

statin,[613] gelonin,[614] and the C36-like glycoprotein of cobra venom[615] to antibodies. The procedure tends to leave the activity of the conjugated protein unimpaired, with greatly enhanced cytotoxicity.

Fluorescent Reagents.—*N*-(1-Pyrenyl)iodoacetamide labels rabbit skeletal-muscle actin at the most reactive sulphydryl.[616] The fluorescence intensity of the product (excited at 365 nm, detected at 407 nm) is enhanced approximately 25-fold upon polymerization of labelled G-actin, giving a highly sensitive probe of conformational changes in the actin–myosin interaction. The binding of meromyosin (or subfragment 1) reduces the intensity to approximately 25%.

The modification of the Ca^{2+}-ATPase moiety of the sarcoplasmic reticulum by another Cys-specific reagent, *N*-(1-anilinonaphthyl-4)maleimide, is bi-phasic, as two Cys residues are affected.[617] Addition of Mg-ATP increases the fluorescence intensity of one of these, reflecting conformational changes induced by Ca^{2+} translocation.

Four new dansyl thiocholine esters have been synthesized as substrates for fibrinoligase (Factor XIIIa) and transglutaminase.[618] The most suitable of these, [dansyl-(*N*-methyl-β-alanyl)]thiocholine, provided kinetic data (*e.g.* the pH-activity profile for enzyme-catalysed acyl transfer, which implicated His as a prototropic group in catalysis) and allowed formation of fluorescent acyl-enzyme intermediates.

The modification of haemoglobins A and S by 1-anilino-8-naphthalene sulphonate and IAEDANS has shown fluorescence anisotropy decay and static fluorescence polarization to be very informative for studying internal motion in proteins. β^A and apo-β^A subunits were modified at Cys-93 or -112, and significant proportions of the polypeptide chain were found to exhibit rotational freedom.[619]

IAEDANS has also been used to investigate ribosome structure and function. The interaction between protein L7 (modified by IAEDANS at the *C*-terminal COOH with 0.29 mole dye per L7 monomer, with considerable retention of function) and yeast $tRNA^{Phe}$ (modified by fluorescein thiosemicarbazide near the anticodon or 3′ end) has been studied in 50S of 70S conjugates *via* fluorescence quenching, steady-state anisotropy, and singlet–singlet energy-transfer measurements.[620] A model in which the four L7/L12 copies are arranged in parallel on the ribosome has been proposed on the basis of these results, and it broadly confirms electron-microscope studies that show L7/L12 protruding into solution but that fail to pinpoint the CO_2H termini.

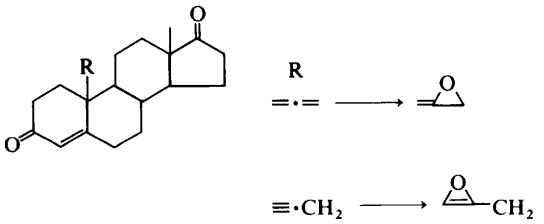
Tritium-labelled IAEDANS was used to study the partial reconstitution of L7/L12-deficient ribosomes with L7/L12 fluorescent protein conjugates.[621] The labelling is semi-specific for the CO_2H terminus, although specific modification

[613] G. Jung, W. Köhnlein, and G. Luders, *Biochem. Biophys. Res. Commun.*, 1981, **101**, 599.
[614] P. E. Thorpe, A. N. F. Brown, W. C. J. Ross, A. J. Cumber, S. I. Detre, D. Caird Edwards, A. J. S. Davies, and F. Stirpe, *Eur. J. Biochem.*, 1981, **116**, 447.
[615] C. W. Vogel and H. J. Müller-Eberhard, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 7707.
[616] T. Kouyama and K. Mihashi, *Eur. J. Biochem.*, 1981, **114**, 33.
[617] K. Miki, T. L. Scott, and N. Ikemoto, *J. Biol. Chem.*, 1981, **256**, 9382.
[618] V. P. Williams, C. Fried, and G. Popjak, *Arch. Biochem. Biophys.*, 1981, **206**, 353.
[619] J. Oton, E. Bucci, R. F. Steiner, C. Fronticelli, D. Franchi, J. Montemarano, and A. Martinez, *J. Biol. Chem.*, 1981, **256**, 7248.
[620] C. C. Lee, B. D. Wells, R. H. Fairclough, and C. R. Cantor, *J. Biol. Chem.*, 1981, **256**, 49.
[621] C. C. Lee, C. R. Cantor, and B. Witlman-Liebold, *J. Biol. Chem.*, 1981, **256**, 41.

has no effect on activity according to analysis of the poly-Phe synthesis and GPTase activity assay results. It is concluded that *C*-terminally modified IAEDANS derivatives are the most suitable fluorescent conjugates available for studies of ribosome structure and function.

Mechanism-based Inhibitors.—Interest in enzyme substrates that utilize the catalytic process to generate reactive intermediates (suicide or mechanism-based inhibition) has continued to grow and the topic has been reviewed.[622] Studies on the β-elimination reactions that follow imine formation between suicide substrates and pyridoxal 5′-phosphate (PLP) have shed light on the mechanism of inhibition of PLP-dependent enzymes. For example, (DL)-βββ-trifluoroalanine was found to be an extremely efficient inhibitor of alanine racemase by virtue of a low partition ratio between turnover and inactivation *via* a β-difluoroenamino-PLP adduct.[623] In the case of 4-aminobutyrate aminotransferase, inactivation by ethanolamine-*O*-sulphate follows elimination of sulphate from the PLP adduct. This enzyme is also efficiently inactivated *in vitro*[624] and *in vivo*[625] by 4-amino-5-halopentanoic acids.

In a second class of suicide substrates, electrophilic enzyme-bound intermediates are generated by rearrangement or oxidation of alkynes, allenes, or cyclopropanes. An interesting case of oxygen insertion occurs in the oxidation of



Scheme

the 10-allenyl and 10-alkynyl steroids by aromatase (see the Scheme).[626] Formation of the allene oxide or oxirene leads to inactivation of the enzyme, which is postulated to occur *via* the cyclopropanone or ketocarbene isomerization products. Alkylation with an allenic ketone is the mechanism for the inactivation of hydroxysteroid dehydrogenase by the allenic secosteroid (4β)-*S*10-secoestra-4-*S*-diene-3β,17β-diol-10-one.[627] Ketosteroid isomerase is also inactivated by pregnyne and estryne through a similar mechanism.[628] In the latter case the initial site of attachment to the protein was determined and shown to be the amide nitrogen of the asparagine side chain.

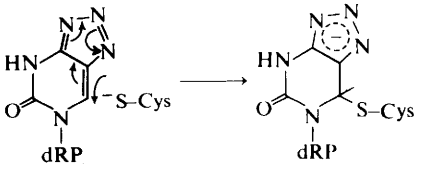
Suicide substrates in which nitrosoamine precursors act as sources of carbonium ions have been investigated further. *N*-Nitroso-1,4-dihydro-6,7-dimethoxy-3(2H)-isoquinoline has been shown to be a substrate of α-chymotrypsin that forms an

[622] B. W. Metcalf, *Annu. Rep. Med. Chem.*, 1981, **16**, 289.
[623] E. A. Wang and C. Walsh, *Biochemistry*, 1981, **20**, 7539.
[624] R. B. Silverman and M. A. Levy, *Biochemistry*, 1981, **20**, 1197.
[625] R. B. Silverman, M. A. Levy, A. J. Multar, and J. D. Hirsch, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 520.
[626] B. W. Metcalf, C. L. Wright, J. P. Burkhart, and J. O. Johnston, *J. Am. Chem. Soc.*, 1981, **103**, 3221.
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[628] T. M. Penning, D. F. Covey, and P. Talalay, *J. Biol. Chem.*, 1981, **256**, 6842.

intermediate acyl-enzyme in which the acyl group can undergo elimination of the nitrosoamine to give an enzyme-bound carbonium ion.[629] The inactivation of acetylcholinesterase by alkyl (acyloxymethyl) nitrosoamines appears to follow a similar pathway except that the nitrosoamine formed is not covalently bound to the enzyme and can rearrange to a diazo compound, which in turn may be photolysed to give a carbene. This process has been termed photosuicide inactivation.[630]

The formation of stabilized acyl-enzymes has been the subject of numerous reports, but the acylating agents used in most active-site titration studies cannot be regarded as true suicide substrates unless a further irreversible reaction pathway is open to the intermediate. In the case of the inhibition of serine β-lactamases by clavulanic acid, olivanic acids, and penam sulphones a series of studies has clarified the general mechanism of inactivation.[631-633] β-Elimination reactions can occur when the first-formed acyl-enzyme contains a suitable leaving group such as the sulphinate moiety in penam sulphones. The products of such elimination may either stabilize the acyl-enzyme further (resulting, ultimately, in turnover) or react with the protein, usually by Michael addition, to give enamine-type structures.

A novel enzyme-generated and covalently bound intermediate that is also a transition-state analogue has been reported.[634] 1-(β-D-2′-Deoxyribofuranosyl)-8-azapurin-2-one 5′-monophosphate (2a) reacts with thymidylate synthetase at an active-site cysteine to generate an anionic triazole derivative (2b) that mimics the carbanionic intermediate of the normal enzyme reaction.



(2a) (2b)

Although, in general, the structural requirements for suicide inactivation reflect the restricted specificity of the target enzyme, *S*-adenosyl homocysteine hydrolase is unusual in that it is inactivated by a wide variety of nucleoside analogues.[635] The inactivation may be mediated by enzymatic oxidation of nucleoside to 3-ketoadenosine derivatives that can give rise to reactive 4-methylene functions by elimination of water from the 5-hydroxymethyl group.

Affinity Labelling.—A review on the affinity labelling of hormone receptors has appeared.[636] There has been considerable interest in chloro- and diazo-methyl

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ketone derivatives of oligopeptides as specific alkylating agents for proteases involved in coagulation and related processes. The specificity conferred by, for example, tripeptides enables a considerable degree of selective inactivation of enzymes with similar primary specificity to be achieved. For example, Pro-Phe-Arg chloromethyl ketone, a specific inhibitor of plasma kallikrein, is a much more effective inhibitor of the thrombin-like snake-venom enzyme crotalase than are Ile-Pro-Arg and Gly-Val-Arg chloromethyl ketones, which are powerful inhibitors of human thrombin.[637] The diazomethyl ketone of Z.Phe-Phe was found to be a specific inactivator of the cysteine protease cathepsin L, although the site of attachment was not identified.[638] N^{α}-Tosyl-L-lysine chloromethyl ketone (TLCK), an affinity label often thought to be specific for histidine in the active centre of trypsin-like serine proteases, has been shown to inactivate cAMP- and cGMP-dependent protein kinases.[639]

An enkephalin analogue, Tyr-D-Ala-Gly-Phe-Leu chloromethyl ketone, irreversibly inactivated a population of enkephalin receptors in rat-brain membrane preparations.[640] Although there must always be doubts about the specificity of chloromethyl ketones in such impure tissue preparations, this affinity label appears to be superior to some photoaffinity labels. Chloromethylphosphonate, an unusual example of an alkylating affinity label for inorganic phosphate binding sites, has been reported to inactivate ATPase.[641] (*RS*)-2-Bromo-3-(5-imidazolyl)propionic acid and iodoacetic acid have been used in a series of studies on affinity labelling of liver alcohol dehydrogenase.[642-644]

The same enzyme was also inactivated by 3-chloroacetylpyridine adenine dinucleotide, but imidazole inhibited the interaction of this affinity label with the enzyme.[645] The mesitoyl moiety has been used as a leaving group in mesitoyl-AMP, an affinity label for tRNA synthetase.[646] *S*-Methanesulphonyl coenzyme A is reported to inactivate succinate thiokinase, probably by exchange of the methanesulphonyl group with an enzyme sulphydryl.[647]

The use of photoactivated affinity labels for the chemical characterization of enzymes and receptors continues to be a very active area of research. Since the preparation of the last report on this topic, the use of aryl azides as photoaffinity labels has been reviewed[648] and specialist reviews on the labelling of cytokinin[649] and purinergic[650] receptors have appeared.

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[650] N. J. Cusack and S. M. O. Houdan, *Receptor Recognition, Ser. B*, 1981, **12**, 325.

The use of hydrophobic photolabels to explore the topology of intrinsic membrane proteins has increased. 3-(Trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)-diazirine has been advocated as a hydrophobic carbene precursor which is less susceptible to photoisomerization (and production of the diazo compound) than either 3-phenyl-3H- or 3-adamantyl-3H-diazirine.[651] 1-Azidopyrene has been used to label retinal-rod outer-segment disk membranes.[652] The labelling appeared to be restricted to the lipophilic surface of rhodopsin. The interactions of vesicular stomatitis virus proteins with lipid membranes were studied using 5-[^{125}I]iodo-1-azidonaphthalene, which provided a labelling pattern sensitive to the effects of temperature-sensitive mutation.[653] A variety of arylazidophosphatidylcholines have been synthesized and used to label ATPase from sarcoplasmic reticulum.[654]

8-Azido derivatives of cAMP and cGMP have been very widely studied, and the former has been used to locate cAMP binding proteins in microsomal fractions[655] and other crude cell extracts.[656] The compounds have also been used to study chemotaxis and cell differentiation.[657] Specifically derivatized proteins have been used to probe protein–protein interactions, and in certain cases the site of crosslinking has been located. For example, linkage of an arylazido cytochrome *c* derivative has been found near the amino terminus of cytochrome *c* peroxidase.[658] Similarly, the corticotropin binding domain of bovine serum has been defined through the use of ACTH specifically labelled at Trp-9 with the 2-nitro-4-azidophenylsulphenyl group.[659]

An example of a photoaffinity reagent of which part can be removed from the target protein after labelling has been reported. The 2-azidoanthraniloyl glycine hydrazone derivative of tetrodotoxin gives a fluorescent derivative when used to label sodium channels. The label may be removed with periodate.[660] The chromium complex of arylazido-β-alanyl ATP has been used to inactivate (Na^+, K^+)-ATPase.[661] In an appendix to this study, it has been pointed out that the specificity of a photoaffinity labelling experiment may be checked by looking for saturation kinetics in the inactivation process.[662]

Benzodiazepine receptors have been located by direct photoaffinity labelling with flunitrazepam.[663] Calmodulin has also been labelled with trifluoroperazine.[664] However, except in the case of triplet-state photoactivation of α,β unsaturated ketones[665] the photochemical basis of these experiments is still

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unclear. An example of pseudo-photolabelling has been reported in which light-induced formation of bridged cyclic peroxide of quercetin gives rise to inactivation of malate dehydrogenase.[666]

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

1 Introduction

The year of 1981 has seen a very varied and interesting range of structure determinations for proteins and amino-acids together with several innovations in data-collection and data-processing methods. One of the more exciting methods has been the use of neutron-diffraction studies to examine the exact locations of hydrogen atoms in enzyme active sites, and this has allowed the possible mechanisms of the enzymes to be described with greater certainty.

A large number of crystallizations have been reported for proteins; these are collated in the Table. The protein-structure reports are grouped under fairly general headings, and the wide range of proteins now being studied has led to the category of 'Other Globular Proteins' becoming the largest. Among these proteins the structures of catalase, catabolite gene activator protein, *cro* repressor protein, and vitamin D-dependent calcium-binding protein are of particular interest. The crystal structure of the influenza-virus glycoprotein and the identification of the four antigenic sites have opened a new field of study, and many more viruses are being studied by *X*-ray methods.

In an attempt to shorten the length of this section the detailed tabulation of amino-acid crystal structures has been omitted – readers interested in such structure determinations are referred to the Structure Data Bank for recent results.

2 Methods and Equipment

General Crystallographic Theory.—Two incorrect formulae in International Tables have been reported.[1,2] Thomas[3] has described a useful algorithm, which can be implemented on a digital computer, for determining unit cells, and Clegg[4] has described a procedure for determining the correct lattice symmetry that avoids errors. Hovmöller[5] has defined a general rule for specifying the origin in any space group, and Lovell and Mitchell[6] have described how the molecular-orientation distribution can be derived from a single arbitrary reflection. A method for the refinement of unit-cell parameters from precession photographs has been reported,[7] and Rogers[8] has critically examined the use of Hamilton's ratio test for the assignment of absolute configuration and has proposed an alternative test.

[666] A. P. Seddon and K. T. Douglas, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 15.
[1] W. Clegg, *Acta Crystallogr., Sect. A*, 1981, **37**, 438.
[2] W. Winter, *Acta Crystallogr., Sect. A*, 1981, **37**, 766.
[3] D. J. Thomas, *Acta Crystallogr., Sect. A*, 1981, **37**, 553.
[4] W. Clegg, *Acta Crystallogr., Sect. A*, 1981, **37**, 913.
[5] S. Hovmöller, *Acta Crystallogr., Sect. A*, 1981, **37**, 133.
[6] R. Lovell and G. R. Mitchell, *Acta Crystallogr., Sect. A*, 1981, **37**, 135.
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[8] D. Rogers, *Acta Crystallogr., Sect. A*, 1981, **37**, 734.

Mitra and Mathur [9] have investigated the polarization ratio for *X*-ray diffraction from a perfect crystal, and Cromer and Liberman [10] have discussed the effect of absorption edges on anomalous-scattering factors. A method for choosing the wavelengths in the anomalous-scattering technique with synchroton radiation has been reported.[11] A way of estimating the required neutron-beam time for crystal-structure analysis has been described,[12] and Mair and Wilkins [13] have shown how accurately to determine atomic positions from *X*-ray and neutron data. A joint *X*-ray and neutron data refinement technique has been reported,[14] and a general thermodynamic approach to the structure analysis of crystals has been discussed.[15]

Beurskens [16] has examined the use and interpretation of rotation and translation functions in reciprocal space. Alternative search functions are also proposed. Wilson [17] has described how central-limit theorems may influence the calculation of structure factors, and Mitchell and Lovell [18] have applied cylindrical distribution functions to wide-angle *X*-ray scattering from oriented polymers. Hiragi and Ihara [19] have described a general procedure for the calculation of radii of gyration and scattering intensities of hollow triaxial bodies. Mitchell [20] has reported a simple procedure for calculating the small-angle component of *X*-ray scattering and illustrates its use. The effect of data truncation on the measurability of Bijvoet differences has been discussed.[21]

Direct Methods.—A new way of solving structures by constrained least-squares refinement has been described by Bianchi and co-workers.[22] Two papers on the application of phase relationships to complex structures, one describing RANTAN-random MULTAN [23] and one describing the MAGEX procedure,[24] have appeared. The influence of systematic errors on the refinement of crystal structures using Guinier camera intensity data has been discussed by Scott.[25]

Data-collection Methods and Equipment.—Clegg [26] has described an extension of the learnt profile method of data collection, which allows faster data collection without loss of precision. A further extension to the method has also been reported.[27] Sjölin and Wlodawer [28] have proposed a technique for improving the precision of *X*-ray data collected either on films or with electronic detectors. The

[9] G. B. Mitra and B. K. Mathur, *Acta Crystallogr., Sect. A*, 1981, **37**, 439.
[10] D. T. Cromer and D. A. Liberman, *Acta Crystallogr., Sect. A*, 1981, **37**, 267.
[11] R. Narayan and S. Ramaseshan, *Acta Crystallogr., Sect. A*, 1981, **37**, 636.
[12] W. Jauch and H. Dachs, *Acta Crystallogr., Sect. A*, 1981, **37**, 162.
[13] S. L. Mair and S. W. Wilkins, *Acta Crystallogr., Sect. A*, 1981, **37**, 264.
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[16] P. T. Beurskens, *Acta Crystallogr., Sect. A*, 1981, **37**, 426.
[17] A. J. C. Wilson, *Acta Crystallogr., Sect. A*, 1981, **37**, 808.
[18] G. R. Mitchell and R. Lovell, *Acta Crystallogr., Sect. A*, 1981, **37**, 189.
[19] Y. Hiragi and S. Ihara, *Acta Crystallogr., Sect. A*, 1981, **37**, 378.
[20] G. R. Mitchell, *Acta Crystallogr., Sect. A*, 1981, **37**, 488.
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[22] R. Bianchi, C. M. Gramaccioli, T. Pilati, and M. Simonetta, *Acta Crystallogr., Sect. A*, 1981, **37**, 65.
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[28] L. Sjölin and A. Wlodawer, *Acta Crystallogr., Sect. A*, 1981, **37**, 594.

Table *Preliminary protein crystallization reports*

Protein	*Source*	*Space group*	*Cell dimensions*			
			a/nm	*b*/nm	*c*/nm	*β*/deg
Ribonuclease St.	*Streptomyces erythreus*	*C*2	8.84	3.30	6.90	98.4
Trypsin inhibitor: trypsinogen complex	Pig : bovine	$P2_12_12_1$	6.71	7.55	6.69	—
EcoRI endonuclease + DNA		$P42_12$	18.32	18.32	4·97	—
Aspartate transcarbamylase (catalytic subunit)		$P4_12_12$ or $P4_32_12$	8.2	8.2	29.8	—
Belladonna mottle virus		*R*3	29.6	29.6	72.9	—
Cowpea chlorotic mottle virus		$P2_12_12$	39.4	38.2	39.7	—
Haemoglobin	*Dicrocoelium dendriticum*	P_1	3.71	3.99	4.90	$\alpha = 88.8$ $\beta = 76.8$ $\gamma = 64.6$
		$P6_2$ or $P6_4$	9.15	9.15	2.83	$\gamma = 120$
λIII Bence-Jones protein Cle.	Human	$P2_12_12_1$	11.3	7.23	4.89	—
VL fragment	Human	$P6_2$ or $P6_4$	8.26	8.26	6.03	—
Bence-Jones protein Wat.		$P6_122$ or $P6_522$	10.83	10.83	10.88	—
Galactose-binding protein	*Salmonella typhimurium*	*C*2	11.97	3.72	8.01	123.4
Haemerythrin	*Phascolosoma arcuatum*	$P3_121$ or $P3_221$	7.81	7.81	15.40	—
Collagenase	*Hypoderma lineatum*	*I*422	11.2	11.2	16.6	—
F_{ab} fragment[o]		$P3_121$ or $P3_221$	13.23	13.23	7.38	—
4Fe–4S ferredoxin	*Bacillus thermoproteolyticus*	*P*1	3.296	3.783	3.982	$\alpha = 118.1$ $\beta = 104.2$ $\gamma = 89.7$
Macromycin[r]	*Streptomyces macromomyceticus*	$P2_12_12_1$	4.892	5.471	10.331	—
		$P2_12_12$	4.61	5.44	4.12	—
Ribosomal proteins B-L8 + B-L13	*Bacillus stearothermophilus*	$P4_12_12$ or $P4_32_12$	10.8	10.8	24.2	—
Parvalbumin III_f	*Opsanus tau*	$P2_1$	2.731	5.875	3.083	98.5
Calotropin DI[v]	*Calotropis gigantea*	$P2_12_12_1$	5.75	8.62	4.03	—
Calotropin DII[v]		*C*2	13.8	3.20	4.77	103.80
Tobacco ringspot virus	—	$P2_12_12_1$	38.8	39.6	40.5	—
Histidine decarboxylase	*Lactobacillus* 30a	$I4_122$	22.2	22.2	10.75	—
		*P*321	10.0	10.0	16.4	—

Mol. wt. and no. of subunits	*Mol. wt. of asymmetric unit*	V_M/ $nm^3\,dalton^{-1} \times 10^3$	*Precipitant*	pH	*Ref.*
11 350 (1)	22 700	2.19	Ammonium sulphate	4.1	*a*
31 000 (1 + 1)	31 000	2.73	Magnesium sulphate	6.7	*b*
31 100 (1)	124 400	—	MPD[c]	7.4	*d*
100 000 (3)	1 000 000	2.50	Ammonium sulphate	8	*e*
20 300 (1)	—	3.50	PEG 6000[f]	4.5	*g*
3 528 000 (180)	3 528 000	3.30	PEG 6000	4.0	*h*
16 750 (1)	33 500	1.90	PEG 2000	4—5	*i*
16 750 (1)	16 750	2.04	PEG 2000	4—5	*i*
23 000 (1)	46 000	2.17	Ammonium sulphate	7.0	*j*
23 000 (2)	23 000	2.49	Ammonium	6.5	*k*
23 000 (2)	34 500	—	sulphate	6.5	*k*
33 000 (1)	33 000	2.25	PEG 6000	6.0	*l*
28 000 (2)	56 000	2.42	PEG 4000	8.0	*m*
24 000 (1)	48 000	2.8	Ammonium sulphate	7.15	*n*
48 000 (1)	—	—	Ammonium sulphate	4.5	*p*
11 000 (1)	11 000	2.34	Ammonium sulphate	7.5	*q*
12 000 (1)	24 000	2.88	MPD	8.0	*s*
12 000 (1)	12 000	2.15	MPD	8·0	*s*
66 000 (4 + 1)	132 000	2.75	Ammonium sulphate	6.2	*t*
10 100 (1)	10 100	2.43	Ammonium sulphate	7.0	*u*
23 400 (1)	23 400	2.13	PEG 20 000	7.0	*w*
24 000 (1)	24 000	2.10	PEG 20 000	7.0	*w*
5.8×10^6	5.8×10^6	2.7	PEG 6000	6.4	*x*
208 000 (6 + 6)	104 000	3·18	Ammonium sulphate	4.8	*y*
208 000 (6 + 6)	69 000	3.40	PEG	4.8	*y*

Table (*cont.*)

Proteins	*Source*	*Space group*	*Cell dimensions*			
			a/nm	*b*/nm	*c*/nm	*β*/deg
Triose phosphate isomerase	Yeast	$P2_1$	6.13	9.84	4.97	90.9
Triose phosphate isomerase + 2 phosphoglycolate[z]	—	$P2_1$	7.42	8.29	3.76	102.0
Purine nucleoside phosphorylase	Human erythrocytes	$R32$	14.38	14.38	16.51	$\gamma = 120$
Aspartate trans-carbamoylase[cc]	*E. coli*	$R32$	13.08	13.08	19.38	$\gamma = 120$
Manganese superoxide dismutase	*Thermus thermophilus*	$P4_12_12$ or $P4_32_12$	14.75	14.75	5.59	—
Chorionic somato-mammotropin	Human	$C2$	12.39	3.03	5.38	119.5
Retinol-binding protein	Rat liver	$P2_12_12_1$	4.009	4.739	6.934	—
Cardiotoxin analogue III	Taiwan cobra	$P222$	3.34	5.46	5.78	—
Phospholipase A2	Taiwan cobra	$P3_112$ or $P3_212$	5.78	5.78	12.14	—
α-Chymotrypsin/β-trypsin/Bowman–Birk inhibitor complex	—	$P1$	5.196	5.634	4.670	$\alpha = 98.83$ $\beta = 97.29$ $\gamma = 97.15$
BL17 ribosomal protein[jj]	*Bacillus stearo-thermo-philus*	Ortho-rhombic	3.75	4.89	13.53	—
Monellin[ll]	—	$P2_1$	3.91	7.15	8.69	107.6
Methaemoglobin Barts.	Human	$P3_121$ or $P3_221$	5.585	5.585	15.910	—
Elongation factor complex EF-Tu/EF-Ts	*E. coli*	$P2_12_12_1$	8.17	11·05	20.60	—
α-Cobratoxin[pp]	Siamese cobra	$P6_522$	7.459	7.459	4.289	$\gamma = 120$

(*a*) Y. Yamamoto, K. T. Nakamura, Y. Iitaka, Y. Mitsui, K. Miyamoto, H. Matsuo, K. Narita, and N. Yoshida, *J. Mol. Biol.*, 1981, **145**, 285. (*b*) M. Bolognesi, A. Coda, M. Guarneri, and E. Menegatti, *J. Mol. Biol.*, 1981, **145**, 603. (*c*) MPD = 2-methyl-2,4-pentane diol. (*d*) T.-S. Young, S.-H. Kim, P. Modrich, A. Beth, and E. Jay, *J. Mol. Biol.*, 1981, **145**, 607. (*e*) A. M. Foote, F. K. Winkler, and M. F. Moody, *J. Mol. Biol.*, 1981, **146**, 389. (*f*) PEG = polyethylene glycol. Number refers to average molecular weight. (*g*) K. L. Heuss, J. K. M. Rao, and P. Argos, *J. Mol. Biol.*, 1981, **146**, 629. (*h*) K. L. Heuss, J. K. M. Rao, and P. Argos, *J. Mol. Biol.*, 1981, **146**, 635. (*i*) J. D. G. Smit and K. H. Winterhalter, *J. Mol. Biol.*, 1981, **146**, 641. (*j*) F. J. Stevens, F. A. Westholm, N. Panagiotopoulos, A Solomon, and M. Schiffer, *J. Mol. Biol.*, 1981, **147**, 179. (*k*) F. J. Stevens, F. A. Westholm, N. Panagiotopoulos, M. Schiffer, R. A. Popp, and A. Solomon, *J. Mol. Biol.*, 1981, **147**, 185. (*l*) T. Alber, M. Fahnestock, S. L. Mowbray, and G. A. Petsko, *J. Mol. Biol.*, 1981, **147**, 471. (*m*) L. C. Sieker, L. Bolles, R. W. Stenkamp, L. H. Jensen, and C. A. Appleby, *J. Mol. Biol.*, 1981, **148**, 493. (*n*) A. Ducruix, B. Arnoux, C. Pascard, A. Lecroisey, and B. Keil, *J. Mol. Biol.*, 1981, **151**, 327. (*o*) Monoclonal antibody fragment specific towards influenza virus neuraminidase. (*p*) P. M. Colman, K. H. Gough, G. G. Lilley, R. J. Blagrove, R. G. Webster, and W. G. Laver, *J. Mol. Biol.*, 1981, **152**, 609. (*q*) T. Tsukihara, K. Homma, K. Fukuyama, Y. Katsube, T. Hase, H. Matsubara, N. Tanaka, and M. Katudo, *J. Mol. Biol.*, 1981, **152**, 821. (*r*) The apoprotein of the antitumour antibiotic auromomycin. (*s*) P. Van Roey, C. M. Weeks, F. E. DeJarnette, T. A. Beerman, and M. McHugh, *J. Mol. Biol.*, 1981, **153**, 183. (*t*) A. Liljas and M. E. Newcomer, *J. Mol. Biol.*, 1981, **153**, 393. (*u*) G. Hamoir, O. Dideberg, and P. Charlier, *J. Mol. Biol.*, 1981, **153**, 487. (*v*) Two cysteine proteases of plant origin. (*w*) G.

Mol. wt. and no. of subunits	*Mol. wt. of asymmetric unit*	V_M/ $nm^3\,dalton^{-1}$ $\times 10^3$	*Precipitant*	pH	*Ref.*
53 000 (2)	53 000	2.82	PEG 4000	6.8	*aa*
53 200 (2)	53 000	2.12	PEG 4000	6.8	*aa*
94 000 (3)	94 000	1.82	Ammonium sulphate	5.3	*bb*
—	—	—	PEG 6000	7.0	*dd*
80 000 (4)	80 000	1.9	Ammonium sulphate	5.7	*ee*
22 300 (1)	22 300	1.97	PEG 1000	6.5	*ff*
15 000 (1)	15 000	2.0	PEG 6000	8.0	*gg*
6700 (1)	13 400	1.97	MPD	6.0	*hh*
13 200 (1)	26 400	2.32	MPD	5—7	*hh*
57 550 (1 + 1 + 1)	57 550	—	PEG 4000	4.6	*ii*
16 068 (1)	—	—	Ammonium sulphate	7.8	*kk*
10 700 (1 + 1)	42 800	2.76	PEG 6000	7.2	*mm*
66 500 (4)	33 250	2.15	Potassium phosphate	9.0	*nn*
80 000 (1 + 1)	160 000	2.90	PEG 6000	—	*oo*
7803 (1)	7803	2.2	MPD	2.83	*qq*

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effects of the procedure are described for several sets of data and show particular enhancement for neutron data. Hope and Nichols [29] have described a method for collecting about 400 *X*-ray reflections per hour using a conventional diffractometer, and they have compared the data collected by the faster method with data counted for longer periods. An apparatus for the collection of small-angle *X*-ray scattering curves at high speed has been reported.[30] The apparatus utilizes a linear-position-sensitive detector. Jennings [31] has examined a number of theories for the predictions of extinction coefficients and polarization ratios with emphasis on the effects errors may have in connection with crystal-monochromated apparatus. Langridge and co-workers [32] have described a real-time colour graphics system that has been developed to study molecular interactions. Both skeletal and surface views can be displayed and manipulated, and the authors present several excellent examples of the sort of still pictures that can be produced on the system. The application of this system to structure analysis and publication will be a great improvement on monochrome graphics systems. Schmid and co-workers [33] have described an oscillation data-collection system for high-resolution protein crystallography. They have discussed the hardware involved and the techniques required for such things as the design of collimators and the determination of crystal orientations.

Crystal Growth.—Thaller and co-workers [34] have described a technique for the growth of large single protein crystals. A small washed crystal is used to seed a protein solution and once growth stops it is then transferred to a fresh protein solution. In some instances isomorphous derivatives have been prepared by the inclusion of heavy-atom reagents in the seeded protein solution. Shaikevitch and Kam [35] have examined the degree of long-range order in protein crystals by *X*-ray diffraction.

Protein Dynamics.—Calculations on the molecular dynamics of pancreatic trypsin inhibitor have been reported [36] using a technique that models the effect of solvent molecules on the protein. Gavish [37] has described studies on the modelling of atomic displacements in proteins that use non-harmonic potentials to describe unusual temperature dependence.

Cryoenzymology.—Fink and Petsko [38] have published a detailed review on *X*-ray cryoenzymology. They describe the possible uses of cryoenzymology and how crystals may be stabilized at sub-zero temperatures. The current results obtained using the technique are reported, and possible future uses are discussed.

[29] H. Hope and B. G. Nichols, *Acta Crystallogr., Sect. B*, 1981, **37**, 158.
[30] T. Hashimoto, S. Suehiro, M. Shibayama, K. Saijo, and H. Kawai, *Polym. J.*, 1981, **13**, 501.
[31] L. D. Jennings, *Acta Crystallogr., Sect. A*, 1981, **37**, 584.
[32] R. Langridge, T. W. Ferrin, I. D. Kuntz, and M. L. Connolly, *Science*, 1981, **211**, 661.
[33] M. F. Schmid, L. H. Weaver, M. A. Holmes, M. G. Grütter, D. H. Ohlendorf, R. A. Reynolds, S. J. Remington, and B. W. Matthews, *Acta Crystallogr., Sect. A*, 1981, **37**, 701.
[34] C. Thaller, L. H. Weaver, G. Eichele, E. Wilson, R. Karlsson, and J. N. Jansonius, *J. Mol. Biol.*, 1981, **147**, 465.
[35] A. Shaikevitch and Z. Kam, *Acta Crystallogr., Sect. A*, 1981, **37**, 871.
[36] W. F. Van Gunstern and M. Karplus, *Nature (London)*, 1981, **293**, 677.
[37] B. Gavish, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 6868.
[38] A. L. Fink and G. A. Petsko, *Adv. Enzymol.*, 1981, **52**, 177.

Protein-structure Determinations.—Harada and co-workers[39] have described a new translation function that should give the absolute position of a known molecular structure in an unknown crystal structure. The determination of the crystal structure of a high-temperature form of hen egg-white lysozyme at 0.6 nm resolution is described and the resulting structure compared with the structure calculated from isomorphous replacement data.

The use of the molecular replacement method to examine the quaternary structure of haemoglobin has been reported.[40] The techniques used are described in detail and the resulting structures are discussed.

Rice[41] has described the structure determination of horse-muscle phosphoglycerate kinase using a combination of isomorphous replacement phases with phases calculated from a partial model of the structure. The procedure gives more readily interpretable electron-density maps, and the relative merits of several different types of difference Fourier synthesis are discussed.

Podjarny and co-workers[42] have reported a matricial direct method for phasing the low-resolution structure factors of yeast $tRNA_f^{Met}$. The method has allowed a structure determination to 0.4 nm resolution, and the authors have examined the accuracy of the method by comparison with isomorphously phased data. In a second paper[43] methods of improving and extending medium- and low-resolution structure factors by density modification have been described. Non-negativity of the electron-density function and constancy within the solvent regions are utilized to improve the phases. The use of the method for yeast $tRNA_f^{Met}$ is described.

The techniques used in the crystal-structure determination of Southern-bean mosaic virus at 0.28 nm resolution have been reported.[44] The authors detail the methods for data collection and processing, heavy-atom site determination and refinement, and the steps in structure averaging and back-transformation. A method for the combination of isomorphous replacement phases with molecular replacement phases is described.

3 Immunoglobulins

Human F_c Fragment.—Deisenhofer has reported the refinement of human F_c fragment at 0.29 nm resolution.[45] The refinement used two different automated procedures and has reached an *R*-factor of 0.22. The structure of the dimer of CH3 domains is seen to resemble closely the CH1–CL aggregate in F_{ab} fragments. There is seen to be no contact between the CH2 domains whereas the CH3–CH3 contact is about three times the size of the CH2–CH3 contact. The carbohydrate moiety, consisting of nine hexose units, covers part of the C-contact face on the CH2 domain and shields the hydrophobic residues on this surface.

[39] Y. Harada, A. Lifchitz, J. Berthou, and P. Jolles, *Acta Crystallogr., Sect. A*, 1981, **37**, 398.
[40] Z. S. Derewenda, E. J. Dodson, G. G. Dodson, and A. M. Brzozowski, *Acta Crystallogr., Sect. A*, 1981, **37**, 407.
[41] D. W. Rice, *Acta Crystallogr., Sect. A*, 1981, **37**, 491.
[42] A. D. Podjarny, R. W. Schevitz, and P. B. Sigler, *Acta Crystallogr., Sect. A*, 1981, **37**, 662.
[43] R. W. Schevitz, A. D. Podjarny, M. Zwick, J. J. Hughes, and P. B. Sigler, *Acta Crystallogr., Sect. A*, 1981, **37**, 669.
[44] C. Abad-Zapatero, S. S. Abdel-Meguid, J. E. Johnson, A. G. W. Leslie, T. Rayment, M. G. Rossmann, D. Suck, and T. Tsukihara, *Acta Crystallogr., Sect. B*, 1981, **37**, 2002.
[45] J. Deisenhofer, *Biochemistry*, 1981, **20**, 2361.

In the same paper the structure of the complex between the F_c fragment and the B fragment of protein A from the cell wall of *Staphylococcus aureus* is described at 0.28 nm resolution. The structure of the fragment B is seen to consist of two α-helices and a random chain. Fragment B forms two contacts with the F_c fragment. A predominantly hydrophobic contact is made between the two α-helices of fragment B and the CH2 and CH3 domains of F_c. The second contact is smaller, mainly polar in nature, includes a sulphate ion, and seems to be just a crystal contact and not a true physiological interaction.

4 Oxygen Carriers and Electron-transfer Proteins

Haem-protein Stereochemistry.—Brown and co-workers[46] have used a graphics display system to explore the orientation of oxygen in oxyhaemoproteins and its implications for the catabolism of haem.

Myoglobin.—A real space refinement of neutron-diffraction data from sperm-whale carbon monoxymyoglobin at 0.18 nm resolution has been reported.[47] Neutron occupancies at the end of the refinement are close to the expected values and it is seen that hydrogen atoms not involved in hydrogen bonds have greater degrees of exchange with deuterium. The oxygen and deuterium co-ordinates for 40 water molecules have been determined. Twenty-seven are involved in bridges between protein atoms and nine are involved in deuterium bonds with main-chain atoms. Deuterium bond angles in the α-helical regions show significant deviations from linearity with a mean ND···O angle of 154° and a mean CO···D angle of 145°.

In another paper[48] on the neutron structure of myoglobin Phillips and Schoenborn have shown that the distal histidine, His-E7, stabilizes bound molecular oxygen by formation of a hydrogen bond. This stabilization is in addition to the steric hindrance offered to linear ligands by the histidine.

Haemoglobins.—*General.* The structures of the binding sites for heterotropic effectors in two fish haemoglobins have been analysed[49] by comparison of amino-acid sequences.

Human Haemoglobin. Stuhrmann and Notbohm[50] have examined the configuration of the four iron atoms in dissolved human haemoglobin. They have used the anomalous dispersion of the iron atom studied using synchrotron radiation, and the mean distance between the iron atoms they calculate, 2.6 nm, agrees well with previously determined values from crystal structures.

Horse Methaemoglobin. The structure of horse methaemoglobin complexed with isothiocyanate has been examined by difference Fourier techniques.[51] The thiocyanate ion is co-ordinated to the iron exclusively by its nitrogen atom, thereby forming the isothiocyanate complex. The ligand is bent as in the similar azide complex and there is no evidence of strain in the ligand pocket, in contrast to

[46] S. B. Brown, A. A. Chabot, E. A. Enderby, and A. C. T. North, *Nature (London)*, 1981, **289**, 93.
[47] J. C. Hanson and B. P. Schoenborn, *J. Mol. Biol.*, 1981, **153**, 117.
[48] S. E. V. Phillips and B. P. Schoenborn, *Nature (London)*, 1981, **292**, 81.
[49] D. Barra, F. Bossa, and M. Brunori, *Nature (London)*, 1981, **293**, 587.
[50] H. B. Stuhrmann and H. Notbohm, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 6216.
[51] Z. R. Korszun and K. Moffat, *J. Mol. Biol.*, 1981, **145**, 815.

the cyanide complex. There are slight structural changes at the haems, the most obvious of which is a slight lengthening of the iron–ligand-nitrogen bond in the β-haem.

The structure of horse methaemoglobin complexed with imidazole has been studied by difference Fourier analysis.[52] All the subunits show extensive changes, these being different for the α- and β-subunits. Unlike all other ligands the imidazole only partially enters the ligand pocket with about 25% occupancy on the α-haems and about 45% occupancy on the β-haems. The structural basis for these differences is discussed.

Mutant Haemoglobins. The crystal structure of haemoglobin A Creteil has been determined at 0.35 nm resolution.[53] The replacement of serine 89β by asparagine causes severe disordering of the β-chain *C*-terminal tetrapeptide leading to destabilization of the quaternary structure. Analysis of the structure suggests that these effects are common to other variant haemoglobins having similar functional abnormalities but very different changes in primary structure. The structure of haemoglobin Nancy in which tyrosine 145β is replaced by aspartate shows the same mechanism of dysfunction.

The crystal structure of haemoglobin A Zürich has been determined at 0.28 nm resolution.[54] The conformation of arginine E7 (residue 63β and a replacement of the distal histidine E7) in the deoxy form differs from that found in the carbon monoxy form, and structural comparison with normal haemoglobin A allows the altered functioning of the protein to be rationalized.

Crocodile Haemoglobin. Perutz and co-workers[55] have considered how the amino-acid substitutions in crocodilian species may explain the altered allosteric control properties seen for these proteins.

Sickling Haemoglobin. Edelstein[56] has discussed the possible molecular topology in crystals and fibres of haemoglobin S.

Haemerythrin.—The crystal structure of metazidohaemerythrin has been determined at 0.22 nm resolution.[57] The iron complex consists of two octahedrally co-ordinated iron atoms bridged by two carboxylate groups and an oxygen atom. The three other ligands are three histidines for one iron atom and two histidines and the azide ion for the other.

Cytochromes.—*General.* The comparison[58] of cytochrome b_{562} and cytochrome *c'* monomers suggests the possible divergent evolution of the two proteins from a common ancestor. Four aromatic side chains, including the haem ligands, and a methionine residue occur in spatially equivalent positions.

Kraut[59] has described the molecular geometry of the complex between

[52] J. A. Bell, Z. R. Korszun, and K. Moffat, *J. Mol. Biol.*, 1981, **147**, 325.
[53] A. Arnone, J. Thillet, and J. Rosa, *J. Biol. Chem.*, 1981, **256**, 8545.
[54] S. E. V. Phillips, D. Hall, and M. F. Perutz, *J. Mol. Biol.*, 1981, **150**, 137.
[55] M. F. Perutz, C. Bauer, G. Gros, F. Leclercq, C. Vandecasserie, A. G. Schnek, G. Braunitzer, A. E. Friday, and K. A. Joysey, *Nature (London)*, 1981, **291**, 682.
[56] S. J. Edelstein, *J. Mol. Biol.*, 1981, **150**, 557.
[57] R. E. Stenkamp, L. C. Siecker, L. H. Jensen, and J. Sanders-Loehr, *Nature (London)*, 1981, **291**, 263.
[58] P. C. Weber, F. R. Salemme, F. S. Mathews, and P. H. Bethge, *J. Biol. Chem.*, 1981, **256**, 7702.
[59] J. Kraut, *Biochem. Soc. Trans.*, 1981, **9**, 197.

cytochrome *c* and cytochrome *c* peroxidase and considered a general model for biological electron transfer.

Cytochrome c. Tuna ferrocytochrome *c* has been refined [60] at 0.15 nm resolution to a final *R*-factor of 0.173 for the 14000 observed reflections. The structure is very much as previously described, but the improved quality has allowed a meaningful comparison to be made with the oxidized molecule.[61] The haem group in ferrocytochrome *c* [60] is seen to be held tightly in place and is deformed slightly into a saddle shape. The iron atom lies in the plane of the haem, and bond lengths and angles are as expected from model compounds. Tentative assignments of function to 24 of the evolutionarily invariant residues have been made.

The refinement of ferricytochrome *c* at 0.18 nm resolution has been reported [61] and has progressed to an *R*-factor of 0.208. Twelve water molecules have been located in both the oxidized and reduced forms, three of these being buried. The oxidized/reduced change leads to a more hydrophobic haem environment in ferricytochrome *c*. The possible mechanisms for the redox changes have been discussed in light of the structures.

Computer simulations of the atomic motions in tuna ferrocytochrome *c* have been reported.[62] The average structure and the magnitudes of the atomic-position fluctuations are in reasonable agreement with *X*-ray diffraction results.[60, 61] However, the simulations show that the atomic displacements are quite anisotropic, the anisotropies correlating with structural features of the protein.

Cytochrome c'. The crystal-structure determination of cytochrome *c'* from *Rhodospirillum molischianum* at 0.25 nm resolution has been reported.[63] The molecule is a symmetric dimer, each subunit having 128 amino-acid residues and a covalently bound protohaem IX prosthetic group. The subunit structure consists of four nearly parallel α-helices that pack closely at one end. The four helices diverge from one end to the other to form the haem pocket. Several features of the haem binding resemble cytochrome *c*, but the iron atom is only five-co-ordinated, a solvent-exposed histidine being the single axial ligand to the iron atom. Subunit contacts are principally interhelix interactions and lead to the exposed regions of both haems lying on the same molecular surface.

Cytochrome c_3. The structure of cytochrome c_3 from *Desulphovibrio vulgaris* has been determined at 0.25 nm resolution.[64] The molecule contains four haem atoms that are exposed on the surface of the molecule; each haem iron is co-ordinated by two histidine residues. There are some α-helices in the structure, and several reverse turns are observed at glycine and alanine residues.

Cytochrome c_4. Cytochrome c_4 from *Pseudomonas aeruginosa* has been crystallized and the low-resolution structure determined at 0.5 nm.[65] The molecule

[60] T. Takano and R. E. Dickerson, *J. Mol. Biol.*, 1981, **153**, 79.
[61] T. Takano and R. E. Dickerson, *J. Mol. Biol.*, 1981, **153**, 95.
[62] S. H. Northrup, M. R. Pear, J. D. Morgan, J. A. McCammon, and M. Karplus, *J. Mol. Biol.*, 1981, **153**, 1087.
[63] P. C. Weber, A. Howard, N. H. Xuong, and F. R. Salemme, *J. Mol. Biol.*, 1981, **153**, 399.
[64] Y. Higuchi, S. Bando, M. Kusunoki, Y. Matsuura, N. Yasuoka, M. Kakudo, T. Yamanaka, T. Yagi, and H. Inokuchi, *J. Biochem.*, 1981, **89**, 1659.
[65] L. Sawyer, C. L. Jones, A. M. Damas, M. M. Harding, R. O. Gould, and R. P. Ambler, *J. Mol. Biol.*, 1981, **153**, 831.

contains two haem molecules one of which can be clearly seen in the 0.5 nm map. Studies are being continued to higher resolution.

Cytochrome c_{550}. A correction[66] to the sequence of cytochrome c_{550} from *Paracoccus denitrificans* has been reported. Five discrepancies are found and the effect on the structure is examined.

Cytochrome b_{562}. A redetermination of the amino-acid sequence of cytochrome b_{562} from *E. coli* has been reported.[67] The newly determined sequence of the molecule has been fitted to a 0.25 nm resolution electron-density map by means of a molecular graphics system. The results indicate 106 residues in the protein rather than the previously thought 110. Proposed sequence changes are confirmed by the electron-density map and some had already been proposed from *X*-ray work.

Ferredoxin.—Ghosh and co-workers[68] have reported the structure of a 7-iron ferredoxin from *Azotobacter vinelandii* at 0.3 nm resolution. The structure consists of an *N*-terminal core, residues 1—50, which forms the iron–sulphur cluster sites, and a *C*-terminal portion, residues 51—107, which wraps around this core. The [4Fe–S] and [3Fe–S] clusters have been described in detail and several techniques used to confirm the geometry of the clusters. A partial refinement at 0.25 nm resolution has been commenced.

5 Lysozyme and Ribonuclease

Lysozyme.—Pincus and Scheraga[69] have carried out conformational-energy studies on substrate binding to hen egg-white lysozyme and compared their results with those from *X*-ray studies. Perkins and co-workers[70] have examined by proton magnetic resonance the binding of monosaccharide inhibitors to hen egg-white lysozyme and, in a later paper,[71] the binding of lanthanide ions and inhibitors to the same enzyme.

Matthews and co-workers[72] have compared the structures of lyzozymes from hen egg-white and bacteriophage T4. They have shown that despite their amino-acid sequences not being homologous the two structures do show very similar backbone conformations, similar substrate-binding modes, and similar specific protein–substrate interactions. It also seems very likely that they have similar modes of catalytic activity, and the authors conclude that the two enzymes have diverged from a common ancestral protein. The authors have presented the work in more detail in a second paper.[73]

Artymiuk and Blake have described the refinement of human lysozyme at 0.15 nm resolution.[74] The refinement has progressed to an *R*-factor of 0.187, and a

[66] R. P. Ambler, T. E. Meyer, M. D. Kamen, S. A. Schichman, and L. Sawyer, *J. Mol. Biol.*, 1981, **147**, 351.
[67] F. Lederer, A. Glatigny, P. H. Bethge, H. D. Bellamy, and F. S. Mathews, *J. Mol. Biol.*, 1981, **148**, 427.
[68] D. Ghosh, W. Furey, jun., S. O'Donnell, and C. D. Stout, *J. Biol. Chem.*, 1981, **256**, 4185.
[69] M. R. Pincus and H. A. Scheraga, *Biochemistry*, 1981, **20**, 3960.
[70] S. J. Perkins, L. N. Johnson, D. C. Phillips, and R. A. Dwek, *Biochem. J.*, 1981, **193**, 553.
[71] S. J. Perkins, L. N. Johnson, D. C. Phillips, and R. A. Dwek, *Biochem. J.*, 1981, **193**, 573.
[72] B. W. Matthews, M. G. Grütter, W. F. Anderson, and S. J. Remington, *Nature (London)*, 1981, **290**, 334.
[73] B. W. Matthews, S. J. Remington, M. G. Grütter, and W. F. Anderson, *J. Mol. Biol.*, 1981, **147**, 545.
[74] P. J. Artymiuk and C. C. F. Blake, *J. Mol. Biol.*, 1981, **152**, 737.

very detailed analysis of the non-bonded and hydrogen-bonded contacts in the molecule has been made. These contact distances were not restrained during the refinement process and all the types of non-bonded interactions show minimum distances close to standard van der Waals' separations. The lengths, angles, and participating atoms for all the intramolecular hydrogen bonds have been analysed and the values obtained agree well with standard values. It is seen, however, that the hydrogen bonds tend to be consistently more linear than those seen in small molecules. A detailed analysis of the α-helices' regularity suggests that the current standard model may need some revision. Nineteen of the twenty-one reverse turns that include a hydrogen bond have conformations which agree closely with four of the five standard types.

The crystal structure of lysozyme from *Streptomyces erythraeus* has been determined at 0.29 nm resolution.[75] This enzyme has 185 amino-acid residues and has an amino-acid composition very different from that of every other known lysozyme. The structure was solved at 0.29 nm resolution using multiple isomorphous replacement methods and has been refined at 0.25 nm resolution. The molecule has three definable domains and contains four α-helices. The structure also shows a very prominent cleft, and two residues, aspartate-7 and glutamate-34, are possible contenders for the catalytically active residues.

Hen egg-white lysozyme with a chemically modified residue, tryptophan-62, has been studied by both nuclear magnetic resonance and *X*-ray crystallography.[76] The results show that although there are only very small conformational changes in the area of modification and the rest of the enzyme is unaltered, the catalytic activity towards the bacterial cell wall is virtually abolished. This lack of activity seems to arise from the obstruction of subsite B by the oxindolealamine residue, preventing productive binding of the substrate.

Anderson and co-workers[77] have described crystallographic studies of the binding of oligosaccharides to T4 bacteriophage lysozyme. *N*-Acetylglucosamine and *N*-acetylmuramic acid bind at a single site analogous to the C subsite of the hen egg-white enzyme, while tri-*N*-acetyl glucosamine binds clearly in sites B and C but is seen less clearly in site A. Model-building studies have allowed some proposals as regards productive substrate binding and the catalytic process to be put forward.

A crystallographic study at 0.4 nm resolution of the structure of monoclinic hen egg-white lysozyme has been reported.[78] The packing of the asymmetric units, each consisting of two lysozyme molecules, has been examined and is described in some detail.

Ribonuclease.—Wlodawer and Sjölin have examined the orientation of histidine residues in ribonuclease A by neutron diffraction.[79] They have confirmed the *X*-ray-derived orientation for histidine-48 but have shown that the other three

[75] S. Harada, R. Sarma, M. Kakudo, S. Hara, and T. Ikenoka, *J. Biol. Chem.*, 1981, **256**, 11 600.
[76] C. C. F. Blake, R. Cassels, C. M. Dobson, F. M. Poulsen, R. J. P. Williams, and K. S. Wilson, *J. Mol. Biol.*, 1981, **147**, 73.
[77] W. F. Anderson, M. G. Grütter, S. J. Remington, L. H. Weaver, and B. W. Matthews, *J. Mol. Biol.*, 1981, **147**, 523.
[78] J. Hogle, S. T. Rao, M. Mallikarjunan, C. Beddell, R. K. McMullan, and M. Sundaralingam, *Acta Crystallogr., Sect. B*, 1981, **37**, 591.
[79] A. Wlodawer and L. Sjölin, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 2853.

histidines needed to be rotated 180° about the C_β—C_γ bonds in order to fit the neutron difference Fourier maps. In their final model histidine-12 is hydrogen-bonded to the carbonyl oxygen of threonine-45 and to the oxygen of the inorganic phosphate. Histidine-119 is hydrogen-bonded to another oxygen on the phosphate and to the carboxylate oxygen of the aspartic acid residue number 121.

Crystallographic studies on chemically modified ribonuclease-*S* have been described.[80] Both the analogues, one lacking residues 16—20 and the other lacking residues 16—20 and also containing 4-fluoro-L-histidine in place of the active histidine (His-12), have backbone conformations and active-site configurations very similar to those of the native protein. The fluorine-substituted histidine assumes the same position as histidine in the native enzyme and this supports the view that the fluoro-enzyme is inactive owing to a change in the imidazole basicity of histidine-12 rather than conformational distortion.

Rosa and Richards[81] have examined hydrogen exchange from identified regions of the *S*-protein component of ribonuclease-*S*.

6 Proteolytic Enzymes

Pancreatic Trypsin Inhibitor.—Levitt[82] has analysed the molecular dynamics of the hydrogen bonds in bovine pancreatic trypsin inhibitor and shown that such calculations give results that agree with experimentally determined solvent-exchange properties. He suggests that molecular-dynamics calculations can complement *X*-ray studies by providing information about the rates and pathways of conformational changes.

Kazal-type Inhibitor Protein.—The third domain of Japanese quail ovomucoid, a Kazal-type inhibitor, has had its structure determined at 0.25 nm resolution.[83] The molecules in the crystal are arranged in two slightly different octamers with approximate D_4 symmetry, the molecules being held together mainly by interactions of the *N*-terminal residues. These interactions form a novel secondary structure termed a β-channel. The individual molecules are globular and approximately 3.5 nm × 2.7 nm × 1.9 nm. They contain a two-stranded antiparallel β-sheet of eleven residues and a twelve-residue α-helix. The reactive residues, lysine-18–aspartic acid-19, are located on an exposed loop close to the *N*-terminus of the helical segment. The authors have discussed the structure in terms of other known Kazal-type inhibitor sequences and also in structural terms compared with trypsin inhibitor and subtilisin inhibitor. The three sorts of inhibitor all have an antiparallel double strand following the reactive site loop.

Proteases.—*General.* Greer has described the results of comparative model-building studies of the structures of mammalian serine proteases.[86] He has examined eleven proteins in all. Kamer and Argos[85] have compared the active-site

[80] H. C. Taylor, D. C. Richardson, J. C. Richardson, A. Wlodawer, A. Komoriya, and I. M. Chaiken, *J. Mol. Biol.*, 1981, **149**, 313.
[81] J. J. Rosa and F. M. Richards, *J. Mol. Biol.*, 1981, **145**, 835.
[82] M. Levitt, *Nature (London)*, 1981, **294**, 379.
[83] E. Weber, E. Papamokos, W. Bode, R. Huber, I. Kato, and M. Laskowski, jun., *J. Mol. Biol.*, 1981, **149**, 109.
[84] J. Greer, *J. Mol. Biol.*, 1981, **153**, 1027.
[85] G. Kamer and P. Argos, *Biochim. Biophys. Acta*, 1981, **669**, 93.

geometries of thermolysin, soybean trypsin–trypsin inhibitor complex, and phospholipase A_2. The results suggest a mechanism for phospholipase similar to that proposed for the serine proteases, and the reactive carbon centres of the three molecules show the same handedness.

Trypsin. A neutron structure analysis at 0.22 nm resolution of the trypsin molecule inhibited with the monoisopropylphosphoryl group has been reported.[86] Since the inhibitor mimics the tetrahedral intermediate structure, the ability to examine directly the protonation states of histidine-57 and aspartic acid-102 is of importance. The work has shown conclusively that histidine-57 is the catalytic base in the transition state, not the aspartic acid. The mechanism has been interpreted, and other aspects of the protein structure observable only by neutron-diffraction analysis are also discussed. These aspects include the definite stereochemistry for well ordered amide side chains, the location and orientation of water molecules, and the hydrogen-exchange properties of the protein.

γ-Chymotrypsin. Cohen and co-workers[87] have described the refined crystal structure of γ-chymotrypsin at 0.19 nm resolution and compared it with other serine proteases. The final *R*-factor is 0.18 for the 15240 *X*-ray reflections to 0.19 mm resolution. The stereochemistry of the three active-site residues, aspartic acid-102–histidine-57–serine-195, agrees well with both trypsin and *Streptomyces griseus* protease A. The γ-hydroxyl of serine-195 does not seem to be hydrogen-bonded to histidine-57. The structure of γ-chymotrypsin is seen to be very similar to that of α-chymotrypsin, the largest backbone differences being located in the calcium-binding loop, residues 75—78, and the autolysis loop, residues 146, 149, and 150. In γ-chymotrypsin residues 149 and 150 are seen to be disordered whereas in α-chymotrypsin they are stabilized by intermolecular contacts. These conformational differences are discussed in terms of the slow, pH-dependent interconversion of α- and γ-chymotrypsin.

Carboxypeptidase A. The refinement of the structure of carboxypeptidase A at 0.175 nm resolution has been reported.[88] In the native enzyme the zinc co-ordination number is five, the five ligands being two imidazole nitrogens, two carboxylate oxygens, and a water molecule. In the complex with the dipeptide glycyl-L-tyrosine this water molecule is replaced by both the carbonyl oxygen and the amino nitrogen of the dipeptide. Therefore it seems that the co-ordination number of the zinc ion varies from five to six, and the authors have discussed the catalytic mechanism in view of these results. The refinement has also shown the existence of three *cis* peptide bonds in the structure, none of which involves proline as the amino-nitrogen donor.

In a later paper[89] the same group of workers have examined the binding of several ligands to carboxypeptidase A. The 39-amino-acid inhibitor from potatoes binds with cleavage of the *C*-terminal glycine residue. This cleavage produces a new carboxylate ion, valine-38, which has one oxygen atom bound to the zinc and

[86] A. A. Kossiakoff and S. A. Spencer, *Biochemistry*, 1981, **20**, 6462.
[87] G. H. Cohen, E. W. Silverton, and D. R. Davies, *J. Mol. Biol.*, 1981, **148**, 449.
[88] D. C. Rees, M. Lewis, R. B. Honzatko, W. N. Lipscomb, and K. D. Hardman, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 3408.
[89] D. C. Rees and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 5455.

the other bonded to tyrosine-248. Tyrosine-248 also receives a hydrogen bond from the amide hydrogen of the residue 37—38 peptide bond, and the authors discuss the implications of this binding for the catalytic activity of the enzyme. The binding of indole-3-acetic acid, glycyl-L-tyrosine, and 2-benzyl-3-*p*-methoxybenzoyl propionate has also been studied and compared with that observed for the inhibitor.

Paslen and Lipkind [90] have described a theoretical conformational analysis of the residues arginine-145, tyrosine-248, and glutamate-270 in carboxypeptidase A, both in the native enzyme and in the complex with glycyl-L-tyrosine. They show that complex formation leads to a rearrangement of the active site and the movement of these residues to functionally active positions.

α-Lytic Protease. Bauer and co-workers [91] have studied the active site of α-lytic protease using a number of synthetic peptides. The data indicate that α-lytic protease has an active site that extends over six subsites. Comparison with the active site of elastase shows major differences, for example subsite S_2 appears most important for α-lytic protease whereas subsite S_4 is of major importance for elastase. The α-lytic protease molecule's tertiary structural homology with *Streptomyces griseus* protease A has allowed detailed model-building examination of the active site of α-lytic protease. This has allowed the comparison of subsites S_1—S_4 of α-lytic protease with other serine proteases.

Pepsin. Andreeva and co-workers [92] have described the arrangement of charged groups in the structure of porcine pepsin. They have demonstrated the existence of several ion pairs and describe their various effects of structural and molecular properties of pepsin.

7 Glycolytic Enzymes

Glycolysis – General.—The proceedings of a meeting devoted to the structure of glycolytic enzymes have been published.[93]

Pyruvate Kinase.—Muirhead and co-workers [94] have described recent studies on the three-dimensional structure and the functioning of muscle pyruvate kinase.

Alcohol Dehydrogenase.—The structure of a complex of liver alcohol dehydrogenase, NADH, and the inhibitor dimethyl sulphoxide has been determined at 0.29 nm resolution.[95] Extensive conformational changes are seen when the structure is compared with the apoenzyme, the main change involving a rotation of 7.5° of the catalytic domain relative to the nucleotide-binding domain. In general the internal structure of the domains remains unaltered but the inter-domain cleft is closed, making the active site less accessible from the surrounding solution. The structures of the two crystallographically independent subunits are

[90] V. V. Paslen and G. M. Lipkind, *Mol. Biol. (Moscow)*, 1981, **14**, 731.
[91] C.-A. Bauer, G. D. Brayer, A. R. Sielecki, and M. N. G. James, *Eur. J. Biochem.*, 1981, **120**, 289.
[92] N. S. Andreeva, A. A. Zdanov, and A. A. Fedorov, *FEBS Lett.*, 1981, **125**, 239.
[93] *Philos. Trans. R. Soc. London, Ser. B*, 1981, **293**, 1.
[94] H. Muirhead, J. P. Grant, M. A. Lawton, C. A. Midwinter, J. C. Nocton, and D. I. Stuart, *Biochem. Soc. Trans.*, 1981, **9**, 212.
[95] H. Eklund, J.-P. Samama, L. Wallén, C.-I. Brändén, A. Åkeson, and T. A. Jones, *J. Mol. Biol.*, 1981, **146**, 561.

very similar and bind the coenzyme and inhibitor identically. The conformational changes bring no new residues into the active site and the active-centre zinc atom is close to the hinge region. Small differences in the zinc-atom co-ordination are not excluded, and the oxygen atom of the inhibitor is bound directly to the zinc atom, suggesting that the proposed mechanism of catalysis is correct.

The structure of the binary complex of alcohol dehydrogenase with 5-methylnicotinamide–adenine dinucleotide at 0.37 nm resolution has been reported [96] and shows that the coenzyme analogue binds with the pyridinium ring pointing away from the active site.

Lactate Dehydrogenase.—Two papers describing the methods involved in [97] and the results observed for [98] the crystal-structure determination of an active ternary complex of pig-heart lactate dehydrogenase have been published. Using the known structure of the dogfish enzyme as a starting point, repeated cycles of molecular replacement and subunit averaging have yielded a final *R*-factor of 0.196 for the 22 000 observed reflections.[97]

The active coenzyme substrate analogue (3*S*)-5-(3-carboxy-3-hydroxypropyl)NAD^+ was bound to the pig lactate dehydrogenase, and the structure was solved at 0.27 nm resolution.[98] The structure observed differs significantly from the abortive ternary complexes of the dogfish enzyme but closely resembles the proposed 'active' structure. The active-site loop is in an overall closed conformation typical of ternary complexes but is arranged slightly differently from the dogfish muscle enzyme complexes. The stronger binding of NAD^+ to the heart isoenzyme does not seem to be accounted for by a single additional hydrogen bond to the pyrophosphate group, but the intersubunit anion-binding sites seen in the dogfish M4 enzyme are also present in the pig H4 enzyme. The authors also propose that a loop arginine residue is involved in modulating the pK_a value of the internal acid/base catalyst, histidine-195.

8 Hormones

Avian Pancreatic Polypeptide.—Blundell and co-workers [99] have described the *X*-ray analysis at 0.14 nm resolution of the 36-residue avian pancreatic polypeptide. The structure determination involved using multiple isomorphous replacement to 0.21 nm resolution and a modified tangent formula to extend the phases to 0.14 nm resolution. The molecule contains an extended polyproline-like helix, residues 1—8, and an α-helix, residues 14—31, which lie roughly antiparallel. Two pancreatic polypeptide molecules form a symmetrical dimer that is stabilized by the interlocking of non-polar groups from the α-helices. These dimers are crosslinked by zinc atoms, three polypeptide molecules co-ordinating each zinc with a distorted trigonal-bipyramidal geometry. The molecule has several features in common with the pancreatic hormones insulin and glucagon, and its biological activity seems to reside in the more flexible carboxyl terminal region.

[96] J.-P. Samama, A. D. Wrixon, and J.-F. Biellmann, *Eur. J. Biochem.*, 1981, **118**, 479.
[97] U. M. Grau, M. G. Rossmann, and W. E. Trommer, *Acta Crystallogr., Sect. B*, 1981, **37**, 2019.
[98] U. M. Grau, W. E. Trommer, and M. G. Rossmann, *J. Mol. Biol.*, 1981, **151**, 289.
[99] T. L. Blundell, J. E. Pitts, I. J. Tickle, S. P. Wood, and C.-W. Wu, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4175.

Hormones and Receptors – General.—The conformations and receptor binding of pancreatic hormones have been discussed,[100] and proposals for the tertiary structure of sand-tiger-shark relaxin have been described.[101] Jörnvall and co-workers [102] have discussed the structural homologies between prealbumin, gastrointestinal prohormones, and other proteins.

9 Other Globular Proteins

L-Arabinose-binding Protein.—The structure of L-arabinose-binding protein has been determined at 0.24 nm resolution.[103] The electron-density map was interpreted initially in an optical comparator and then using a computer graphics system. The protein molecule is an ellipsoid and consists of two globular domains, each domain being composed of two separate polypeptide-chain segments. The arrangements within the two domains are very similar, both containing six-stranded β-sheets; one is all parallel and the other has a sixth antiparallel strand, the sheets being flanked by two α-helices on each side. Despite showing structural homology the two domains show no sequence homology. The sugar-binding site appears to be located in a cleft lined by hydrophilic residues.

The structure of the complex with L-arabinose has been solved at 0.24 nm resolution,[104] and the sugar, in the C1 pyranose chair conformation, has been fitted to the difference electron density. All sugar hydroxyls are hydrogen-bonded to side-chain residues, and the inaccessibility of the bound L-arabinose to the external solution indicates that protein structural change accompanies the binding.

Using small-angle *X*-ray scattering, the radius of gyration of L-arabinose-binding protein has been examined [105] in the free and sugar-complexed forms. The sugar-induced decrease in the radius of gyration suggests a substrate-induced cleft closure of the bilobal molecule. Argos and co-workers [106] have examined the amino-acid sequences of several sugar-binding proteins and have found regions of homology.

Catalase.—The crystal structure of beef-liver catalase has been determined at 0.25 nm resolution [107] and the known sequence fitted to the electron-density map. Each subunit consists of a large antiparallel β-sheet domain with helical insertions followed by a smaller domain containing four α-helices. The haem group is buried 2 nm below the molecular surface and is accessible by a channel lined with hydrophobic residues. The proximal haem ligand is a tyrosine residue with a histidine and an asparagine on the distal side. The structure determination and the molecular architecture have been described in greater detail in a second paper.[108]

[100] T. L. Blundell, J. E. Pitts, I. J. Tickle, and S. P. Wood, *Biochem. Soc. Trans.*, 1981, **9**, 31.
[101] L. K. Gowan, J. W. Reinig, C. Schwabe, S. Bedarkar, and T. L. Blundell, *FEBS Lett.*, 1981, **129**, 80.
[102] H. Jörnvall, A. Carlström, T. Pettersson, B. Jacobsson, M. Persson, and V. Mutt, *Nature (London)*, 1981, **291**, 261.
[103] G. L. Gilliland and F. A. Quiocho, *J. Mol. Biol.*, 1981, **146**, 341.
[104] M. E. Newcomer, G. L. Gilliland, and F. A. Quiocho, *J. Biol. Chem.*, 1981, **256**, 13213.
[105] M. E. Newcomer, B. A. Lewis, and F. A. Quiocho, *J. Biol. Chem.*, 1981, **256**, 13218.
[106] P. Argos, W. C. Mahoney, M. A. Hermodson, and H. Hanei, *J. Biol. Chem.*, 1981, **256**, 4357.
[107] T. J. Reid, tert., M. R. N. Murthy, A. Sicignano, N. Tanaka, W. D. L. Musick, and M. G. Rossmann, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4767.
[108] M. R. N. Murthy, T. J. Reid, tert., A. Sicignano, N. Tanaka, and M. G. Rossmann, *J. Mol. Biol.*, 1981, **152**, 465.

The crystal structure of catalase from *Penicillium vitale* has also been reported.[109] The enzyme subunit has three domains, the first two in common with the beef-liver enzyme and an extra *C*-terminal α/β domain. This third domain has a topology similar to that seen for flavodoxin.

Catabolite Gene Activator Protein.—The crystal structure of catabolite gene activator protein, also known as cyclic AMP receptor protein, from *E. coli* has been determined at 0.29 nm resolution.[110] The dimeric protein is composed of two identical subunits each of which is composed of two domains, the *N*-terminal domain binding the cyclic AMP and the *C*-terminal domain probably binding to DNA. Model-building studies suggest that the protein binds to left-handed B-DNA, contacting the DNA molecule's major groove *via* two α-helices.

***cro* Repressor Protein.**—The structure of bacteriophage λ *cro* repressor protein has been determined at 0.28 nm resolution.[111] The molecule consists of a three-stranded antiparallel β-sheet and three α-helices. From the structure it seems that a dimer of *cro* molecules binds to B-form DNA with the two-fold axis of the repressor dimer coincident with the two-fold axis of the DNA. A pair of two-fold, related α-helices of the repressor, lying within successive major grooves of the DNA, seem to be the recognition and binding mechanism. A *C*-terminal extended arm also seems to be involved in the binding.

Crambin.—The crystal structure of crambin, a 46-residue hydrophobic protein from Abyssinian cabbage seeds, has been solved at 0.15 nm resolution[112] by means of the anomalous-scattering effects of the sulphur atoms. The secondary structure consists of 46% α-helices and 17% β-sheet, and the molecule contains three disulphide bridges. The structure shows that the molecule's six charged groups are segregated on the molecule away from the hydrophobic surface groups.

Glutathione Reductase.—The 0.2 nm resolution structure of the FAD-containing enzyme glutathione reductase has been reported.[113] The two identical subunits both contain four domains with a flexible 18-residue segment at the *N*-terminus. Each subunit contains 11 α-helices, 31% of all residues, with 32% β-structure in five β-sheets. An intersubunit disulphide bridge has been detected in the crystal structure.

Alkaline Phosphatase.—The crystal structure of alkaline phosphatase has been determined[114] in the presence of several different metal ions in the functional metal sites. Each subunit contains a seven-stranded β-sheet flanked by helices, which comprise 27% of the 459 residues. Replacement of the zinc and magnesium ions by cobalt or cadmium reveals seven metal-binding sites per dimer. The active sites are 3.2 nm apart and each contains two metal ions, 0.49 nm apart. There seem to be several protein structural changes associated with the metal binding, but these are located within 1 nm of the active sites.

[109] B. K. Vainshtein, W. R. Melik-Adamyan, V. V. Barynin, A. A. Vagin, and A. I. Grebenko, *Nature* (*London*), 1981, **293**, 411.
[110] D. B. McCay and T. A. Steitz, *Nature* (*London*), 1981, **290**, 744.
[111] W. F. Anderson, D. H. Ohlendorf, Y. Takeda, and B. W. Matthews, *Nature* (*London*), 1981, **290**, 754.
[112] W. A. Hendrickson and M. M. Teeter, *Nature* (*London*), 1981, **290**, 107.
[113] R. Thieme, E. F. Pai, R. H. Schirmer, and G. E. Schulz, *J. Mol. Biol.*, 1981, **152**, 763.
[114] J. M. Sowadski, B. A. Foster, and H. W. Wyckoff, *J. Mol. Biol.*, 1981, **150**, 245.

Phospholipase A2.—The refined structure of phospholipase A2 from bovine pancreas has been reported.[115] Based on a 0.24 nm structure the model has now been refined at 0.17 nm resolution to a final *R*-factor of 0.171. The essential calcium ion is located in the active site and stabilized by a carboxyl group as well as a loop of peptide containing several totally invariant residues. A total of 106 water molecules were located, the majority in a two-layer shell around the surface. Structural mobility has been examined from the individual temperature factors and the active centre is seen to be fairly rigid.

The 0.25 nm resolution structure of phospholipase A2 from the venom of a rattlesnake has been reported.[116] The structure of the dimeric molecule shows a subunit architecture similar to that of the monomeric bovine-liver enzyme. The subunit contact site is extensive and involves the catalytic and the calcium-binding sites. Access to the central cavity formed by the active-centre regions is quite restricted.

Dihydrofolate Reductase.—The binding of the inhibitor trimethoprim to dihydrofolate reductase has been determined[117] at 0.28 nm resolution. The structure is similar to the enzyme–methotrexate complex with a central eight-stranded β-sheet. Binding interactions between enzyme and inhibitor have been tentatively assigned but it is not yet possible to explain the different binding properties of trimethoprim and methotrexate.

Stone[118] has reviewed the structure and function of bacterial and mammalian dihydrofolate reductase.

Elongation Factor Tu.—The GDP-binding site on elongation factor Tu from *E. coli* has been located by *X*-ray analysis.[119] The binding site is located on the surface of the head domain.

The structural features of the GDP-binding site have been examined in an improved electron-density map.[120] The guanine base is bound in a hydrophobic pocket with no stacking interactions. Specific hydrogen bonds between guanine atoms and the protein explain the EF-Tu preference for guanine. The ribose is partly buried and the α- and β-phosphates are hydrogen-bonded to protein side-chain and main-chain atoms.

Vitamin D-dependent Calcium-binding Protein.—The structure of bovine-intestine vitamin D-dependent protein has been reported at 0.23 nm resolution.[121] The molecule contains two calcium-binding domains similar to those seen in parvalbumin, one domain having a larger and slightly rearranged calcium-binding loop. This protein is a member of the calmodulin family, and the possible structural relationships of the family are discussed.

[115] B. W. Dijkstra, K. H. Kalk, W. G. J. Hol, and J. Drenth, *J. Mol. Biol.*, 1981, **147**, 97.
[116] C. Keith, D. S. Feldman, S. Deganello, J. Glick, K. B. Ward, E. O. Jones, and P. B. Sigler, *J. Biol. Chem.*, 1981, **256**, 8602.
[117] D. J. Baker, C. R. Beddell, J. N. Champness, P. J. Goodford, F. E. A. Norrington, D. R. Smith, and D. K. Stammers, *FEBS Lett.*, 1981, **126**, 49.
[118] D. Stone, *Biochem. Soc. Trans.*, 1981, **9**, 271.
[119] D. Suck and W. Kabsch, *FEBS Lett.*, 1981, **126**, 120.
[120] J. R. Rubin, K. Morikawa, J. Nyborg, T. F. M. La Cour, B. F. C. Clark, and D. L. Miller, *FEBS Lett.*, 1981, **129**, 117.
[121] D. M. E. Szebenyi, S. K. Obendorf, and K. Moffat, *Nature (London)*, 1981, **294**, 327.

Inorganic Pyrophosphatase.—The location of the substrate-binding sites in yeast pyrophosphatase has been determined [122] by use of uranyl ions. Three uranyl ions bind to each subunit at a site 2.0—2.5 nm from the molecular two-fold axis. Kinetic experiments have also been performed to determine the physiological significance of these sites.

Wheatgerm Agglutinin.—The locations of the two histidine residues present in isolectin 2 have been determined by difference Fourier analysis.[123] One lies at the intersubunit interface; the other forms part of the primary saccharide-binding site. This latter histidine also acts as a major target for the binding of platinum and mercury heavy-atom compounds.

Crystallins.—The 0.26 nm resolution structure of bovine γ-crystallin II has been reported.[124] The protein has a two-domain β-structure folded into four identical Greek-key motifs. The molecule shows a very high degree of internal symmetry, and the effect of possible destabilization of the structure caused by the arrangement of the disulphide bridges is discussed.

The structure of β-crystallin has been predicted by comparison with γ-crystallin.[125]

The structure of γ-crystallin IIIb from calf lens has been reported at 0.3 nm resolution.[126] The structure is homologous to the γ-crystallin II molecule,[124] being a two-domain β-protein.

L-3-Hydroxyacyl-coenzyme A Dehydrogenase.—The binding of coenzyme to L-3-HADH has been examined in solution and in the crystal.[127] Only one tight binding site is seen in the crystal whereas solution studies suggest two identical binding sites.

Ferritin.—Changes in the crystal form of ferritin have been shown to correspond to the appearance of ferritin oligomers.[128]

Ferredoxin-NADP$^+$ Oxidoreductase.—The crystal structure of ferredoxin-NAD$^+$ oxidoreductase has been determined at 0.37 nm resolution[129] and shows the molecule to be composed of two domains. At this preliminary stage it has been possible to determine the molecular shape, a tentative polypeptide-chain tracing, and the location of the NADP$^+$ binding site.

Actinoxanthin.—The growth-inhibiting protein actinoxanthin from *Actinomyces globisporus* has been crystallized and its structure determined at 0.4 nm resolution.[130] The molecule is composed of two different-sized domains separated by a clearly identifiable cleft. The larger domain is mostly β-structure whereas the smaller domain is mostly irregular.

[122] I. P. Kuranova, E. A. Smirnova, V. V. Makhaldiani, A. A. Voronova, E. G. Arutyunyan, W. Höhne, and G. Hansen, *Dokl. Akad. Nauk SSSR*, 1981, **258**, 178.
[123] C. S. Wright, *J. Mol. Biol.*, 1981, **145**, 453.
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[125] G. Wistow, C. Slingsby, T. Blundell, H. Driessen, W. De Jong, and H. Bloemendal, *FEBS Lett.*, 1981, **133**, 9.
[126] Y. N. Chirgadze, Y. V. Sergeev, N. P. Fomenkova, and V. D. Oreshin, *FEBS Lett.*, 1981, **131**, 81.
[127] H. M. Holden, L. J. Banaszak, C. Frieden, and D. J. McLoughlin, *FEBS Lett.*, 1981, **132**, 15.
[128] S. A. Saeed and T. R. C. Boyde, *FEBS Lett.*, 1981, **123**, 111.
[129] S. Sherriff and J. R. Herriott, *J. Mol. Biol.*, 1981, **145**, 441.
[130] V. Z. Pletnev, A. P. Kuzin, S. D. Trakhanov, P. V. Kostetsky, V. A. Popovich, and I. N. Tsigannik, *Biopolymers*, 1981, **20**, 679.

Actin: Pancreatic DNase I.—The crystal structure of skeletal-muscle actin complexed with bovine pancreatic DNase I has been determined at 0.6 nm resolution.[131] The actin molecule is 6.7 nm × 4.0 nm × 3.7 nm in size whereas the DNase molecule is 5.0 nm × 5.0 nm × 4.0 nm. The actin molecule is composed of two unequal domains both containing what seems to be β-sheets flanked by helices. A possible site for the bound ATP lies between the two domains in a cleft. Two high-affinity Ca^{2+}-binding sites have been located on the surface of the DNase I molecule.

10 Nucleic Acids and Related Proteins

Tyrosyl tRNA Synthetase.—Rubin and Blow[132] have described the preparation of a crystalline complex of tyrosyl adenylate with tyrosyl tRNA synthetase. A different Fourier calculated at 0.27 nm shows that the tyrosyl adenylate straddles the central β-sheet of the structure with the tyrosine and adenosine moieties lying on opposite sides of the sheet. This binding is also accompanied by extensive movements of the protein. Puromycin, when soaked into crystals of the synthetase, binds in the tyrosine site but produces only small structural changes elsewhere. Arsenate substitution of the mother liquor has identified sulphate-binding sites both on the surface of the molecule and in the binding site. Pyrophosphate has also been shown to occupy the position of the binding-site sulphates.

Methionyl tRNA Synthetase.—The structure of *E. coli* methionyl tRNA synthetase has been described at 0.25 nm resolution[133] and has allowed for a complete tracing of the polypeptide chain. The most salient structural feature is the central core, which shows the topological characteristics of the nucleotide-binding domain, and this is suggested as further evidence for the divergent evolution of nucleotide-binding proteins from a common ancestral nucleotide-binding protein.

Transfer RNA.—Harvey and McCammon[134] have examined the possibility of intramolecular flexibility in phenylalanine tRNA. They show that the molecule appears to show a large degree of flexibility, bending with two degrees of freedom through angles up to 30° at little energetic cost. Since the energy involved is comparable to that of a few hydrogen bonds it is suggested that solvent conditions and interactions with other molecules are important in modulating structure.

DNA/RNA.—Of the many papers published on the crystal structures of DNA and RNA fragments two of note are a preliminary report on the crystallization of DNA/RNA hexanucleotide complex[135] and the structure determination of a left-handed DNA molecule containing six base pairs.[136]

[131] D. Suck, W. Kabsch, and H. G. Mannherz, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4319.
[132] J. Rubin and D. M. Blow, *J. Mol. Biol.*, 1981, **145**, 489.
[133] J. L. Risler, C. Zelwer, and S. Brunie, *Nature (London)*, 1981, **292**, 384.
[134] S. C. Harvey and J. A. McCammon, *Nature (London)*, 1981, **294**, 286.
[135] S. R. Holbrook, I. Tinoco, jun., T.-S. Young, S.-H. Kim, and F. H. Martin, *J. Mol. Biol.*, 1981, **153**, 837.
[136] A. H.-H. Wang, G. J. Quigley, F. J. Kolpak, G. van der Marel, J. H. van Boom, and A. Rich, *Science*, 1981, **211**, 171.

11 Viruses

Influenza-virus Glycoprotein.—The crystal structure at 0.3 nm resolution of the haemagglutinin membrane glycoprotein from influenza virus has been reported.[137] The protein is a trimer of molecular weight about 225 000, each of the three polypeptide chains being glycosylated at seven sites with a total carbohydrate content of about 20% by weight. The overall shape of the trimer is an elongated cylinder, 13.5 nm long, with a triangular cross-section varying from 1.5 to 4.0 nm in radius. There is a triple-stranded α-helical coiled coil, extending about 8.0 nm from the membrane, on top of which sits a globular region containing an eight-stranded sheet structure. It is this globular structure that contains the receptor-binding site and the variable antigenic determinants. The seven carbohydrate sites have been identified and the authors discuss the biological functioning of the molecule.

In an accompanying paper [138] four antigenic sites on the haemagglutin molecule are identified, and it is shown that at least one amino-acid substitution in each site seems to be required for the production of new epidemic virus strains between 1968 and 1975.

Tobacco Mosaic Virus.—Tobacco mosaic virus coat protein has been shown to form two helical aggregates with $16\frac{1}{3}$ and $17\frac{1}{3}$ subunits per turn. Both these forms have been studied and compared with the intact virus.[139] Both the aggregates and the protein in the intact virus are very similar to each other even in the low-radius region, although this low-radius region has a very different structure in the crystalline protein disk. In the helical forms the RNA is replaced by at least one bound anion, and it is suggested that the conformation of one or more of the RNA-binding arginine residues may influence the structure's formation.

By means of computer model-building techniques the structure of RNA in tobacco mosaic virus has been examined [140] and a model was derived that fits the electron density much better than previous models.

Filamentous Viruses.—Caspar and Makowski [141] have considered the geometry and helical symmetry of the two classes of filamentous bacteriophage particles.

A fibre-diffraction study of the fd filamentous bacterial virus has been reported [142] and has allowed a clearer understanding of the protein and DNA structures in the virion.

The preparation of two heavy-atom derivatives of Pf1 filamentous bacterial virus and the subsequent analysis of fibre-diffraction patterns have been described.[143] The results show that the virus has 5.4 protein subunits per 1.5 nm pitch and the positions of the iodine and mercury atoms have been located. The orientation of the DNA in the Pf1 virus has also been studied.[144]

[137] I. A. Wilson, J. J. Skehel, and D. C. Wiley, *Nature* (*London*), 1981, **289**, 366.
[138] D. C. Wiley, I. A. Wilson, and J. J. Skehel, *Nature* (*London*), 1981, **289**, 373.
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[142] D. W. Banner, C. Nave, and D. A. Marvin, *Nature* (*London*), 1981, **289**, 814.
[143] C. Nave, R. S. Brown, A. E. Fowler, J. E. Ladner, D. A. Marvin, S. W. Provencher, A. Tsugita, J. Armstrong, and R. N. Perham, *J. Mol. Biol.*, 1981, **149**, 675.
[144] R. E. Webster, R. A. Grant, and L. A. W. Hamilton, *J. Mol. Biol.*, 1981, **152**, 357.

Cowpea Mosaic Virus.—A study, using electron microscopy and *X*-ray diffraction, of crystals of cowpea mosaic virus has been reported.[145] The crystal packing shows large, open solvent channels, and proposals for the particle orientation and packing are discussed.

Alfalfa Mosaic Virus.—The crystallization of partially digested alfalfa mosaic virus coat protein has been described.[146] Two crystal forms have been produced, one of which diffracts *X*-rays to at least 0.34 nm resolution.

Other Viruses and Viral Products.—An *X*-ray diffraction study of oriented sols of bacteriophage T2L tail-tubes has been reported.[147] Small-angle neutron-scattering studies of alfalfa mosaic virus [148] and a complex of the fd DNA-gene 5 protein [149] have been described.

12 Muscle and Muscle Proteins

The use of a synchrotron radiation source for the recording of low-angle *X*-ray diffraction patterns from frog sartorius muscle with a time resolution in the millisecond range has been described.[150] Offer and co-workers [151] have examined the arrangement of cross-bridges in insect flight muscle in the rigor state using *X*-ray analysis. Luther and collaborators [152] have used diffraction analysis to study the three-dimensional structure of vertebrate-muscle A-band. Taylor and Amos [153] have studied the binding of myosin cross-bridges to muscle thin filaments. Pepe and co-workers [154] have examined changes in the structure of the myosin filament by using diffraction methods. Bennett has studied the structure of paracrystals of light meromyosin.[155] Yagi and co-workers [156] have described studies on the hexagonal net of light meromyosin. The use of *X*-ray diffraction to study the structure of a tactoid of a light meromyosin has been reported,[157] as have studies on the structure of actin paracrystals induced by nerve growth factor.[158]

13 Membranes and Membrane Proteins

Hayward and Stroud [159] have reported the projected structure of the purple membrane at 0.37 nm resolution, and Rogan and Zaccai [160] have described neutron-diffraction experiments on the purple membrane in a wide range of

[145] J. E. Johnson and C. Hollingshead, *J. Ultrastruct. Res.*, 1981, **74**, 223.
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[157] N. Yagi and G. W. Offer, *J. Mol. Biol.*, 1981, **151**, 467.
[158] L. Castellani and E. J. O'Brien, *J. Mol. Biol.*, 1981, **147**, 205.
[159] S. B. Hayward and R. M. Stroud, *J. Mol. Biol.*, 1981, **151**, 491.
[160] P. K. Rogan and G. Zaccai, *J. Mol. Biol.*, 1981, **145**, 281.

relative humidities. The phase transitions of the purple membrane and the brown holo-membrane have been investigated by a range of techniques including *X*-ray diffraction.[161] The locations of the platinum-binding sites on bacteriorhodopsin have been determined[162] as a first step in the use of this derivative as an isomorphous heavy-atom derivative. Kataoka and Ueki[163] have described *X*-ray diffraction studies on the photoreaction unit isolated from *Rhodospirillum rubrum*. A brief report of a workshop on the structure of membrane proteins has appeared.[164] The structure of a complex between ubiquinol and cytochrome *c* reductase from *Neurospora* has been examined in membrane crystals,[165] and the results have been compared with known chemical properties of the components. The same group of workers has also examined freeze-fractured samples of the complex and has confirmed the unequal distribution of protein on the membrane surfaces.[166] Hovmöller and co-workers[167] have examined membrane crystals of mitochondrial cytochrome reductase containing cytochromes *b* and c_1, and Skriver and co-workers[168] have described the formation of two-dimensional crystals of membrane-bound Na^+,K^+-ATPase suitable for structural analysis. Mannella has described an *X*-ray analysis of the outer mitochondrial membrane from plant tissues.[169]

Diffraction studies on yolk-platelet crystals from teleosts and amphibia[170] and from hagfish[171] have been reported, as has an *in vivo* evaluation of the electron-density profile of the frog-rod outer-segment disc-membrane.[172] Welti and co-workers[173] have examined the gel-phase phospholipid in the plasma membrane of mouse LM cells that have been sterol-depleted, and Hauser and Shipley have described the lithium-induced crystallization of phosphatidylserine bilayers.[174]

14 Fibrous Proteins

Collagen.—The meridional *X*-ray diffraction pattern from collagen fibrils in corneal stroma has been interpreted, and a comparison of the electron-density profiles of rat-tail tendon and corneal collagen has been made.[175] The results suggest that hydroxylysine residues may be the major binding sites of sugar binding. Lim[176] has proposed a model for collagen in which the three helices of the collagen molecule interact *via* hydrogen bonds through water molecules. The

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[169] C. A. Mannella, *Biochim. Biophys. Acta*, 1981, **645**, 33.
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[176] V. I. Lim, *FEBS Lett.*, 1981, **132**, 1.

structure of a collagen-like polypeptide, (Pro-Pro-Gly)$_{10}$, has been determined and refined at 0.22 nm resolution.[177]

Fibroin.—Lim and Steinberg[178] have proposed a novel structural model for silk fibroin that differs totally from earlier proposals.

Synthetic Polypeptides.—The conformation of poly(β-phenethyl-L-aspartate) has been found to be a left-handed π-helix.[179] The structure of poly(γ-methyl-L-glutamate) has been examined by small-angle *X*-ray scattering.[180]

15 Other Biological Structures

Ribosomes.—Kuckuk and Hosemann[181] have reported an *X*-ray study of crystallized 70S ribosome lamellae. The dimensions of the macrocells have been determined as 42 nm × 42 nm × 52 nm, and a model for the structure of the lamellae has been proposed. The use of solvents of variable density to study the 50S ribosomal subunit of *E. coli* has been described.[182]

Nucleosome Cores.—Crystals of intact nucleosome cores from a variety of sources have been grown[183] and show very similar unit cells with one core particle per asymmetric unit. The data are consistent with a previously proposed model for the particle in which the 146 base pairs of DNA are wound in about $1\frac{3}{4}$ turns of superhelix around the histone octamer core. The DNA is in the B form and is not fixed firmly to the histone core all along the length of the superhelix path. These results correlate well with the known differential susceptibility of the DNA to nuclease digestion.

Neutron-diffraction studies on the same crystals have been reported at 2.5 nm resolution,[184] using contrast variation methods. The proposed model consists of 1.8 turns of a DNA superhelix, of pitch 2.75 nm and radius 4.2 nm, wound around a protein core.

Bacterial Pili and Flagella.—The structures of polar pili from *Pseudomonas aeruginosa* have been studied by *X*-ray diffraction,[185] and a three-dimensional reconstruction of the flagellar hook from *Caulobacter crescentus* has been reported.[186]

The structure of Streptococcal M protein, which forms the fimbriae on the bacterial surface, has been examined by *X*-ray diffraction.[187] The structure determined for these fimbriae is the first α-helical coiled-coil conformation to be

[177] K. Okuyama, K. Okuyama, S. Arnott, J. Takayanagi, and M. Kakudo, *J. Mol. Biol.*, 1981, **152**, 427.
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demonstrated for bacterial surface projections. The general conformations of bacterial spikes and fimbriae have also been discussed.[188]

Prothrombin/Clotting Factor Xa.—Greer[189] has modelled the possible interactions between prothrombin and blood-clotting factor Xa and suggests that between the two molecules several salt-bridges are formed that may be partially responsible for the enzyme's high degree of specificity.

Antibiotics and Plant-growth Substances.—The structures of des-*N*-tetramethyl triostin A,[190] a quinoxaline antibiotic analogue, and ferric pseudobactin,[191] a siderophore that promotes plant growth, have been described. Butters and co-workers[192] have considered the possible structures for the *N*-terminus of alamethicin.

Small Molecules of Biological Interest.—*X*-Ray studies on the structures of peptide substrates and inhibitors of the Zn^{2+} G and serine R61 D-alanyl-D-alanine peptidases,[193] model complexes of flavin coenzymes,[194] and an adenine : indole-complex-containing compound[195] have been reported.

16 Small-angle Scattering

General Methods.—Miller[196] has discussed the use of small-angle *X*-ray and neutron scattering for the investigation of biological macromolecules. Fedorov[197] has calculated theoretical scattering curves for proteins and discussed the determination of globular-protein volume in solution by small-angle *X*-ray scattering. The use of synchrotron radiation to determine fluctuation *X*-ray scattering from biological particles in frozen solution has been described from the experimental and analytical points of view.[198] Ramakrishnan and Moore[199] have described techniques for processing neutron-distance data in order to derive information about the three-dimensional structure of macromolecular assemblies.

Results from Small-angle Scattering Experiments.—Ribosomal protein S1 has been located on the small ribosomal subunit on the basis of neutron-distance measurements.[200] The S1 molecule also shows a radius of gyration that suggests an axial ratio of 10 : 1, the same as seen for the free molecule in solution. Twelve of the other 21 proteins of the 30S ribosomal subunit have been mapped based on

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neutron scattering.[201] Zipper and Durschschlag[202] have used small-angle *X*-ray scattering to study the *X*-ray-induced aggregation of malate synthase, and a small-angle study of the *E. coli* DNA-dependent RNA polymerase holoenzyme has been reported.[203] Cser and co-workers[204] have described the shape of pig antibody to dinitrophenyl and its conformational changes induced on hapten binding. Neutron small-angle scattering studies of nitrogenase from *Clostridium pasteurianum* have been published,[205] and Paradies and co-workers[206] have described *X*-ray small-angle studies on *E. coli* ATP synthetase. Solution *X*-ray scattering experiments on Ca^{2+}-dependent ATPase have been reported,[207] and Serdyuk and co-workers have shown that ribosomal protein S4 has a compact structure when examined by *X*-ray small-angle scattering.[208] Charlier and co-workers[209] have proposed a low-resolution model for the lac repressor based on small-angle neutron studies. They have studied the native protein as well as the isolated tryptic core and the S1-residue *N*-terminal head-piece and describe the implications of the structure for the interaction with the lac operator DNA. Neutron-scattering studies on selectively deuteriated human-plasma low-density lipoproteins[210] and the acetylcholine receptor dimer[211] have been published.

17 Protein Conformations – Analysis and Predictions

Rossmann and Argos[212] have reviewed the general topic of protein folding, and Ptitsyn[213] has proposed a general physical model for how a protein folds. Schulz[214] has considered the process of protein differentiation and has discussed how new proteins have evolved.

Lesk and Rose[215] have described a method to identify all compact, contiguous-chain, structural units in a protein from the *X*-ray co-ordinates. These units can then be used to describe the hierarchy of folding pathways for the molecule.

Rashin[216] has reported a method, based on surface-area measurements, for the location of domains in protein structures. Wodak and Janin[217] have also used surface-area measurements to define structural domains and present examples of its use.

[201] V. R. Ramakrishnan, S. Yabuki, I.-Y. Sillers, D. G. Schindler, D. M. Engelman, and P. B. Moore, *J. Mol. Biol.*, 1981, **153**, 739.
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Hol and co-workers [218] have discussed the dipoles of the α-helix and β-sheet and discussed the role of the dipoles in protein folding. Thornton [219] has examined the presence of disulphide bridges and analysed their distribution, topology, conformation, and conservation. Disulphide bridges are generally found to be very well conserved, whereas free cysteines mutate rapidly. Levitt [220] has used conformational-energy calculations to study the role of the proline residues in the folding of bovine pancreatic trypsin inhibitor.

The specific interactions involving the guanidyl groups observed in crystal structures have been described,[221] and the radial distribution of clusters of hydrophobic and hydrophilic amino-acids has been studied.[222] Wada and Nakamura [223] have examined the nature of the charge distribution in proteins and shown that the charges in the proteins are, on average, surrounded by charges of opposite sign. The effects of the helix dipoles are also considered.

The packing of 50 helix pairs in ten proteins of known structure has been analysed.[224] The results support a model in which helix–helix packing consists of the intercalation of the helix-surface ridges and grooves. The model explains the predominance of helix packings with an interhelical angle of $-50°$.

Salemme and Weatherford [225] have examined the geometry of idealized, twisted, parallel β-sheets and compared their properties with known structures. The comparison shows that naturally occurring sheets are closely approximated by the models. In a second paper [226] the authors expand the analysis to antiparallel and mixed β-sheets. They show that antiparallel structures can assume a much greater diversity of conformations than occur in parallel sheets. The third paper in the series [227] summarizes and contrasts the geometrical and conformational properties of parallel and antiparallel β-sheets. In either case it is shown that the surfaces formed correspond to isotropically stressed configurations.

Chothia and Janin [228] have analysed the face-to-face packing of β-sheets in proteins. The authors propose a model to explain the observed predominance of an angle of $-30°$ between the sheets. The model shows that this orientation is a consequence of the rows of side chains being approximately aligned and the β-sheet having a right-handed twist. In several proteins examined the amino-acid composition of the interface makes the contact surfaces essentially smooth and hydrophobic.

Cohen and co-workers [229] have examined the tertiary structures of ten β-sandwich structures and have detailed the standard packing geometry they have

[218] W. G. J. Hol, L. M. Halie, and C. Sander, *Nature* (*London*), 1981, **294**, 532.
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observed. These observations have been incorporated into an algorithm to predict the fold of β-sandwiches from primary and secondary structure.

Kopple [230] has described a method for the location of β-turns in structures, and Némethy and co-workers [231] have reported conformational-energy calculations of *cyclo*-(L-alanylglycyl-ε-aminocaproyl), a cyclized dipeptide model of a β-bend.

Rao and Argos [232] have reported an examination of the structural stability of halophilic bacterial proteins. The suggested principal mode of structural stabilization is the utilization of many external carboxyl groups while halophilic functionality is preserved through avoidance of additional negative charge at the active-site surface.

Némethy and Scheraga [233] have examined the interactions between disulphide derivatives and aromatic groups in peptides and proteins. Wako and Scheraga [234] have described a simple method to assess the information that is provided by distance constraints when a protein is folded. Rapaport and Scheraga [235] have examined the evolution of the conformations of a short polypeptide by computer simulation. The use of a high-speed array processor has allowed simulations on protein chain lengths not possible on conventional large computers. Meirovitch and Scheraga [236] have described an approach that can overcome the problem of multiple minima in protein-folding simulations, and Rackovsky and Scheraga [237] have analysed single-site and nearest-neighbour distributions and the nucleation of protein folding.

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

Edited by R. H. Pain, with contributions by B. Adams, T. Brittain, R. Devonshire, D. P. E. Dickson, P. D. Jeffrey, L. W. Nichol, H. W. E. Rattle, N. K. Rogers, R. M. Stephens, M. J. E. Sternberg, and D. J. Winzor

1 Theoretical Aspects of Protein Conformation

Contributed by N. K. Rogers and M. J. E. Sternberg

Introduction.—This year there have been two general reviews of the analysis and prediction of protein structure.[1,2] The proceedings of two conferences also provide many useful papers.[3,4] In this review we will concentrate on the primary literature.

Energy calculations have received considerable coverage in applications to conformational analysis, hydrogen-bond analysis, electrostatic interactions,

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[2] M. G. Rossmann and P. A. Argos, *Annu. Rev. Biochem.*, 1981, **50**, 497.
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Monte Carlo and statistical mechanical calculations, and, increasingly, molecular dynamic simulations.

Energy Functions and Conformational Analysis.—If energy calculations are to continue to yield useful results, then the energy functions used must be improved and extended. Peters and Peters have continued their quantum-mechanical analysis of the various hydrogen bonds found in small model peptides [5,6] and α-helices.[7] They have also examined the quality of different basis sets [8] in describing these bonds. A mathematical expression [6] is given that may help future classical or semi-classical energy calculations. Classical analyses have been carried out by Mao *et al.*[9] on the interaction of polar and non-polar atoms, where penetration of Van der Waals' radii seems common, and by Némethy and Scheraga [10] on the interaction of disulphide derivatives with aromatic groups.

Long-range resonance and electrostatic effects have been examined by Ohsaku *et al.*[11] Jernigan *et al.*[12] have considered the role of electrostatic forces in the stabilization of protein structures, as have Hol *et al.*[13] Two other contributions to the conformational energy have been discussed: entropy and a method for its calculation by Karplus and Kushick [14] and the hydrophobic effect by Ponnuswamy and Prabhakaran.[15] Meirovitch and Scheraga [16] have examined short-range restrictions on long-range interactions governing the folding of bovine pancreatic trypsin inhibitor (BPTI), having started from a near-native conformation.

Conformational analysis has continued on a number of model small peptides by the use of both classical [17–21] and quantum [22–23] mechanical methods. Rao *et al.*[24] have reformulated the energy-minimization techniques of Goel and Ycas [25] in terms of interatomic distances instead of angles and have applied [26] it to a model tripeptide, using standard non-bonded and electrostatic functions. Manavalan and Momany have performed conformational-energy studies on *N*-methyl-

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[6] D. Peters and J. Peters, *J. Mol. Struct.*, 1980, **68**, 255.
[7] D. Peters and J. Peters, *J. Mol. Struct.*, 1980, **69**, 249.
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[23] D. Cabrol, H. Brock, and D. Vasilescu, *Biopolymers*, 1981, **20**, 269.
[24] G. S. Rao, R. S. Tyahi, and R. K. Mishra, *J. Theor. Biol.*, 1981, **90**, 377.
[25] N. S. Goel and M. Ycas, *J. Theor. Biol.*, 1979, **77**, 253.
[26] G. S. Rao, R. S. Tyahi, and R. K. Mishra, *J. Theor. Biol.*, 1981, **90**, 391.

ated analogues[27] of small polypeptides and enkephalin.[28] Further to these, energy calculations were applied to α-helix docking [29] and structure refinement for collagen models.[30]

Active-site analysis has been applied to a number of different protein–substrate complexes, using known native co-ordinates. Pincus and Scheraga [31] have examined (GluNAc–MurNAc) polymers complexing with hen egg-white lysozyme. Paslen and Lipkind [32] have worked on ester and peptide complexes of carboxypeptidase A. Day *et al.*[33] have carried out *ab initio* calculations on model protein–DNA interactions and found a predominant electrostatic term in the interaction energy. De Tar [34] has examined the relative substrate activities of the D and L forms of Ac-(L or D)-Trp-X with relation to chymotrypsin and found the electrostatic differences between the enantiomorphs to be the dominant factor. Sufrin *et al.*[35] have carried out a steric mapping of the L-methionine binding site of ATP:L-methionine *S*-adenosyl transferase.

Water Structure and Electrostatics.—A major problem in electrostatic calculations is the dielectric constant and whether this macroscopic concept can be used on microscopic calculations. For this and other reasons the water structure around globular proteins has been studied theoretically and experimentally with regard to enzymic function, electrical properties, and more precise energy calculations.

Marchese *et al.*[36] have analysed a potential function for water–water interactions by using electron–nuclei interaction energies. Goodfellow *et al.*[37] have been more specific to proteins and amino-acids in extensive computer simulations of solvent structure. Experimental work has been carried out by Gascoyne and Pethig [38, 39] on electric-dipole moments of bound water and on dissociation as a function of hydration. They found a discontinuity in enzymic activity and the electrical properties at 10% hydration. Bryan [40] has examined the thermodynamic properties of protein–water systems.

Electrostatic calculations have been applied to functional analysis [41] of haemoglobin and its tetramer formation,[42] active-site potential mapping of human carbonic anhydrase,[43] folding of secondary-structure units,[13] radial-distribution analysis of residue types,[44] crystal packing of model and amides,[45] transmem-

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[28] P. Manavalan and F. A. Momany, *Int. J. Pept. Protein Res.*, 1981, **18**, 256.
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brane charge transfer,[46] and the mimicking of secondary-structure dipoles by simpler electronic arrangements.[47] So it is seen that the implications of electrostatic calculations are diverse, but development is hampered by the problems of solvent and dielectric modelling and insufficient dynamical knowledge. As water structure and dynamics simulations become better understood, then electrostatic (electrodynamic?) calculations may well shed new light on function, activity, and mechanism.

Kollman and Hayes[46] have carried out *ab initio* calculations on the protonation of model serine and cysteine proteases. Rees[48] has conducted experiments on the dielectric properties of cytochrome *c* and found a very high value of $\rho_r \simeq 50$ within the protein, compared with previously accepted values of 3—5.

Helix–Coil Transitions.—The helix–coil transition has been studied in detail for a number of model polypeptides by a variety of techniques. Pear *et al.*,[49] McCammon *et al.*,[50] and Birshtein *et al.*[51] used simplified time-extended dynamic simulations and Monte Carlo methods. Scheraga[52–55] and co-workers continued their statistical analysis of experimental results, Amiya *et al.*[56] have developed theoretical work from experimental data, and Mattice[57] has examined the changes in the molecular inertia tensor during transitions.

Molecular Dynamics.—Molecular dynamics simulations have been carried out on a number of different proteins by a number of different methods. The work on BPTI has been considerable: Levitt[58] considered the overall atomic displacements in the production of his 'movie' and in the examination of hydrogen-bond fluctuations.[59] In this latter paper he has correlated his calculations with *X*-ray diffraction and n.m.r. data. McCammon and Karplus[60] have considered the tyrosine ring rotations. van Gunsteren and Karplus[61] have done a 25 ps simulation with explicit solvent interactions calculated; their results seem to indicate considerable differences from *in vacuo* calculations especially at the protein surface. Northrup *et al.*[62] have continued their analysis of anisotropic motion in a dynamic simulation of tuna ferrocytochrome *c*. Phillips[63] has reviewed *X*-ray diffraction studies on

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various known lysozymes, and Gavish [64] has modelled the *X*-ray data in terms of non-harmonic potentials where the mean-square displacement is proportional to T^{μ} for $-2 \leqslant \mu + 20$, T being the absolute temperature.

Protein dynamics methods have been applied to hinge bending [65] in yeast $tRNA^{Phe}$, the calculation of conformational entropies,[14] helix–coil transitions,[49, 50] and transmembrane [66] ion transport.

Analysis and Prediction of Secondary Structure.—The relationship between local sequence and local structure has continued to be examined.[67–71] As noted in last year's review, the work on the prediction of secondary structure from sequence has been on the application of existing methods rather than the development of new and better algorithms. The accuracy of secondary-structure prediction therefore remains around the 70% level.

The main-chain dihedral angles observed in globular proteins have been examined by several workers.[67–70] Chain reversals have also been analysed.[71]

Several workers have carried out secondary-structure predictions on proteins of unknown structure.[72–78] The method most often used is that of Chou and Fasman.[78] The proteins include the nicotinic acetylcholine receptor,[72] fish protamines,[73] glutamate dehydrogenase,[74] apamin and related bee-venom peptides,[75] beta-lactamases and penicillin-sensitive D-alanine-carboxypeptidases,[76] and *Lens culinaris* lectin.[77]

Analysis of Tertiary Structure.—The complexity of the three-dimensional structure of globular proteins is reflected by the variety of types of analyses performed. These can be divided into studies on the following: the conformation and packing of α-helices and β-strands,[79–89] the role of the disulphide

[64] B. Gavish, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 6868.
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[85] M. G. Rossmann, *Philos. Trans. R. Soc. London, Ser. B*, 1981, **293**, 191.
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bridge,[1,90-92] the relationships between different proteins,[93-95] domains and exons,[96-108] and the location of residue types in the globular protein.[15,44,109-114]

In 1976 Levitt and Chothia[115] classified globular proteins into four types depending on the packing of their regular secondary structures: α/α, β/β, α/β, and $\alpha + \beta$. Comprehensive summaries of the known protein structures in terms of this classification are given in reviews by Richardson[1] and by Rossmann and Argos.[2] Hol *et al.*[13] considered the peptide dipole in α-helices and β-sheets and suggested that electrostatic interactions between these dipoles are important in the determination of the alignment of secondary structures in globular proteins. The stacking of two β-sheets in β/β proteins such as immunoglobulin domains has been examined by Cohen *et al.*[79] and by Chothia and Janin.[80] The geometry of stacking is explained as a result of the twisted nature of the β-sheet.[116] There have been analyses of and reviews on the packing of α-helices against a β-sheet in α/β proteins such as the glycolytic enzymes (refs. 81—85). Several topological and geometric features of α/β packing result from the twist observed in β-sheets.[116] This aspect of β-sheet structure has been examined by Salemme and Weatherford,[86-88] who constructed idealized β-sheet with different twists and examined resultant stereochemical features such as hydrogen bonds. Ashida *et al.*[89] have considered the twisted nature of β-sheets found in several oligopeptide crystals.

The polypeptide chain is often crosslinked by disulphide bridges. Thornton,[90] Richardson,[1] and Pirtskhalava *et al.*[90] have analysed these bridges according to their distribution, topology, conformation, and conservation. Klapper and Klapper[92] considered the types of disulphide linkages that will produce a knot in the polypeptide chain.

[90] J. M. Thornton, *J. Mol. Biol.*, 1981, **151**, 261.
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[115] M. Levitt and C. Chothia, *Nature (London)*, 1976, **261**, 552.
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Several workers have considered the evolutionary significance of structural similarity between different proteins or protein domains. The studies considered the four α-helical haem proteins,[93] hen egg-white and bacteriophage T4 lysozymes,[94] and the two domains of rhodanese.[95]

It is well known (*e.g.* ref. 117) that many globular proteins fold into two or more spatially distinct regions known as domains. Several workers[96-99] have developed algorithms that identify domains from the atomic co-ordinates of a protein. The results of the different algorithms are in broad agreement. However, Richardson[1] has, on an intuitive basis, classified the domains in the known structures, and her view is quite different from the results of these algorithms. Clearly further work is required to achieve a consensus as to what constitutes a domain.

One of the reasons for the current interest in domains is that their locations could be related to the exon structure of the gene.[100-108] The present evidence is confusing. In an immunoglobulin each domain (*e.g.* V_H or C_{H1}) is coded for by one or more different exons.[100] But in other proteins the relationship often is less obvious. Artymiuk *et al.*[101] have shown that exons 2 and 3 of hen egg-white lysozyme code for the part of the protein structure that resembles T4 lysozyme – a protein that has a different overall fold. Go[102] has related the division of the β-haemoglobin gene into three exons to a plot that shows distant Cα–Cα separations in the tertiary fold. This work had a remarkable predictive value as it suggested that in some globins there might be four exons, and their presence was subsequently found in leghaemoglobin.[103, 104]

There have been several analyses of the location of residue types within globular proteins.[15, 44, 109-114] Often the radial distribution of hydrophobic and hydrophilic residues from the centroid of protein is considered. Wada and Nakamura[44] have considered the charge distribution in proteins. They found that often charges are surrounded by charges of the opposite sign. In this study and in the work of Hol *et al.*[13] there is a major problem of accurately modelling the dielectric constants of the protein and the surrounding medium. Kazim and Atassi[114] have investigated the nearest neighbours to antigenic sites in sperm-whale myoglobin.

Prediction of Three-dimensional Structure.—Although many different approaches are being explored to predict protein structure from sequence,[81, 82, 118-134] this

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year there has been an emphasis on prediction based on homology or analogy with a known fold.[118-127] In addition the pathway of folding has been modelled.[135-140]

Blundell and his co-workers [118, 119] have used interactive computer graphics to model the atomic structures of casiragua insulin from the known co-ordinates of porcine insulin and of β-crystallin from γ-crystallin. Other studies are less detailed and suggest an overall fold for an unknown protein based on the resemblances of its sequence and its predicted secondary structure with a protein of known structure. For example Sternberg and Cohen[120] have suggested a tertiary model for interferon based on a secondary-structure prediction that suggests four α-helices and on a proposal that these α-helices pack into a four-fold α-helical bundle. Two studies have tried to assess the success of predictions based on structural analogy. Greer[126] investigated comparative model-building for the mammalian serine proteases. Olsen[127] suggested criteria for judging the correctness of structures predicted from sequence data and applied them to the proposed dinucleotide binding fold of glutamate dehydrogenase.

Several more general approaches have been explored. Cohen *et al.*[81] have developed a combinatorial algorithm to dock α-helices and β-strands in α/β proteins. Ptitsyn and Finkelstein[82] have demonstrated the power of topological restrictions in the prediction of the fold of α/β proteins. The use of distance geometry to fold a protein was considered by Wako and Scheraga[128] and by Crippen *et al.*[129] Rackovsky and Scheraga[130] found good agreement between the recently determined *X*-ray structure of gramicidin S and a structure predicted by energy minimization.

Various workers have investigated the pathway or pathways along which a protein folds. Levitt[135] considered the effect of the *cis–trans* isomerization of proline residues during the refolding of ribonuclease. He finds that only one of the four proline residues must be in the native (*i.e. trans*) conformation prior to the rest of the protein acquiring its native structure. Go and Abe[136, 137] have explored the use of two-dimensional lattices to model protein renaturation.

Conclusion.—This year there has been an increasing emphasis on the role of electrostatic interactions in determining the structure and function of proteins. Improvement in the modelling of these effects might lead to greater accuracy in the application of energy functions to model molecular dynamics and to predict protein structure based on homology with a known fold.

2 The Stability and Mechanism of Folding in Globular Proteins

Contributed by B. Adams

Stability.—Privalov[141] has reviewed the stability of small globular proteins.

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[133] R. Rosen, *Int. J. Syst. Sci.*, 1980, **11**, 527.
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[135] M. Levitt, *J. Mol. Biol.*, 1982, **145**, 251.
[136] N. Go and H. Abe, *Biopolymers*, 1981, **20**, 991.
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[139] V. L. Hess and A. Szabo, *Biophys. Chem.*, 1980, **12**, 243.
[140] D. Kristofferson, T. L. Karr, and D. L. Punch, *J. Biol. Chem.*, 1980, **255**, 8567.
[141] P. L. Privalov, *Adv. Protein Chem.*, 1979, **33**, 167.

The Effect of Amino-acid Composition. The guanidinium hydrochloride-induced unfolding of yeast iso-2-cytochrome *c*, which differs from horse cytochrome *c* by 46% of its amino-acid sequence, has been studied.[142] The difference in conformational stability between these two proteins is in agreement with the predicted difference calculated from the free energies of transfer of buried hydrophobic groups. The kinetics of refolding are broadly the same as those observed for horse cytochrome *c*,[143] suggesting that the quantitative features of protein-folding reactions are conserved during evolution.

A comparison of the amino-acid composition of twenty proteins from closely related mesophilic and thermophilic micro-organisms[144] is consistent with the suggestion[145] that proteins from thermophilic bacteria have a greater hydrophobicity index, a lower ratio of polar-to-non-polar volumes, an increased arginine and lysine content, and more α-helix- and β-sheet-forming amino-acid residues. A study of point mutation in bacteriophage T4 lysozyme[146] shows that thermolabile proteins are associated with changes in both entropy and enthalpy, causing little net change in the Gibbs free energy of unfolding.

Proteins from halophilic bacteria[147] contain fewer bulky amino-acid side chains in their interior and additional acidic residues on their surfaces when compared to normal proteins. It is suggested that the increase in surface acid residues will help the protein compete with salt for hydration water and the reduced bulkiness of internal residues will prevent inactivation in high concentrations of antichaotropic potassium chloride.

The Effect of Charge. Wada and Nakamura[148] have analysed the distribution of ionic groups and the apparent charges at the termini of α-helices for 14 proteins. They conclude that charges tend to be surrounded by charges of the opposite sign, each charge contributing several kcal to the conformational stability. The role of α-helix and β-sheet dipoles in protein folding has been studied.[149] The alignment of these secondary-structure dipoles appears significant in determining the three-dimensional structure of proteins.

Modification of methionine-52 of bovine pancreatic trypsin inhibitor by methyl iodide, giving a positive charge, had no effect on the native protein but slowed its refolding kinetics.[150]

The Effect of Disulphides. A study of disulphide bridges in globular proteins[151] shows a strong preference for links between cysteines close to one another in the amino-acid sequence. The role of disulphides in globular proteins is discussed and it is suggested that they stabilize but do not dictate folding.

A study of the refolding of reduced ribonuclease by trapping disulphides[152]

[142] B. T. Nall and T. A. Landers, *Biochemistry*, 1981, **20**, 5403.
[143] P. J. Hagerman, *Biopolymers*, 1977, **16**, 731.
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[145] P. Argos, M. G. Rossman, U. M. Grau, H. Zuber, G. Frank, and J. D. Tratshin, *Biochemistry*, 1979, **18**, 5698.
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[150] T. E. Creighton, *J. Mol. Biol.*, 1981, **146**, 375.
[151] J. M. Thornton, *J. Mol. Biol.*, 1981, **151**, 261.
[152] A. Galat, T. E. Creighton, R. C. Lord, and E. R. Blout, *Biochemistry*, 1981, **20**, 594.

shows the presence of a three-disulphide intermediate with a native-like structure. Other trapped intermediates had no apparent structure, suggesting disulphides are formed at random.

The Effect of Ligands. Phosphate has been shown to protect yeast 6-phosphogluconate dehydrogenase from proteolysis and to stabilize it against inactivation by disulphide-exchange reagents, SDS, and urea.[153] This could be due to the formation of stabilizing 'phosphate bridges'.

Calcium has been shown to induce and stabilize folding in synthetic peptides similar to the calcium-binding site three of troponin C.[154] A c.d. study on gastrin-related peptides indicates that the active *C*-terminal tetrapeptide folds into its active structure only on interaction with a receptor.[155]

The limited unfolding caused by the removal of haem from human α-globin has been characterized.[156]

The Effect of Solvent. The equilibria of amino-acid side-chain groups between their dilute aqueous solutions and the vapour phase have been determined by dynamic vapour-pressure measurements.[157] The resulting 'hydration potentials' are more closely correlated with the relative tendencies of various amino-acids to appear on the surface of globular proteins than with values from earlier studies on free amino-acids.

By studying lysozyme in reverse micelles with differing water content, alpha-helix content and enzymic activity have been shown to be dependent on the protein's hydration state.[158] This is thought to be due to the change in protein–water hydrogen bonding. A hydration-dependent change in the mobile charges of bovine serum albumin is observed by conductivity measurements.[159] The thermal stability of chymotrypsin [160] is shown to be dependent on hydration, by studies on crystals of differing water content. This gave results similar to the behaviour of lysozyme.[161]

The effect of molecular crowding by other proteins on glyceraldehyde 3-phosphate dehydrogenase [162] and myoglobin [163] has been studied. In both cases molecular crowding leads to protein association, probably because of the smaller exposed surface area requiring hydration in the associated proteins.

The effect of glycerol [164, 165] and sucrose [166] on the stability of many proteins has been studied. Both are thought to be excluded from the protein–water interface bringing about a reduction in the entropy of protein hydration. This destabilizes both the native and the unfolded proteins but has a greater effect on

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[154] R. E. Reid, J. Gariepy, A. K. Saund, and R. S. Hodges, *J. Biol. Chem.*, 1981, **256**, 2742.
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[166] J. C. Lee and S. N. Timasheff, *J. Biol. Chem.*, 1981, **256**, 7193.

the unfolded state because of its larger exposed surface area. Sucrose has also been shown to cause a change in the tertiary structure and aggregation of α-crystallin.[167] This could be similar to the effect seen for molecular crowding (see above).

The interaction of nine proteins with urea has been studied by measuring their partial specific volume in 8M urea.[168] The results were in good agreement with a model in which one urea molecule binds each pair of peptide units and one to each aromatic side chain. There was no correlation with protein hydrophobicity.

Protein Association. Ross and Subramanian[169] state that as the free-energy, entropy, enthalpy, and heat-capacity changes associated with protein association are all negative then association cannot be driven by hydrophobic interactions alone. They suggest a strengthening of hydrogen bonding and van der Waal's interaction occurring in the hydrophobic interior of the protein. The role of hydrogen bonding on the dimerization and receptor interaction of insulin has been investigated using a thermodynamic model, based in part on the *X*-ray crystallographic data.[170] It appears that hydrogen bonding does play an important role in these interactions and it is suggested that this could apply as a general rule for other protein associations.

Evidence for the mutual stabilization of subunits on protein association comes from beef cardiac troponin,[171] lactate dehydrogenase hybrids,[172] *Drosophila melanogaster* alcohol dehydrogenase heterodimers,[173] and the binding of ribonuclease S peptide to the S-protein.[174, 175] Evidence for the formation of native monomers during refolding of a dimeric protein comes from work on creatine kinase.[176]

The association of lactate dehydrogenase dimers to give tetramers has been shown to have the same kinetics as tetramer formation from the unfolded protein.[177] This indicates that dimer association is the rate-limiting step in the refolding of this protein.

Conformational Dynamics.—Gurd and Rothgeb review motions in proteins and discuss their significance.[178] A theoretical study on the pressure dependence of aromatic-ring rotation[179] concludes that this dependence does not imply a physical volume in which the residue can rotate, as suggested earlier,[180, 181] but

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[172] J. Muller and C. Klein, *Biochim. Biophys. Acta*, 1981, **671**, 38.
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[175] A. M. Labhardt, *Biopolymers*, 1981, **20**, 1459.
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[177] R. Jaenicke, W. Vogll, and R. Rudolph, *Eur. J. Biochem.*, 1981, **144**, 525.
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that the occurrence of small packing defects is pressure dependent and that these defects could initiate ring rotation, as previously suggested.[182]

A nuclear gamma resonance absorption study on metmyoglobin crystals[183] indicates two distinct temperature regions with differing motional behaviour. Computer modelling of the temperature dependence of motion in proteins[184] shows that the constituent groups spend part of their time in weakly bound states or traps and the rest of their time almost free in a restricted volume. Below its transition temperature a particle is trapped and above its transition temperature it is free.

Cooper[185] suggests that many observed transitions in globular proteins may not be changes in the mean conformation but temperature-dependent dynamical processes. He argues that an observed fluorescence quenching by acrylamide in proteins[186] could be due to surface-tryptophan-residue quenching having a different dependence on solvent viscosity from that of buried tryptophan residues.[187] An earlier, more complex explanation is that acrylamide could be preferentially binding to some proteins.[188]

A simulation of the dynamics of hydrogen bonds in bovine pancreatic trypsin inhibitor[189] based on the *X*-ray structure shows that different hydrogen bonds have different stabilities as indicated by the mean bond lengths and length fluctuations. The most stable hydrogen bonds involve the hydrogen atom observed to exchange most slowly with solvent in n.m.r. experiments.

There has been a study of amino-acid side-chain dynamics of bacteriorhodospin in *Halobacterium halobium* by high-field deuterium n.m.r. spectorscopy.[190] This is the first observation of side-chain dynamics in a membrane protein.

Fluorescence-depolarization studies on the α-subunit of F_1-ATPase from the thermophilic bacterium PS3 show a low anisotropy.[191] This is said to be due probably to rotation between domains in the subunit.

Measurement of the spin–spin coupling constants for many previously assigned residues was used to compare the crystal and solution conformation of the basic pancreatic trypsin inhibitor.[192] Although the centre of the protein was similar in both forms, the protein surface showed a lower mobility in the crystallized protein. Computer simulation of atomic motion in tuna ferrocytochrome *c* showed the mean atomic positions and mobility about these atomic positions to be in agreement with the *X*-ray data.[193] The degree to which motion is limited to one axis and the preferred direction of atomic displacement showed correlation with structural features of the protein.

[182] J. A. McCammon and M. Karplus, *Biopolymers*, 1980, **19**, 1375.
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Mobility and Protein Activity. Infrared spectra of the carbon monoxide complex of bovine-heart myoglobin as a function of temperature and pH indicate four discrete and rapidly interconverting conformers at the active site.[194] N.m.r. has been used to show the co-existence of two conformers of the *Lactobacillus casei* dihydrofolate reductase–trimethoprim–$NADP^+$ ternary complex[195] and conformational heterogeneity in the haem pocket of ferrocytochrome *c*.[196]

Mobility in the ox-heart pyruvate dehydrogenase complex has been attributed to large regions of polypeptide carrying the lipoic acid residues.[197,198] This would enhance the mobility of the lipoic acid groups, essential for catalysis by this enzyme complex.

Mechanisms of Folding.—In general, a nucleation-driven hierarchic mechanism for the folding of globular protein is emerging. This involves nucleation events followed by the formation around the nucleation sites of structural units leading to the formation of folded domains. These domains would then arrange themselves to give the native structure.

The Nucleation Event. Go and Abe [199] support the suggestion of Karplus [200] that the initial stage in protein folding could be the formation of non-interacting portions of native-like structure along the polypeptide chain. These sites then grow by a 'growth-merge' mechanism to give the native protein. This differs from the proposal of Lesk and Rose,[201] who suggest that hydrophobic nucleation sites lead to the formation of folding clusters. A study on the refolding of reduced bovine serum albumin shows that backbone c.d. forms five-to-ten times faster than ligand binding.[202] Also, antigenic determinants on some portions of the molecule return faster than others. They suggest that this is due to independent folding of structural regions from multiple nucleation sites.

Formation of Domains. Proteolytic fragments of proteins have been shown to refold to give native-like structure in bovine serum albumin,[202] myoglobin,[203] tropomysin,[204] ovomucoid,[205-207] isocitrate dehydrogenase,[208] lac repressor protein,[209] thioredoxin,[210] and mollusc haemocyanin.[211] There is an excellent review on the folding of protein fragments.[212]

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Proteins shown to contain domains from analysis of their unfolding transitions are tropomyosin,[204] ovomucoid,[207] isocitrate dehydrogenase,[208] T_4 lysozyme,[213] apomyoglobin,[214] aspartokinase homoserine dehydrogenase,[215] pepsin and pepsinogen,[216] bovine prothrombin,[217] soybean β-amylase,[218] and rabbit-muscle pyruvate kinase.[219] The unfolding of the α-subunit of tryptophan synthase,[220] which is apparently not two-state, may involve the separate folding of two subdomains.

Wodak and Janin [221] have used surface-area measurements to find segments of polypeptide chains making a minimum of interactions with the rest of the proteins. These areas fit well with structural regions observed from inspection of protein models. A prediction for globin domains based on buried surface area [222] correlates with those observed by Wetlaufer,[223] but not with sequences separated by introns. Structural units predicted by stereodiagrams and the Cα-to-Cα distance map for haemoglobin [224] give a good correlation with DNA exonic regions. This correlation is supported by Eaton [225] and discussed by Blake.[226]

An examination of the degree of sequence homology between domains of bovine-liver rhodanese [227] supports the idea that they are the intraspecies products of a partial internal duplication of an ancestral gene. There is a review on the occurrence and role of structural domains in proteins.[228]

Joining of Domains. A compact folding intermediate with a native-like secondary structure and slowly fluctuating tertiary structure has been observed for α-lactalbumin.[229] This could involve the shuffling of secondary structure suggested by the authors, or even the association of domains and subdomains. Measurement of the exchange rate of tryptophan in lysozyme with deuteriated water [230] suggests a 'premelting' of the protein surface before unfolding.

The presence of competitive inhibitors during the refolding of immobilized trypsin has been shown to increase the yield greatly.[231] It is suggested that these inhibitors are binding to a folding intermediate that has a section of native structure. The irreversible loss of activity of acetylcholinesterase occurs at a lower guanidine concentration than the major changes in the c.d. and difference

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spectra.[232] This could be due to the separation of folding units about the active site.

Proline Isomerization. Under conditions where the native structure of ribonuclease A is only marginally favoured, the kinetics of the return of native tyrosine fluorescence coincide with those of all of the parameters measured. When the kinetics are measured in conditions strongly favouring the native state, fluorescence changes are slower than absorbance and 2′-CMP binding changes, the fluorescence return showing the properties of proline isomerization.[233] It is suggested that the refolding is blocked in a nearly native intermediate, whose further folding requires a proline isomerization event, but that this intermediate is unstable at low denaturant concentrations. The non-native isomer of proline-93 is responsible for this blocking.[234] Konishi *et al.*[235] have examined the pre-rate-limiting steps in the refolding of ribonuclease by trapping SH groups. Myer *et al.*[236] have used stopped-flow absorbance measurements to show fast and slow phases in the refolding of heart ferricytochrome *c*. As the first step is urea dependent and the second urea independent they suggest that the slow phase is limited by proline isomerization. The slow step apparently involves haem co-ordination and structural rearrangements in the tryptophan-containing domains. Experiments following the refolding of iron(III) cytochrome *c*[237] show that in the fast refolding species the return of native absorbance at 695 nm precedes the return of activity whereas the two parameters superimpose in the slow refolding species. It was previously thought that one unfolded species was responsible for the biphasic unfolding of this protein.[238]

The two slow steps of refolding of the α-subunit of tryptophan synthase have been characterized.[239] The slower step could be limited by proline isomerism as it is independent of urea concentration. It has an apparent activation energy of $14.5 \pm 2.6\,\text{kcal mol}^{-1}$.

The folding of hen egg-white lysozyme can be biphasic.[240] The kinetics of the slow refolding phase are surprisingly dependent on guanidine concentration but, as the rate of proline isomerization is dependent on the bulkiness of adjacent side chains and peptide interaction, the nature of the solvent could affect the kinetics.

Wood *et al.*[241] suggest that the dimerization of mitochondrial malate dehydrogenase monomers could have a rate-limiting step involving isomerization.

Branching of Folding Pathways. Five proteins with enolase activity were obtained from worm body fluids after electrophoresis on cellulose polyacetate strips.[242] Two were shown to have the same subunit structure, the same electrophoretic mobility in 8M urea, and the same-sized distribution of peptides produced on

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protease digestion but differing sensitivities to thermal denaturation. Treatment of one of the purified forms with acid, alkali, urea, guanidine, or iodide ions resulted in its conversion to the other form.

Refolding of bovine pancreatic trypsin inhibitor leads not only to native protein but also to other native-like states.[243] Gentle heating results in the conversion of all native-like states to a conformation indistinguishable from that of the native protein. It is suggested that these metastable states resulted from events early in the refolding pathway.

Irreversible thermal denaturation of ribonuclease results in five components on Sephadex G-75.[244] One of these states is virtually indistinguishable from the native protein, while the others show low activity, which is enhanced by addition of the S-peptide.

3 Nuclear Magnetic Resonance

Contributed by H. W. E. Rattle

N.m.r. continues to open up as a technique for the biochemist: 500 MHz commercial spectrometers are delivered and working, and the burgeoning field of topical magnetic resonance offers new and unique opportunities of studying metabolism *in vivo*. Structural studies of smaller peptides and proteins will be greatly facilitated by the two-dimensional methods described below. Reviews of the field include a new volume in the series *Biological Magnetic Resonance*[245] and reviews of earlier peptide and protein n.m.r.[246] and of applications of two-dimensional methods to proteins.[247]

Methods.—A sequence of four papers[248-251] presents the first examples of the full assignment of a protein-proton magnetic-resonance spectrum using the two-dimensional methods developed over the past five years or so, together with an experimental strategy that may ultimately lead to full three-dimensional structures of smaller proteins in solution. A multi-pulse experiment is performed in which the sample is subjected to a pulse sequence of the general form [90° pulse–evolution time t_1–90° pulse–data-acquisition time t_2]. Data are collected as a function of t_2, but this does of course also depend on the value of t_1, and, if several hundreds of experiments are performed, each for a different value of t_1, a matrix of data points is obtained, each point being a function of both t_1 and t_2. This matrix is Fourier-transformed twice, along the t_1 direction and then along the t_2 direction, to yield a new matrix that may be presented as a square 'contour map' (see Figure) in which the main spectrum appears along the diagonal. Any intensity away from this diagonal reveals a 'connectivity' between two of the resonances on the diagonal; such a connectivity might, for example, be the spin-coupling between N*H* and

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C*H* protons adjacent along the peptide backbone. Starting from one known resonance, peaks may thus be assigned one by one along the entire backbone. Side-chain peaks may be assigned in the same way, ultimately leading to a full assignment of the spectrum. Spectra of this type are known by the acronym COSY (for correlated spectroscopy). An extension of this method, in which a third 90° pulse is inserted midway between the others, leads to NOESY, in which the

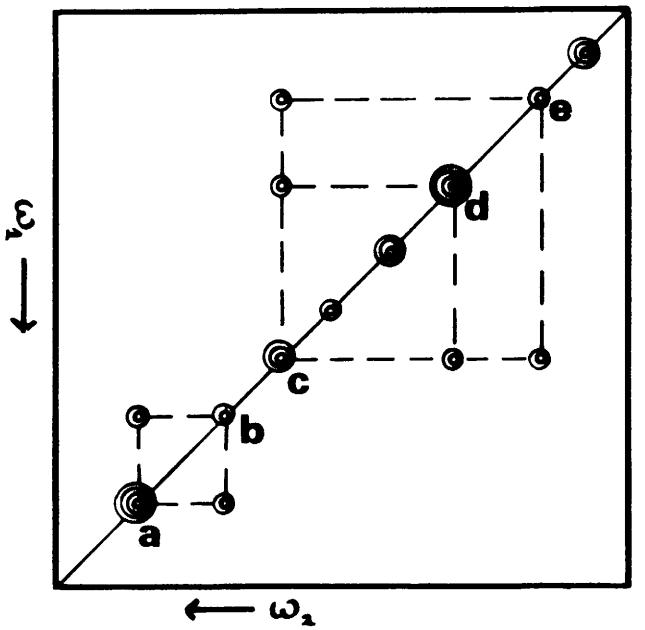


Figure *Schematic representation of a two-dimensional COSY or NOESY contour-map spectrum. The peaks of the conventional proton magnetic resonance spectrum appear along the diagonal, and cross-peaks reveal the connectivity between resonances a and b, and between c, d, and e*

connectivities revealed are due to the across-space nuclear Overhauser effect between nuclei that are in close spatial proximity to one another. The majority of protons which satisfy this condition are on the same or on contiguous residues, and, since the effect is distance sensitive, estimates of the distances between the α-C*H* proton of the *i*th residue and the backbone N*H* of the $(i + 1)$th, between backbone N*H* protons of adjacent residues, and between β-C*H* or β-CH_2 of residue *i* and N*H* of residue $(i + 1)$ may be obtained. These distances, in sets of three, are entirely equivalent to the Ramachandran angles ϕ, ψ, and χ, thus opening the possibility of an entirely n.m.r.-based structural study of protein molecules in solution, at least for molecules of up to 60 or 70 residues and for proteins that maintain a stable conformation. These methods are applied to basic pancreatic trypsin inhibitor (58 residues) in solution and to the 29-residue peptide hormone glucagon in its membrane-bound form.[250, 251] Further information

about the methods employed and preliminary experiments may be found in references 252—256.

Methods continue to advance in other aspects of protein n.m.r. as well. Synthetic introduction of ^{17}O into various sites in oxytocin[257] and of deuterium into the egg-white proteins of Japanese quail have been reported;[258] >80% incorporation of selected deuteriated amino-acids into lysozyme was achieved using a synthetic diet. A ^{13}C–^{15}N double-label method has been used to estimate a protein half-life of some 30 days in soybean leaves,[259] while a simple multi-nuclear multi-pulse technique[260] is described that enables the collection of the spectrum of only those protons that are directly bonded to ^{13}C atoms in ^{13}C-enriched samples. The use of high-resolution solid-state ^{13}C n.m.r. in biopolymers, including proteins and whole viruses, using magic-angle spinning is reviewed.[261] The transfer-of-saturation method is of increasing importance; theoretical calculations of the effects to be expected in a three-site exchange situation are presented.[262]

Amino-acids and Small Synthetic Peptides.—Isolated amino-acids are a natural starting-point for the development of techniques applicable to proteins. The use of a u.v. excimer laser will enable a number of new photoreagents to be used in chemically induced dynamic nuclear polarization (CIDNP) experiments, and it has been tested by use of solutions of histidine, tyrosine, and tryptophan.[263] Normally excited CIDNP measurements on tryptophan[264] reveal details of the unpaired spin-density distribution in the Trp radical cation. Analysis of coupling constants, NOE effects, and lanthanide perturbations reveals no less than six conformers in solutions of DL-tryptophan,[265] and solvent dependence of tyrosine and tryptophan side-chain conformations has been discussed.[266] The interactions of lanthanide ions with various amino-acids have been studied.[267, 268] Ca^{2+} and Mg^{2+} bind to aspartic acid and asparagine;[269] both cations interact with the carboxyl groups of the amino-acids but only Mg^{2+} binds to the amino group. Other amino-acid studies are recorded.[270–273]

The influence of proline on peptide conformation continues to arouse interest.

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360 MHz proton n.m.r. was used to investigate the high *cis* : *trans* ratio observed in Gly . Pro . Phe tripeptides;[274] an electrostatic interaction involving the π-electrons of the Phe side chain and the Gly . Pro peptide bond appears to destabilize the *trans* conformer. A series of peptides with α-aminoisobutyric acid residues adjacent to proline reveals the propensity of this combination to form β-bends[275, 276] and also provides an n.m.r. parameter to help determine whether the β-bend at the amino-terminal end of alamethicin is retained.[277] A number of one-bond α-^{13}C–^{1}H coupling constants for amino-acids and small peptides with variations of substituents and pH are presented for use in spectrum prediction and assignment.[278] Conformational studies are also reported for histidine-containing peptides,[279] acetyl . Ala . Ala . NHMe,[280] and the methylamides of the four lysine and/or tyrosine dipeptides.[281] The racemization of benzoyl dipeptide methyl esters has been investigated.[282]

Polypeptides are no longer the vital protein models they once were; however, they can still prove useful in the study of some aspects of protein origin and behaviour. A comparison of poly(aspartic acid) prepared by common methods[283] and by thermal polycondensation[284] revealed that the latter had β-peptide bonds in a mole fraction of about 0.8, which may have some significance in the study of protein evolution. The relaxation behaviour of poly(γ-benzyl L-glutamate) is explained in terms of internal rotations about Cα—H and Cα—Cβ bonds.[285, 286] The relative stabilities of the poly[proline II] helix formed by poly[Gly . $(Pro)_n$] (n = 3 or 4) have been determined; the polypeptide is a model for a proline-rich human salivary protein.[287]

Returning to smaller synthetic peptides, we find an interesting example of a β-turn locked by a salt bridge;[288] the peptide was Boc . Arg . Ala . Gly . Glu . NHEt, and the Arg-to-Glu hydrogen bonds forming the β-turn were considerably reinforced by the Arg^{+}-to-Glu^{-} interaction. One of the best studied synthetic peptides is the repeat pentapeptide of elastin, $(Val . Pro . Gly . Val . Gly)_n$. Cyclic mono- and oligo-mers of this have been prepared[289, 290] in order to determine

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which cyclic oligopeptide would have a structure matching the β-spiral of the linear polypentapeptide. Cyclic oligopentapeptides with $n = 3$ and $n = 6$ met this requirement very well, and energy calculations [291] for the former of these, together with the three-fold symmetry revealed by n.m.r., allowed the construction of probable models of the structure in solution. Cyclic peptides provide a useful vehicle for the investigation of localized conformation; two papers [292, 293] describe the synthesis and analysis of *cyclo*(L-alanylglycyl-ε-aminocaproyl) as a model for β-bends, while a series of cyclic hexapeptides having the sequence $(X\text{-L-Pro-Y})_2$ produced a set of rules for determining whether a type I, type II, or type II′ β-turn would be formed according to the sequence around the proline residue. The rules derived were in accordance with data from known protein structures.[294] Solid-state n.m.r. of crystals of two cyclic peptides containing proline show that sufficient conformationally dependent spectral data are obtained to compare effectively crystal with solution structures.[295] A cyclic octapeptide that mimics the zinc-binding site of carboxypeptidase A has been synthesized;[296] it forms a 1 : 1 complex with zinc, with the imidazoles of both histidines and the carboxyl side chain of the single glutamic residue complexed to the metal ion. Other n.m.r. studies of cyclic peptides are listed.[297-302]

Small Naturally Occurring Peptides.—Preliminary n.m.r. results in solution and in intact erythrocytes reveal that the intracellular tripeptide reducing agent glutathione effectively complexes trimethyl-lead [303] and so may prove of value in the treatment of organolead poisoning. Earlier work on enkephalins has been reviewed.[304] Interest is now centred on studying the basis of the enkephalin conformation by using modified and analogue molecules. The adduct formed by Met[5]-enkephalin and acetaldehyde does not take up the folded conformation characteristic of native enkephalins in $[^2H_6]$DMSO,[305] whereas enkephalins dansylated at the *C*-terminus remain very flexible in a variety of solvents,[306] and the conformational equilibrium of the molecules is greatly shifted by complexing with zinc.[307] Elimination of the *C*-terminal leucine [308] yields a tetrapeptide

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that has a more rigid structure than Leu-enkephalin, while the analogues (D-Met^2,Pro^5)-enkephalin and -enkephalinamide,[309] which show high specificity for the μ-receptor site (guinea-pig ileum), appear to derive their conformational stability from their high content of hydrophobic side chains rather than from the more usual β-turn. A strong correlation was shown to exist[310] between the specificity of enkephalin derivatives for μ- or δ-(mouse vas deferens) receptor sites and the acid or amide form of the *C*-terminal carboxyl group.

A low-molecular-weight analogue of human growth hormone, in which residues 32—46 of the normal hormone are missing, has been shown[311] to fold in a similar, but not identical, manner to the normal 22 000 dalton protein and to occur most frequently as a heterologous dimer with it. The much smaller somatostatin, synthesized in the hypothalamus, can inhibit the release of insulin and glucagon as well as growth hormone; two-dimensional n.m.r. techniques have permitted almost complete assignment of the proton spectrum of the cyclic part of this hormone.[312]

The contribution of intramolecular hydrogen bonding to the solution structure of oxytocin has been evaluated by measuring amide deuteriation rates;[313] evidence was found for some hydrogen bonding involving the cystyl residues, especially Cys-6. Slow exchange at Asp-5 was attributed to steric hindrance to solvent access, although in another paper[314] the slowness is considered to be the result of hydrogen-bond formation in (Glu^4)-oxytocin. A conformationally restricted analogue of oxytocin, 1-penicillamine 2-leucine-oxytocin, is a more potent hormone antagonist than the equivalent analogue without the leucine modification;[315] this is assumed to be due to a greater similarity to native oxytocin at residues 2 and 3. Studies of the rather similar hormone vasopressin are reported.[316, 317]

Insulin has been the subject of a number of studies; its histidine residues have been assigned[318] and a Ca^{2+}-binding site, specific for calcium and separate from the two Zn^{2+} sites of the hexamer, has been demonstrated by use of ^{113}Cd n.m.r.[319] Removal of the *C*-terminal octapeptide of the B-chain appears to destroy the three-dimensional structure of the head end of the A chain[320] and also destroys biological activity. The hypertensive octapeptide angiotensin, which stimulates the smooth muscle of blood vessels, has been assigned various solution conformations in the literature. These are discussed in the light of lanthanide-ion

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perturbations of the molecule,[321] and some peptide exchange rates in analogues and agonists have been compared.[322] Analogues involving replacement of Ile^5 exhibit a strict requirement for a β-branched residue,[323] and other studies[324] indicate that it may be possible to produce potent angiotensin II analogues that are more resistant to enzymic degradation than is the native molecule. The conformational mobility of the hormone and some analogues is discussed.[325]

On the basis of a number of 270 MHz proton-resonance studies of synthetic fragments of alamethicin,[326] a largely 3_{10} helical folding pattern is postulated for the hydrophobic segment from residue 1 to 17, with a polar, flexible *C*-terminal tripeptide. A helical or possibly β-sheet conformation is supported in a study of natural alamethicin.[327]

The long neurotoxins (72 residues) of snake venom provide a fascinating set of variant active peptides with some invariant features, such as tryptophan-29. N.m.r. reveals some structural differences between the crystal and solution conformations of erabutoxins,[328] and proton resonance at various frequencies has shown structural similarities and differences between toxins from several species.[329, 330] As might be expected, the functionally invariant part of the molecule is more rigid than the tail, forming a three-strand antiparallel β-sheet as shown by *X*-rays for related molecules. These results are borne out by work on neurotoxin III from *Naja mossambica mossambica* from two other laboratories.[331, 332] The unfolding of a cobra neurotoxin is reported;[333] other toxins investigated include six from Latin American scorpions, which, like the snake toxins, include extensive β-structure,[334] toxin 401 from bee venom,[335] cholera toxin,[326] toxin II from *Anemonia sulcata*, which, like the related polypeptide anthopleurin A, has heart-stimulating activity,[337] and the cytotoxic depsipeptides known as the didemnins.[338]

A number of papers on the basic pancreatic trypsin inhibitor were mentioned in the introductory section on two-dimensional n.m.r. techniques. Theoretical

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calculations of the internal mobility predicted results in striking agreement with n.m.r. data on the conformational stability of backbone residues.[339] Internal solvent water was included in the calculations, and it will be interesting to compare the results with those from deuterium-exchange studies of internal amide protons[340] in which reduction of the S—S bond 14—38 increased some NH exchange rates between 100- and 1000-fold. Spin–spin splittings revealed by two-dimensional *J*-resolved spectroscopy[341] show that internal residues have their side chains locked into unique orientations identical to those found for the crystal, with differences between crystal and solution side-chain orientations being common for surface residues. Rapid fluctuations were found even for the locked internal side chains, and another theoretical paper[342] points out that such picosecond fluctuations must be accounted for when interpreting T_1 longitudinal relaxation measurements in terms of overall molecular motion. Finally, some photo-CIDNP[343] and titration[344] studies of the aromatic residues of *Streptomyces* subtilisin inhibitor show that tyrosine-7 is always well exposed, Tyr-93 buried, and Tyr-75 in a variable microenvironment, more restricted in solution than it apparently is in the solid crystalline form.

Enzymes.—*Oxidoreductases.* Metal substitution at the catalytic site of liver alcohol dehydrogenase (LADH) has formed the basis of several studies. Manganese ions[345] bind not at the active site, but at two other types of site, from which they are displaced, respectively, by Zn^{2+} or Cd^{2+} ions, the zinc binding possibly being an intermediate for the return of zinc to its normal binding site. Cu^{2+} and Co^{2+} substitution[346] shows that because of a strong spin–orbit interaction of the electronic spin of Co^{2+} no true paramagnetic effects of Co^{2+} on solvent relaxation were detectable; earlier studies may thus need to be re-interpreted. E.s.r. results[347] reveal the Co^{2+} to be tetra-co-ordinated in the free enzyme but penta-co-ordinated in binary enzyme–NAD and higher complexes; co-ordination of Cd^{2+} in LADH has been reported.[348] In lactate dehydrogenase the proton signals of tyrosine-237 have been assigned; the residue is on the enzyme surface and has considerable freedom of motion.[349] Phosphorus n.m.r. of the glycoprotein glucose oxidase shows it to contain a disubstituted phosphorus residue, with the phosphorus moiety bound to the protein at a point remote from the flavin coenzyme and possibly involved in a phospho bridge linking two amino-acid residues.[350] Substrate binding to galactose oxidase, studied by competition between fluoride and cyanide ions and by ^{19}F relaxation as a function of substrate

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concentration,[351-353] gives a dissociation constant for the anaerobic binding of dihydroxyacetone substrate of 0.059 mol dm^{-3}. Remarkably mobile regions of polypeptide chain are reported to be in the large multi-enzyme complex pyruvate dehydrogenase,[354] and also in the similarly large 2-oxoglutarate dehydrogenase;[355] the regions involved may be those encompassing the lipoyl-lysine residues.

Dihydrofolate reductase continues to arouse considerable interest. Complexes of the enzyme with an inhibitor, trimethoprim, have been shown by proton [356] and ^{31}P [357] resonance to exist in two slowly interconverting forms; the trimethoprim itself, like another inhibitor, methotrexate, is protonated when bound to the enzyme.[358] Few of the histidine resonances of dihydrofolate reductase are affected by coenzyme binding; the ligand-induced conformational changes appear different for NADP and NADPH.[359] Only one of the diastereoisomers of folic acid binds to the enzyme in its biologically active form;[360] the 6*S*,α*S*-isomer has a binding constant of 1.3×10^{-8} mol^{-1} dm^3,[361] some 10^4 times stronger than the 6*R*,α*S*-isomer. The dihydrofolate reductase–$NADP^+$–folate complex appears to exist in three slowly interconverting forms.[362] Other studies of dihydrofolate reductase cover specific labelling with [γ-^{13}C]tryptophan [363] and the effect of chloride ion on the reduction of dihydrofolate to tetrahydrofolate catalysed by the enzyme.[364]

Relaxation measurements in soybean lipoxygenase inhibitors and substrates that had been selectively deuteriated indicate considerable internal motions.[365] The binding of water [366] and several different anions [367] to superoxide dismutase (a copper–zinc protein also known as erythrocuprein) has been reported; all anions bind at the Cu site, though high pH is needed for OH^- to bind. In a similar report [368] addition of anions caused downfield shifts of resonances from histidines liganded to the Cu ion but had no effect on Zn ligands in the same molecule, except

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in the case of the enzyme extracted from yeast,[369] which seems to have a slightly different Zn site from enzymes found in vertebrates. With superoxide dismutase in steady-state turnover, it appears from ^{19}F relaxation that Cu^{+} and Cu^{2+} are present at the active site in equal proportions.[370]

Transferases. The surface exposure of a number of residues in α-lactalbumin (one of the two proteins forming lactose synthase) from several sources has been studied by CIDNP methods;[371] several differences were noted, but all species have a common buried tyrosine. Glucose and glucose 6-phosphate appears to bind to brain hexokinase quite differently, with the former apparently close to a site that will bind Mn^{2+}.[372] The roles played by histidine residues in the catalytic activity of pyruvate kinase (237 000 daltons) have been studied at 250 MHz; substrate binding altered the pK of only one histidine, due either to a stronger interaction of the cation activators with the histidine or to some substrate-induced conformational change in its vicinity.[373] A similar study[374] of the smaller creatine kinase showed three histidine resonances affected by substrate binding, a conclusion reinforced by the results of experiments using the paramagnetic substrate analogue Cr^{3+}–ATP. ^{31}P resonance permitted equilibrium constants and interconversion rates to be measured for creatine kinase-catalysed reactions.[375] The interesting effect of different isotopes of oxygen on the chemical shift of phosphorus aided in characterizing the course of the reactions; adenosine (γ-*S*-$^{16}O^{17}O^{18}O$)-triphosphate was used as a substrate, and the phosphorylation reaction was defined as an associative in-line transfer of the phosphoryl group.[376] Saturation-transfer ^{31}P resonance in intact frog muscle[377] reveals that the enzyme is active even with the muscle in the resting state. In the phosphoenolpyruvate-dependent phosphotransferase system of *Staphylococcus aureus*, proton resonance showed that Factor III formed a complex with phosphocarrier protein HPr, the interaction not surprisingly being abolished when both proteins were phosphorylated.[378] The protein kinase from bovine heart binds specific heptapeptides; if there is any absolute requirement for the conformation of these peptides, it is not any of the normal (α-, β-, β-turn) secondary structures.[379] The interaction of ATP with RNA polymerase has been reported.[380]

Hydrolases. Values of the parameters of calcium binding to porcine pancreatic prophospholipase A_2, as measured by ^{43}Ca n.m.r., are reported.[381] Inhibitors bound to acetylcholinesterase from *Electrophorus electricus* show considerable

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conformational flexibility, which is reduced when the active-site serine is modified.[382] The reduction in mobility is proportional to the size of the inhibitor, indicating that it binds in a large anionic pocket near the serine residue. Inhibitor and fluorescent probe binding to acetylcholinesterases is compared with that to cholinesterase from horse serum.[383] Binding of Mn^{2+} at a structural site and a nitroxide spin label at a SH site permitted some active-site mapping in fructose bisphosphatase;[384] acid phosphatase from sweet-potato tubers already contains manganese(III), which has permitted ^{17}O and ^{31}P resonance experiments to reveal direct metal–phosphate interaction and also the course of P—O cleavage.[385]

Thermal denaturation of RNase A[386, 387] leaves considerable structured regions even at elevated temperatures, hydrophobic effects being responsible for the retention of structure. The interactions of ribonuclease A with nucleotides are reported by a number of workers;[388–391] evidence is presented for an interaction between lysine-41 and a histidyl residue.[391] In ribonuclease T_1, on the other hand, it appears that each histidyl group interacts with the carboxyl group of an aspartate or glutamate residue,[392] and the same appears true of ribonuclease S_t, although a normal p*K* argues the presence of a positively charged group in the vicinity as well.[393] Similar studies are reported for the guanyl-specific ribonuclease from the fungus *Penicillium brevicompactum*.[394]

Simultaneous binding of lanthanide-shift reagents and *N*-acetylglucosamine inhibitors to hen egg-white lysozyme revealed one of two or more sugar binding sites to be subsite E of the enzyme[395] and another to be subsite C;[396] the indole rings of tryptophan-62 and -63 rotated towards subsite C on binding. *N*-Bromosuccinimide modification of tryptophan-62[397] inactivated the enzyme, although the p*K* values of catalytically important carboxyl groups Glu-35 and Asp-52 were unchanged by the modification. The modification appears to obstruct subsite B. The hydration of lysozyme has been studied by two groups;[398, 399] in the latter case ^1H measurements showed that every gram of lysozyme is associated with 0.3 g of 'unfreezable' water, which accords with the amount that can be directly linked with polar sites on the surface of the molecule.

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N.m.r. studies of the active sites of serine proteinases have been reviewed.[400] A method for preparing [ε-^{13}C-methionine-192]-chymotrypsin is described.[401] ^{19}F experiments on monofluorocinnamoyl-chymotrypsin[402] reveal that the presence of the fluorocinnamoyl group stabilizes the protein towards urea denaturation. Temperature and pH effects on human prothombin fragment I have been studied.[403] A very low-field resonance found in aqueous solutions of serine proteases and their zymogens, characteristic of the hydrogen bond between imidazolium and aspartate groups of the catalytic triad Ser-His-Asp, is not visible in native subtilisins but appears in their thiol derivatives; the stable hydrogen bond should be more important during catalysis than in the substrate-free enzyme.[404] Evidence from fluorimetric and ^{1}H titration studies of papain and some methylthio derivatives lends support to the involvement of histidine-159 in the deacylation step in papain catalysis.[405] In a sheep-kidney medulla sodium–potassium ATPase, with lithium substituted for potassium, titration with Cr^{3+}-ATP gave a Cr^{3+}–Li^+ distance of 4.8 Å.[406] A K^+-sensitive phosphorylated intermediate of (Na^+,K^+)-ATPase from the salt gland of the duck is reported, following ^{31}P resonance studies in which a resonance +17 p.p.m. from phosphoric acid was attributed to formation of an acyl phosphate at an aspartyl residue of the catalytic site.[407]

Lyases, Isomerases, and Synthetases. The C-2 proton resonances of 6 of the 10 histidyl residues in yeast aldolase have been detected at 360 HMz. Their behaviour[408] is consistent with co-ordination of the enzyme-bound metal (Zn^{2+}) by three imidazole ligands, which, with a previously reported rapidly exchanging water ligand, would provide all four necessary ligands. The inhibition of yeast enolase -Mn^{2+} by fluoride ions[409] and inorganic phosphate appears to be due to the formation of a quaternary enzyme–Mn^{2+}–F^-–P_i complex that isolates the catalytic site, thus preventing the dehydration of 2-phosphoglycerate and inhibiting glycolysis. Inhibition of carbonic anhydrase has been studied by using proton n.m.r. with the Co^{2+}-substituted enzyme[410] and also by ^{17}O resonance with Cu^{2+} substitution.[411] Metal substitution was also used[412] to study human erythrocyte glyoxalase I; manganese in the active site caused enhanced relaxation rates in solvent water that were reduced on inhibitor binding, consistent with inhibitor being bound to the enzyme through the metal ion. The stereochemistry of

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lysine-2 3-aminomutase has been established using ^{2}H n.m.r. of its products,[413] as has the activity of mitochondrial carbamoyl-phosphate synthetase by following the fate of phosphate metabolites identified by oxygen-isotope shift effects with ^{18}O perturbing ^{31}P resonance.[414] In the enzyme ATP_A–HCO_3–ATP_B complex there is reversible transfer of the γ-PO_3 group of ATP_A to HCO_3 without dissociation of products.

Haem Proteins.—A new technique for the investigation of haem protein crystals is described.[415] Microcrystals of the protein, suspended in nearly saturated ammonium sulphate solution, are perfectly aligned in strong magnetic fields; the orientation of specifically labelled residues in the crystal may then be directly determined by combining n.m.r. with powder *X*-ray results. The dynamic behaviour of exchangeable protons in the haem pocket of myoglobins[416,417] and a number of histidine titrations in myoglobins and derivatives have been reported.[418]

Binding of inositol hexaphosphates (IHP) to human haemoglobin is reported.[419,420] Apparently human low-spin metHb can be switched from the R to the T quaternary structure by the binding of IHP; a similar conclusion is reached regarding a conformational change in carboxy haemoglobin on binding of myoinositol hexakisphosphate.[421] Comparison of infrared and ^{13}C n.m.r. measurements of ^{13}CO bound to haemoglobin shows rapid interconversion between conformers.[422] N.m.r. may be used to determine the fraction of polymerized Hb in sickle-trait erythrocytes,[423] and internal water can act as a spin carrier in spin relaxation in paramagnetic Hb molecules.[424]

Solvent-exchange dynamics in soybean ferric leghaemoglobin *a* show the haem to be more accessible in this molecule than in vertebrate ferric myoglobin or haemoglobins;[425] the absence of a hyperfine-shifted exchangeable N*H* peak for the distal histidine suggests either a very different orientation for this ligand or a faster exchange rate with bulk solvent than found in metmyoglobin.[426] Further studies of the monomeric insect haemoglobin from *Chironomus thummi thummi* confirm earlier reports that there are two haem orientations possible in the molecule; ligation on-rates may depend on both pH and haem orientation.[427]

Investigations of the structure and mechanisms of cytochromes continue to occupy a number of laboratories. The resonances of Phe-82 and Phe-10 of horse

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cytochrome *c* have been reassigned,[428] and modification of methionine-65 has been shown to produce an extremely small structural perturbation in one part of the molecule.[429] The source of the asymmetric electron-spin density distribution in cytochrome *c* has been investigated using the active-site haem octapeptide as a model system, showing that the orientation of the axial methionine is an important determinant of the electronic structure of the haem.[430] The octapeptide was also used in experiments leading to a model for anion binding to the active site,[431] and an outline structure is presented for the haem undecapeptide.[432] Comparison of a number of cytochrome *c* samples from different sources reveals that those which have a phenylalanine at position 46 exhibit a temperature-dependent linewidth of a hyperfine-shifted haem methyl resonance, while those with tyrosine in this position do not.[433] Ligation states in cytochrome *c'* from *Rhodospirillium rubrum* are reported.[434] Labelling of the methionyl groups of cytochrome *c* with either ^{13}C or ^{2}H [435] have enabled the acid and alkaline unfolding of cytochrome *c* to be followed in some detail; labelling by conversion of lysine residues to ^{13}C-labelled homoarginine in cytochromes from a number of sources, with full retention of electron-transport reactivity with cytochrome oxidases, may also provide a useful structural probe in the future.[436] Another lysine modification experiment, in which lysine-13 or lysine-72 was altered to 4-carboxy-2,6-dinitrophenyl-lysine, revealed two rapidly exchanging conformers in which conformation of Met-80 (ligand 6) and Phe-82 depends on the 'on' or 'off' position of lysine-13 in its salt bridge with Glu-90.[437] Other studies of cytochrome *c* include a partial delineation of homologies of polypeptide conformation near the haem group between horse ferrocytochrome *c* and cytochrome *c*-552 from *Euglena gracilis*,[438] kinetic studies of the oxidation of horse-heart ferrocytochrome *c* by (penta-ammine)pyridine ruthenium^{3+},[439] and the binding of copper, probably to His-33, to cytochrome *c*.[440]

In the cytochromes *b*, unlike the *c* group, the haem is not bound covalently to the protein. Reconstitution of cytochrome b_5 apoprotein with specifically deuteriated haemin[441] shows that two haem orientations, related by a 180° rotation about the $\alpha-\gamma$-*meso* axis, are possible. The tendency for a haem to exhibit multiple orientations appears to be attributable to the 4-vinyl group; pemptohaemin yields two components, isopemptohaemin only one. Model compounds for cytochrome

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P-450, another member of the '*b*' group, are discussed.[442,443] A number of features of solvent relaxation found in cytochrome oxidase are reported[444] to be similar to those found for both microbial and microsomal cytochrome P-450.

The haem-containing enzymes cytochrome peroxidase and horseradish peroxidase are compared.[445] Formation of the initial oxidized intermediate, compound I, with H_2O_2 causes drastic changes in the hyperfine-shifted proton n.m.r. spectrum of the former, but not the latter. The electronic structure of horseradish peroxidase compound I[446] and of compound II of horseradish peroxidase and catalase have been studied.[447] Haem asymmetry in reconstituted cytochrome peroxidase is described;[448] twice the expected number of proton resonances are seen in the spectrum.

Proteins Associated with Nucleic Acids.—N.m.r. and model-building experiments[449] indicate that direct interactions between the backbone atoms of peptide molecules and the base pairs in single- and double-stranded polynucleotides may play a role in protein–nucleic acid recognition. Addition of kirromycin, an inhibitor of protein synthesis, to the complex between elongation factor Tu and GDP alters its conformation as monitored by ^1H n.m.r. to one similar to the elongation factor–GTP complex,[450] which probably explains the inhibitory effect. Preliminary n.m.r. results on the *E. coli* translational initiation factors IF1, 2, and 3 are presented.[451] A much more detailed analysis of the 500 MHz proton spectrum of the helix-destabilizing gene V protein encoded by coliphage M13 indicates that the phenylalanyl and two tyrosyl residues are involved in its interaction with DNA, a conclusion reinforced by nuclear Overhauser evidence for the proximity of these aromatic rings to DNA bases.[452] Some rather general conclusions regarding the folding of ribosomal proteins S2,[453] L25,[454] and L11[455] have been reported.

Studies of chromosomal proteins have been extended to H1 from the sperm of *Sphaerechinus granularis*[456] in which a lack of conservation of secondary and tertiary structures in the evolution of H1 histones is demonstrated. The recently discovered $H1^0$ histone associated with absence of mitotic activity in mammalian cells appears to share a number of structural features with the H5 of avian erythrocytes and possibly to bind at the point of exit of DNA from the nucleosome in a more stable way than does H1.[457] Progress towards characterization of the

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phosphorylation of histone H4 *in vivo*[458] follows ^{31}P studies of enzymically and chemically phosphorylated H4, with the phosphorylated residues appearing to be histidines-18 and -75. Trout testis contains two non-histone chromosomal proteins of the high-mobility group. One of these, termed H6, has been shown[459] to behave structurally like the homologous calf high-mobility group proteins HMG14 and HMG17, but another, HMG-T, shows major structural differences. Proton resonance of an active pentapeptide fragment of ubiquitin, which is found in the unusual covalently branched A24 complex with histone H2A, is reported.[460]

A considerable number of papers are now appearing on studies of *lac* repressor protein. The DNA-binding site of the protein appears to lie in the first 51 residues, termed the headpiece, which can be removed from the rest of the protein with little apparent alteration in its secondary and tertiary structure. Assignments have been made of the four tyrosine residues[461] and of several methyl resonances[462] in the headpiece, permitting its thermal unfolding to be followed[463] and compared with calorimetric results.[464] A general unfolding of the headpiece appears to occur above pH 10[465] and selective Overhauser and other effects indicate that the molecule has a structure that folds back on itself, with residues in the *N*- and *C*-terminal portions near to each other.[466] Exchange studies are reported,[467] and the interaction of headpiece with synthetic poly-d(A-T) leads to a significant shift of many resonances in the headpiece spectrum.[468] Another group reports on a method of genetically introducing specific modifications and labels into *lac* repressor;[469] ^{19}F resonances were introduced at position 44 by substituting 3-fluorotyrosine for the normal glutamic acid. The two sections of the molecule (*N*-terminal headpiece, which is flexible, and the more rigid *C*-terminal region, which is $\frac{5}{6}$ of the molecule) have been studied,[470] as have a set of headpieces in each of which one of the four tyrosines is missing, thus permitting accurate assignment of the aromatic resonances of the molecule.[471]

Other Proteins.—*Muscle Proteins.* Comparison of the 400 MHz proton spectra of native and denatured rabbit skeletal muscle G-actin shows that a large proportion of the aromatic residues are motionally constrained, consistent with the known existence of a compact, globular proteinase-resistant core in the protein, contain-

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ing some 80% of the residues.[472] Actin quenches line-sharpening molecular motions in myosin,[473] and a proton resonance study[474] establishes that the mobile regions are internal to myosin and reside mainly inside the subfragment 1 moiety. Presumably the quenching results from a structural change in the myosin. Two different conformations of the myosin head S1 molecule, at least in the region of its SH groups, are reported.[475] A theory for the evaluation of rate constants for ATP-hydrolysing enzymes, using ^{18}O labelling of ^{31}P resonances,[476] agrees very well with previous measurements of ^{18}O exchange catalysed by myosin S1. Further experimental measurements of S1 kinetics have been made,[477] and confirmation of the conclusion mentioned earlier that there appear to be two actin-independent conformations of myosin S1, both having ATPase activity, is reported following a comprehensive study of S1 nucleotide complexes using ^{31}P resonance without ^{18}O labelling.[478] *In vivo*, the interaction between actin and myosin is modulated by tropomyosin; the co-operativity visible in the titration of histidines-153 and -276 of this protein in its polymerized form disappears when monomeric tropomyosin is prepared, arguing for some allosteric mechanism between tropomyosin monomers.[479] The trimeric protein troponin is also essential to the action of tropomyosin; the tyrosine assignments and calcium-induced structural changes of the calcium-binding troponin C component of bovine cardiac troponin have been compared[480] to those of two homologous proteins, rabbit skeletal troponin C and bovine brain calmodulin. There are many structural similarities, as might be expected from the high degree of primary-sequence homology. Laser CIDNP comparisons of the same three proteins[481] revealed which tyrosine residues were exposed in solution; tyrosines-5, -11, and -150 were exposed in cardiac troponin apoprotein, becoming buried as Ca^{2+} was bound, and similar behaviour was seen for tyrosines-10 and -109 of skeletal troponin and -99 of calmodulin. Binding constants for Ca^{2+} and Mg^{2+} on skeletal troponin C have been determined by ^{43}Ca and ^{25}Mg resonance measurements,[482] and the drug trifluoroperazine binds to calmodulin apparently close to a methionine-rich region of the protein.[483] Another calcium-binding protein, carp parvalbumin, has been extensively studied by replacing Ca^{2+} by the paramagnetic lanthanide ion ytterbium. Yb^{3+} sequentially replaces the two bound calcium ions of the molecule and causes very large chemical-shift effects, with proton resonances ranging from -32 p.p.m. to $+19$ p.p.m.[484-486] The binding of calcium in the

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human salivary proline-rich phosphoproteins A and C appears to be in the *N*-terminal tryptic peptide and to involve an aspartic acid and a phosphoserine residue, as shown by ^{45}Ca, ^{31}P, and ^{1}H resonance.[487]

Immunoglobulins. Strategies for spectral assignments in antibody fragment F_v of the murine antibody M315 involve feeding mice on a diet that incorporates deuteriated tryptophan.[488] Binding of dinitrophenyl compounds to the V_L dimer of protein 315 causes spectral perturbations in about 10 resonances; comparison of these with shifts caused by DNP binding to the F_v fragment is interpreted to mean that the binding is specific rather than fortuitous,[489] being determined by the size and shape of a largely non-polar combining site. Conformation of the hinge region of the IgG1 immunoglobulin[490] and the correspondence between structure and function in the various IgG subclasses have been studied.[491]

Lipid–Protein Complexes. In the field of protein–lipid interactions, a combined n.m.r. and e.s.r. spin-label experiment on the orientation of glucagon in mixed micelles with dodecylphosphocholine shows the glucagon backbone to lie parallel with the micelle surface, with apolar and polar side chains pointing respectively into and out of the lipid.[492] Melittin appears to assume the same conformation in a self-aggregated tetramer as in its monomeric form bound to micelles.[493] The lipid-binding site on porcine colipase is proposed[494] to be a surface domain formed from regions 49—57 and 77—86, β-sheet fragments brought into proximity by the protein folding. Specific deuteriation of lipids has been used[495] to show that protein had little effect on order in an *Acholeplasma laidlawii* B membrane system but large effects on *E. coli* membranes. Above the gel–liquid-crystal transition temperature in model membrane systems protein has far greater effect on lipid mobility than does cholesterol, although the situation reverses below the transition, probably due to a phase separation.[496] In a study of the interaction between deuteriated model membrane and cytochrome oxidase[497] the results were consistent with weak, short-lived protein–lipid interactions, with 0.18 mg phospholipid mg^{-1} protein necessary to cover the surface of the enzyme.

Specific labelling with ^{2}H of the protein bacteriorhodopsin from the photosynthetic purple membrane of *Halobacterium halobium* permitted observation of individual amino-acid side chains of the proteins in the intact membranes.[498,499]

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The side chains are rather rigid, with the principal motions being methyl-group rotation and discontinuous benzene-ring flipping. Refolding of denatured bacteriorhodopsin is possible if phospholipids, cholate, and retinal are added to the protein in the presence of SDS, which is subsequently dialysed out, leaving vesicles fully active in light-driven proton translocation.[500]

Evidence for multiple ion occupancy in malonyl gramicidin transmembrane channels is presented.[501] Another channel-forming peptide, suzukacillin, appears to contain a large amount of 3_{10} helix, at least if the whole protein behaves like its peptide fragments.[502-504]

Application of magnetic resonance to the study of high-density lipoproteins has been reviewed.[505] Both the mobility[506] and order–disorder transition[507] of cholesteryl esters in low-density lipoprotein have been reported, and low-density lipoproteins have been further studied.[508] Other lipid-associated proteins covered in the 1981 literature include peripheral nerve myelin,[509] the histidine-binding protein J of *Salmonella typhimurium* and its role in the high-affinity transport of L-histidine across the cytoplasmic membrane,[510] and some lauroyl dipeptide derivatives whose immunostimulating properties are linked to the presence of the lauroyl chain, presumably because it provides access to cell-membrane constituents.[511] 360 MHz proton n.m.r. indicates that the heterogeneity of chick ovalbumin glycopeptides AC-C and AC-D is greater than has previously been reported;[512] the structures of four glycopeptides have been confirmed.[513] ^{13}C labelling in a chondrocyte culture system for chick-limb bud cells produced a proteoglycan core protein whose ^{13}C relaxation behaviour suggests considerable flexibility.[514] Cation binding to multi-chain and single-chain glycosaminoglycan peptides has also been investigated,[515] as has the microheterogeneity in a glycopeptide fraction derived from human plasma α_1-acid glycoprotein; previously unreported variation in the position of attachment of fucose was found.[516]

Internal Motions and Water.—The coat protein of virus fd shows evidence of folding but with significant internal mobility for the two tyrosine rings and two of

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the three phenylalanines.[517] Qualitative comparisons may be made with the intact virus, whose DNA is shown by solid-state ^{31}P n.m.r. to be immobilized by the coat protein.[518] Evidence is presented [519] for considerable internal mobility in the coat protein of intact alfalfa mosaic virus, the mobile residues apparently being in the *N*-terminal region of the molecule. Molecular motion in solid proteins has been measured for polycrystalline insulin,[520] in which most of the relaxation was found to be attributable to methyl rotation, and for crystalline enkephalin, in which the tyrosyl ring flips at some 5×10^4 flips s^{-1} at room temperature.[521] Internal motions in seven different enzymes were shown to be more intense than in four non-enzymic proteins studied at the same time;[522] water effects may produce fluctuations important in catalytic activity. Cross-relaxation effects between water and protein protons have been considered.[523] The water-insoluble and highly thermostable protein crambin has been studied with variations in temperature and solvent composition;[524] it retains most of its structure at 105 °C in dimethylformamide.

Miscellaneous.—^{13}C resonance has been used to study the anion-binding sites of transferrin, in particular leading to the conclusion that the anion-binding ligand at the B site is probably the guanidino group of arginine, whereas that at the A site may be the ε-amino group of lysine.[525] In ovotransferrin, by contrast, three histidines are reported to be involved in each binding site, one being involved in binding to anions and two to metal ions.[526] The electron-transfer reaction between ferrocyanide ion and the blue copper protein stellacyanin has an activation energy of 17 kJ mol^{-1}, as indicated by ^{13}C line broadening of the oxidant.[527] Other protein studies that should be mentioned include evidence for different co-ordination of the three-iron centres in ferredoxin I from *Azotobacter vinelandii* and ferredoxin II from *Desulfovibrio gigas*,[528] details of the structure and mechanism of the phosphocarrier protein of *Staphylococcus aureus*,[529] and examination of the aspirin acetylation site of human serum albumin,[530] the mechanism of binding of the radio sensitizers metronidazole and misonidazole to the same protein,[531] and studies of wheat gliadins[532] and of the cobalt(III) complex of the pseudo-tetrapeptide A of bleomycin,[533] this last being claimed as an important structural model for the biologically important Fe-bleomycins.

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4 Infrared and Raman Spectroscopy

Contributed by R. M. Stephens

Model Compounds.—Further evidence that the β-turn structure in peptides can be observed using Raman spectroscopy has been reported. The spectra of crystalline H-Pro-Leu-Gly-NH_2, which has a type II β-turn, crystalline *S*-benzyl-Cys-Pro-Leu-Gly-NH_2, which has a type I turn, and crystalline gramicidin S, which has 2 β-turns and a β-sheet structure, reveal that both the amide I and amide III absorption frequencies are conformationally sensitive with respect to these β-turn structures.[534] The i.r. and Raman spectra of *cyclo*(L-alanylglycylaminocaproyl), a cyclized dipeptide, have been analysed with the aid of a normal mode analysis. In the solid state the spectra are found to be consistent only with a type II β-bend. In solution the same conformation is predominant, but the presence of large amounts of other low-energy bend conformations (types I, II, I′, and III′) was inconsistent with one or several of the observed vibrational frequencies.[535] The Raman-active lattice modes of deuteriated α-glycine measured at room temperature and 85 K complement recent inelastic neutron-scattering results. A lattice dynamical model is described that has eigenvalues in reasonable agreement with the observed frequencies.[536]

Polypeptides.—Different alternating poly-DL-peptides have been synthesized and used as models of linear gramicidin. These were poly(DL-alanine) (I), poly(γ-benzyl-DL-glutamate) (II), (D-leucyl-L-tryptophanyl)$_n$ (III), (L-tryptophanyl-L-leucyl)$_n$ (IV), and (D-phenylalanyl-L-leucyl)$_n$ (V). Tentative correlations were made between their structural properties and those of gramicidin in different solvents. Each of the polypeptides was tested for its eventual ion-transport properties. No significant conduction was found for I and II, whereas single channels were detected in black lipid membranes for III, IV, and V. In nearly all instances the channel conductance was less than for gramicidin under similar conditions.[537] Poly(L-alanylglycyl-L-alanylglycyl-L-alanylglycyl-L-serylglycine) has been used as a model for silk fibroin. This sequential polypeptide gave a similar i.r. pattern to the crystalline part of *Bombyx mori* silk fibroin, which indicated antiparallel β-conformation. Dialysis of this polymer in 60% aqueous LiBr against water changed the polymer conformation to the α-form.[538] The normal modes of various isotopic species of polyglycine I in the antiparallel-chain rippled-sheet structure were calculated as a test of the force field for the parent polypeptide. Small modifications made to a previous force field resulted in a general improvement of predicted frequencies, particularly of CH_2 wag and CH_2 twist modes, and accounted very satisfactorily for the i.r. bands of the isotopic molecules.[539] The i.r. of *N*-deuteriated β-poly(L-alanine) was obtained and used, together with an improved transferable force field for polyglycine I, to make a significant improve-

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ment in the force field and band assignments of β-poly(L-alanine). Assignment of the unperturbed N—D stretch frequency confirmed previous analysis.[540]

The far-i.r. spectra of glycine, L-alanine, polyglycine, poly(L-alanine), lysozyme, myoglobin, and albumin have been discussed with reference to the chemical binding of TFA. One band, occurring at $\sim 268\,cm^{-1}$ for all samples treated with TFA, was assigned as a rocking mode of the CF_3 group of TFA. (The same band occurs in pure liquid TFA, but at a lower frequency.) Bonding configurations between TFA and the polypeptide are proposed.[541]

Proteins.—*Cytochromes.* Resonance Raman spectra and excitation profiles of cytochrome b_{562} and cytochrome *c* have been obtained with a sensitive vidicon multi-channel Raman spectrometer. Comparison of the spectra reveals that two Raman lines at 690 and $700\,cm^{-1}$ in cytochrome *c* may be assigned to the S—S stretch of the thioether bridges, which are absent in cytochrome b_{562}. Four sets of doublets between 320 and $430\,cm^{-1}$ are unique in cytochrome *c* among various haemoproteins and may indicate the existence of 2 subtly distinct haem structures in the ground state of the *c* form. This unique feature was not observed in the reduced form of the haem octapeptide.[542] Raman difference spectroscopic techniques allow reliable detection of very small frequency differences ($0.01\,cm^{-1}$), and the technique was applied to twenty-four species of cytochrome *c*. The differences in the spectrum of the porphyrin of any two species are small, confirming that all of the cytochromes have the same cytochrome fold; however, systematic differences were detected, which indicated structural differences among these proteins. These differences could be classified into three groups and interpreted in terms of structural variations resulting from specific differences in the amino-acid sequences. First, direct interaction between near-haem residues and the porphyrin influences the electron density in the π-orbitals of the porphyrin macromolecule. Second, variation in the residue at position 92, far removed from the haem, affects the frequency of the core-size marker line at $1585\,cm^{-1}$. Third, the conformation near cysteine-14 affects the shape of the Raman mode, which is sensitive to the pyrrole-ring substituents ($\sim 1313\,cm^{-1}$).[543] The main features of the spectra of cytochromes *c* and *b* in *Paracoccus denitrificans* membrane were shown to be similar to those of the analogous cytochrome b–c_1 complex derived from pigeon-breast muscle mitochondria. Differences in the $1300\,cm^{-1}$ region were interpreted in terms of marker bands for haem types and spectral coupling of the haems on the membranes. It was difficult to explain the results without invoking sharing of electronic and vibrational wave function among the haems.[544] Surface-enhanced resonance Raman spectra (SERRS) and resonance Raman spectra were used to study the structure of the haem *c* and haem d_1 components of cytochrome *cd*, a haem protein with nitrate reductase activity. Of general interest is the application of SERRS techniques to large proteins (120 000 daltons). Tentative assignments for cytochrome cd_1 indicate carbonyl, formyl, and ester modes in the haem d_1 moiety. Comparison between the SERRS of cytochrome cd_1

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from different species showed substantial differences, thus illustrating the usefulness of the method for studying extremely scarce samples.[545]

Lysozyme. Previous measurements on the OH stretching behaviour in lysozyme solution have not always been in agreement. Careful Raman-scattering measurements obtained from an isotopic substitutional technique have shown that the interaction between lysozyme and H_2O does not contribute significantly to the Raman spectra of the OH stretching vibration.[546] Comparison of computer-processed Raman spectra of lysozyme in D_2O and H_2O showed that deuteriation affects a band at 750—760 cm^{-1} due to tryptophan as well as the amide bands. Several of the amide frequencies were assigned to specific dihedral angles in the polypeptide backbone. Comparison of the rates of deuteriation of various amides showed that the α-helical regions of lysozyme are more accessible to solvent than are the β-sheet regions.[547]

Haemoproteins. Infrared spectra of the CO complex with myoglobin isolated as the oxygenyl species from bovine heart muscle were obtained in the C=O stretching region as either the pH or the temperature was varied. Deconvolution of these spectra into Gaussian-shaped bands suggests the presence of four bands near 1938 (I), 1944 (II), 1954 (III), and 1965 (IV) cm^{-1} with half-bandwidths of 18, 9, 9, and 10 cm^{-1}, respectively. The relative intensities of the four bands varied with changes in pH or temperature. Other physical studies indicate that the four C=O bands could come from four discrete, rapidly interconverting conformers. Thus the structure at the ligand-binding site of bovine myoglobin is highly flexible and dynamic even when ligands are bound.[548] Splittings related to the stretching vibration of bound oxygen in haemoprotein have been detected by resonance Raman spectroscopy. With excitation at 406.7 nm, three isotope-sensitive lines in oxycobaltmyoglobin or in oxycobalthaemoglobin A were observed near 1103, 1137, and 1153 cm^{-1}. The first two frequencies arise from resonance interaction between a ν(O—O) mode and a degenerate porphyrin-ring mode, whereas the third frequency represents an unperturbed ν(O--O) vibration from a different species. The same resonance interaction may also occur in oxyiron myoglobin. The presence of two ν(O—O) vibrations but only one ν(Co—O) mode at $\simeq$ 538 cm^{-1} means that the two species in oxyCoMb or oxyCoHbA have the same Co—O bond lengths but different O—O bond lengths. Bound molecular O_2 in a bent end-on configuration may have two allowed orientations.[549] The Fe—C bond in (carbon monoxy)-haemoglobin (HbCO) and -myoglobin (MbCO) has been identified for the first time by resonance Raman spectroscopy. The Fe—CO stretching, Fe—C—O bending, and bound C—O stretching vibrations were detected at 507 (512), 578 (577), and 1951 (1944) cm^{-1}, respectively, in human (carbon monoxy)-HbA and sperm-whale MbCO on excitation at 406.7 nm with a Soret band. These assignments were made on the basis of frequency shifts with the

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isotopes $^{13}C^{16}O$, $^{12}C^{18}O$, and $^{13}C^{18}O$. Calculated isotopic shifts according to the model imidazole —Fe—C—O agree well with the observed data.[550] The resonance Raman spectrum of photolysed carbonyl-Hb in the R state, formed during a 0.3 μs laser photolysis interval, was observed. This time-period was prior to the R → T quaternary structural transition of Hb. The band due to Fe—imidazole stretching was more symmetrical and was shifted to higher frequency relative to that of Hb. The band characteristic of the oxidation state was shifted to a lower energy (1355 cm^{-1}) as compared with that of stable Hb (1357 cm^{-1}). The bands indicative of the porphyrin core size, however, were essentially at the same frequencies as those for Hb, indicating that the previously observed short time-scale shifts are associated with tertiary rather than quaternary structure and reflect a molecular process different from the R → T transition.[551] The low-frequency regions of resonance Raman spectra of various low-spin ferrous forms of normal human Hb, soybean legoglobin *a*, and horse myoglobin are reported. Differences observed among the spectra of oxygenated and nitrosyl forms of these haemoproteins show that their globins impose various low-spin haem structures. A quantitative correlation between the variable frequency of resonance Raman band II (215—271 cm^{-1}) and the Fe atom–haem plane distance was observed for haemoproteins and haem models, either ferrous or ferric and of high or low spin. From this correlation the Fe atom–haem plane distance should be 0.3 Å in nitrosyl and oxymyoglobin, whereas the Fe position should be near to or in the haem plane for nitrosyl and oxy forms of Hb and legoglobin.[552] The low frequency of the resonance Raman spectra of deoxygenated ferrous forms of soybean leg-Hb *a*, horse myoglobin, sperm-whale myoglobin, *Aplysia* myoglobin, stripped, normal human Hb (T quaternary form), and the stripped *N*-ethylsuccinimide derivative of human Hb subjected to the action of carboxypeptidase B (R quaternary form) has been reported. Differences observed among these spectra show that the globins of these haemoproteins impose various haem structures. In particular, the variable frequencies of band II (210—224 cm^{-1}) and band Ib (121—163 cm^{-1}) show that an increase in O_2 affinity corresponds to a decrease in Fe—N bond length.[553] Resonance enhancement spectra of low-frequency vibrational modes in soybean ferric leg-Hb *a* (Lb), horse-heart myoglobin (Mb), and their deoxy derivatives indicated no significant differences between the strength of the Fe—O_2 bond in oxyLb and oxyMb or of the Fe—N_6 bond in the deoxyproteins. The principal difference in haem structure between Lb and Mb was in the strength of the in-plane Fe—N bonds, these bonds being stronger in Lb. It was proposed that haem ruffling in Lb, rather than a change in Fe-histidine binding, leads to a strengthening of the ligand field and stabilization of the O_2 complex, thus causing a decrease in the O_2 dissociation rate of LbO_2 relative to MbO_2.[554]

Rhodopsin and Derivatives. Resonance Raman studies of visual pigment analogues regenerated with 10-D, 11,11-D_2, 11-D, 11,12-D_2, 12-D, 15-D, and 20,14-D_4 isotopic derivatives of retinal were used to assign the intense H out-of-plane

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[553] A. Desbois, M. Lutz, and R. Banerjee, *Biochim. Biophys. Acta*, 1981, **671**, 177.
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(HOOP) vibrations in the spectra of rhodopsin, isorhodopsin, and bathorhodopsin. Low-temperature, photostationary steady-state experiments show that the unique 853, 875, and 921 cm^{-1} lines of native bathorhodopsin are due to isolated 14, 10, and 11 HOOP wagging modes, respectively. The 12 HOOP wag modes could not be directly observed but must lie in the 830—860 cm^{-1} region, an unusually low frequency for this vibration. Room-temperature rapid-flow experiments similarly demonstrate that the 969 cm^{-1} line in rhodopsin is due to the coupled $C_{11}H{:}C_{12}HA_2$ HOOP. The 959 cm^{-1} line in isorhodopsin is attributed to a $C_7H{:}C_8HA_2$ HOOP. For bathorhodopsin, the small shift of the 11 HOOP wag with 12-D substitution clearly indicates that the 11 and 12 HOOPs are weakly coupled. This contrasts sharply with the strong coupling observed between these wags in rhodopsin, isorhodopsin, and all-*trans*-retinal. The bathorhodopsin chromophore is thus likely to have a twisted 11,12-*trans* structure that is significantly perturbed near C_{12} by its interaction with an opsin residue. Interpretation of the rhodopsin and bathorhodopsin HOOP intensities according to vibrational theory shows that the excited states of these pigments relax by an initial out-of-plane distortion of $\simeq 10°$ along the enhanced HOOP co-ordinates. Because intense HOOP modes are not seen in model compounds, these out-of-plane distortions are induced by opsin.[555] The Raman spectra of visual pigment analogues formed with 11,12-dideuterio- and 10-monodeuterio-retinals demonstrate that the three intense bands of bathorhodopsin at 853, 875, and 920 cm^{-1} can be assigned to chain vinyl H-atom out-of-plane bends. Calculations show that the intensity of these vibrations can be explained by introducing ground-state twists along the polyene chain.[556] The picosecond and nanosecond resonance Raman spectra of bacteriorhodopsin (bR) were detected by microbeam and flow techniques. The 40 ps pulses were generated by a synchronously pumped, mode-locked, cavity-dumped dye laser. The pulse width was extended to 15 ns by CW pumping the cavity-dumped dye laser. The spectrum of the picosecond transient in the fingerprint region resembled the $bR_{550}DA$ spectrum. The retinal in the latter species is believed to be in the 13-*cis* configuration. These results suggest that the retinal changed from an all-*trans* to a 13-*cis* type of configuration in <40 ps. Slight differences were observed between the picosecond and nanosecond retinal spectra. These observations suggest that more than one species exist in the 40 ps to 100 ns time-scale.[557] The possible interaction of a second lysine with the retinylidine Schiff base of bacteriorhodopsin was investigated by specific incorporation of ^{15}N into the ε-NH_2 groups of the lysine residues. Comparison of resonance Raman spectra of bacteriorhodopsin grown on 0%, 50%, and 100% labelled lysine demonstrated that ^{15}N-isotope effects on the Schiff-base vibration can be accounted for by ^{15}N labelling only at the Schiff-base N. The data also provide *in situ* confirmation of the linkage of the retinal chromophore with the ε-NH_2 nitrogen of lysine.[558] The resonance Raman spectrum of the primary photoproduct K of bacteriorhodopsin was obtained with a novel low-temperature spinning-

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sample technique. Purple membrane at 77 K is illuminated with spatially separated actinic and probe laser beams. The 514 nm pump beam produced a photostationary steady-state mixture of bacteriorhodopsin and K. This mixture is then rotated through the red (676 nm) probe beam, which selectively enhances the Raman scattering from K. The essential advantage of the successive pump-and-probe technique is that it prevents the fluorescence excited by the pump beam from masking the red-probe Raman scattering. K exhibits strong Raman lines at 1516, 1292, 1194, 1012, 957, and 811 cm^{-1}. The effects of C15 deuteriation on K fingerprint lines correlate well with those seen in 13-*cis* model compounds, indicating that K has a 13-*cis* chromophore. However, the presence of unusually strong low-wavenumber lines at 811 and 957 cm^{-1}, attributable to H out-of-plane waves, indicates that the protein holds the chromophore in a distorted conformation after *trans* → *cis* isomerization.[559] Resonance Raman spectra of photostationary-state mixtures of bR 568, L, L′, and M forms of light-adapted bacteriorhodopsin, were recorded at −80 °C. The approximate Raman spectra of L and L′ were obtained by computer subtraction of the Raman spectra of bR 568 and M from the photostationary-state spectra. These results were compared with those of previous studies to elucidate the photoreaction scheme of bacteriorhodopsin.[560]

Serum Albumin. I.r. and laser-Raman spectroscopy have been used to follow secondary-structure changes during the heat-set relation of a number of aqueous (D_2O) globular-protein solutions. Measurements in the amide I region for bovine serum albumin (BSA) gels of varying clarity and texture showed that the very considerable variations in network structure underlying these materials are not reflected in obvious differences in secondary structure. In all cases aggregation is accompanied by development of β-sheet of a kind common in fibrous-protein systems, but for BSA at least this does not appear to vary significantly in amount from one gel type to the next. I.r. studies of gels formed from other protein systems have confirmed this tendency of β-sheet to develop during aggregation, and the tendency is further substantiated by laser-Raman evidence, which provides the extra information that in most of the samples studied α-helix content simultaneously falls.[561]

Chymotrypsin. Resonance Raman spectra obtained by u.v. laser excitation from ten acyl-chymotrypsins and SDS-denatured acyl-enzymes show that acyl groups bound to serine-195 adopt two conformations, characterized either by strong hydrogen bonds to the carbonyl oxygen or by a non-bonding hydrophobic environment about the carbonyl group. It is also likely that in solution the ester and acid analogues of the acyl group adopt more than one conformation about the acryloyl linkages. Thus the measured spectral parameters such as the ethylene double-bond stretching frequency ν_{C-C} in the resonance Raman spectra should be considered as a weighted mean.[562] Changes in the resonance Raman spectra of 4-amino-3-nitrocinnamoyl and 5-methylthienylacryloylchymotrysin with varying

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pH show that at active pH a reduction in π-electron polarization throughout the chromophore occurs before deacylation. The reduction in polarization leads to a blue shift in λ_{max} and an increase in the $\nu_{C=C}$ frequency, which may be caused by generation of a negative or partial negative charge near the acryloyl carbonyl at active pH. The relation between the spectral changes and the activation of the carbonyl group is discussed. The carbonyl profiles or C=C stretching profiles in the resonance Raman spectra of acyl-enzymes indicate that the enzyme–substrate interactions perturb the geometry and π-electron distribution of the enzyme-bound chromophore. It is proposed that steric hindrance in the active site, caused by substrate 3-Me or 2-Me groups, leads to low rates of deacylation.[563]

Adrenodoxin. The Raman spectrum of adrenodoxin has been measured with particular emphasis on the Raman doublet due to tyrosine-82, a residue that emits anomalous fluorescence. The tyrosine doublet is located at 860 and 832 cm^{-1} and the doublet relative-intensity ratio is approximately 2—10. The very high frequency of the 860 cm^{-1} band and the very low relative-intensity ratio I_{860}/I_{832} suggest that the single tyrosine residue in adrenodoxin is unusually strongly hydrogen-bonded to a neighbouring carboxylic acid residue. Comparison of the adrenodoxin Raman doublet with those for ionized tyrosine both in aqueous solution and in the anhydrous potassium salt indicates that the tyrosine is not ionized, although there is probably a very great electron density on the tyrosyl OH group. The present results together with the Chou–Fasman calculation of predicted secondary structure provide evidence that the tyrosine-82 OH group is very strongly hydrogen-bonded to a nearby glutamic acid residue. Backbone-sensitive Raman bands confirm the presence of α-helical and β-pleated-sheet segments in the apoadrenodoxin secondary structure.[564]

Flavin. The normal modes of lumiflavin have been analysed with a valence force field constructed with bond-length-stretching force constants and interaction force constants transferred from small ring molecules. Observed resonance Raman bands of flavin are assigned to calculated modes on the basis of frequency and isotope-shift matching. The normal mode patterns confirm previous inferences based on selective effects of chemical substitutions. These results are used to interpret the observed variability of the prominent resonance Raman bands among different fluoroproteins on the basis of protein–isoalloxazine interactions.[565]

Phenylalanine/Tyrosine Intensity Ratios in Proteins.—The use of phenylalanine/tyrosine Raman spectral-line intensity values for predicting the phenylalanine/tyrosine ratio in proteins is not universally valid as indicated by literature data on the Raman spectra of proteins of known amino-acid composition. Of sixty-four spectra examined only 28 (44%) were within 25% of the actual phenylalanine/tyrosine ratio calculated from amino-acid compositions.[566]

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5 Fluorescence

Contributed by R. Devonshire

This report covers the literature of 1980 and 1981. The major restriction, as in previous years, is that the studies are concerned with isolated and purified components. There is no coverage of specific developments in the instrumentation for luminescence measurements because of pressure on space, but a small section on luminescence techniques has been retained.

The report reveals a continuing healthy trend towards the use of intrinsic chromophore luminescence, a growing interest in the use of long-lived luminescence probes, and a strong interest in the use of lanthanide-ion probes.

Review Articles and Books.—A comprehensive survey of the methodology of time-resolved fluorescence spectroscopy and its applications in biochemistry and biology has been published.[567] This timely and interesting collection of review articles results from a NATO Advanced Study Institute gathering held in March 1980. The articles provide coverage of the instrumentation and data analysis associated with the technique, the kinetics of fluorescence decay and depolarization, and results from selected biochemical and biological systems. The proceedings of the Eighth Katzir Conference held in June 1980 include a number of papers on the theory and applications of luminescence techniques to biological and synthetic macromolecules[568a] as do the proceedings of a meeting on fluorescent probes held at the Royal Institution of Great Britain in November 1979.[568b] The study of protein structure and dynamical behaviour by the quenching of intrinsic fluorescence and phosphorescence with added solutes has been critically reviewed.[569] The authors emphasize the difficulties associated with the interpretation of the results from this straightforward experimental technique. The excited electronic states of proteins have been discussed in terms of the properties of their aromatic amino-acid components.[570]

An extensive review (363 references) of the optical properties and the membrane interactions of fluorescence probes has been published.[571] The applications of fluorescent probes in the study of the structure and function of living cells,[572] of photosynthetic membranes,[573] and in the study of biopolymers[574] have been reviewed. A review of the application of metal ions as donors and acceptors of fluorescence in the study of the structure and function of biopolymers illustrates the strong and growing interest in the use of these probes in recent years (see the section in this report on non-covalent probes).[575] There are a number of examples in the recent literature of the covalent attachment of fluorescence probes to

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[575] B. Holmquist, *Adv. Inorg. Biochem.*, 1980, **2**, 75.

biopolymers by use of the techniques of photoaffinity labelling. The fluorescence probe is attached to a chemically inert but photochemically labile group that can be activated at will to attach itself (often non-specifically) to nearby groups. This approach, for example, can lead to the covalent attachment of a ligand carrying a fluorescent probe to a biopolymer in the vicinity of the natural ligand-binding site. The increasing sophistication of photoaffinity-labelling techniques is shown by a number of papers presented at a recent conference.[576]

The continued strong interest in bioluminescence and chemiluminescence is indicated by the number of extensive reviews that have appeared during the past two years. These reviews have concerned the biochemical and physiological aspects of bioluminescence,[577] the control and ecological significance of bacterial bioluminescence,[578] energy-transfer processes in bioluminescence,[579] and chemiluminescent reactions,[580] the latter having the intriguing title 'Photobiochemistry in the Dark'. The analytical uses of chemiluminescence and bioluminescence have been the subject of a review[581] and a book.[582]

The fluorescence and optical characteristics of reduced flavins and flavoproteins have been discussed.[583] The oxidized forms are well characterized spectroscopically but the reduced forms much less so because of their generally low yields of fluorescence and their sensitivity to oxygen. The paper contains a useful table comparing the optical properties of flavoproteins in their oxidized and reduced states. Fluorescence studies of the flexibility of the active sites in proteinases have been reviewed.[584]

Fluorescence Techniques.—Synchrotron radiation is an important source of excitation for time-resolved fluorescence studies. It is continuously tunable throughout the ultraviolet and visible regions of the spectrum, is purely linearly polarized, and is produced as a regular sequence of nanosecond-duration regularly shaped pulses at high repetition rate. Coupled with photon-counting detection it is a powerful technique for studying time-dependent fluorescence intensity and anisotropy. The technique has been discussed in a review by workers involved with the new SERC Daresbury Synchrotron Facility,[585] and the particular application of the technique to fluorescence studies in biochemical systems has also been discussed.[586] The use of laser-excited fluorescence for determining trace quantities of substances has been described, examples of its application being a number of biologically important fluorophores.[587] Tryptophan, for example, can be detected at concentrations as low as 2.5×10^{-10} mol dm^{-3}, which is a factor of 10^2

[576] 'Applications of Photochemistry in Probing Biological Targets', *Ann. N.Y. Acad. Sci.*, **346**, ed. A.M. Tometsko and F. M. Richards, New York Academy of Sciences, New York, NY, 1980.
[577] W. W. Ward, *Photochem. Photobiol.*, 1981, **33**, 965.
[578] K. H. Nealson and J. W. Hastings, *Microbiol. Res.*, 1979, **43**, 496.
[579] W. W. Ward, *Photochem. Photobiol. Rev.*, 1979, **4**, 1.
[580] G. Cilento, *Photochem. Photobiol. Rev.*, 1980, **5**, 199.
[581] A. K. Campbell, J. Simpson, and A. Steven, *Tech. Life Sci., Sect. B*, 1979, **213**, 1.
[582] 'Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications', ed. M. A. DeLuca and W. D. McElroy, Academic Press, New York, NY, 1981.
[583] S. Ghisla, *Methods Enzymol.*, 1980, **66**, 360.
[584] J. S. Fruton, *Mol. Cell. Biochem.*, 1980, **32**, 105.
[585] R. H. Bisby and I. H. Munro, *UV Spectrom. Group Bull.*, 1980, **8**, 60.
[586] D. M. Jameson and B. Alpert, *NATO Adv. Study Inst. Ser., Ser. A*, 1979, **25** (Synchrotron Radiat. Appl. Biophys. Biochem. Res.), 183.
[587] J. H. Richardson, *Methods Enzymol.*, 1980, **66**, 416.

lower than the limits of conventional procedures. The measurement of chemiluminescence has been reviewed,[588] as has that of the circular dichroism and fluorescence-detected circular dichroism of macromolecules.[589]

An interesting review of the application of linearly polarized absorption and emission spectroscopy in studies of the orientation and mobility of molecules in membranes contains much information of relevance to protein studies.[590] Included in the review is a discussion of time-resolved-fluorescence, phosphorescence, and delayed-fluorescence measurements. The two latter types of measurements, which extend the time domains accessible to time-resolved luminescence measurements to microseconds and milliseconds from the nanosecond region covered by fluorescence emissions, have been discussed in detail.[591] The ratio of monomer-type to excimer-type fluorescence emission from polymer chains labelled with fluorescent probes is a measure of probe-to-probe interactions and can therefore be used to monitor conformational mobility and the mutual interpenetration of the chains. Measurements on synthetic polymer chains using this and other luminescent properties have been discussed.[592]

A paper has considered in detail the criteria for choosing between two methods used to construct nanosecond time-resolved emission spectra (TRES).[593] The direct method involves the setting up of a well defined electronic time 'window' in which to accept counts in a photon-counting instrument. A wavelength scan then results in a spectrum at the chosen time, and a repeat of this procedure at different times leads to the TRES. The problem with this relatively simple procedure is the wavelength and time-dependent distortions that arise from the convolution of the excitation light pulse with the fluorescence decay. The alternative procedure is to produce deconvoluted decay curves at a number of emission wavelengths and to construct the TRES. In effect the former method constructs the TRES from distorted 'slices' in wavelength at fixed time and the latter uses undistorted 'slices' in time at fixed wavelength. The analysis of complex relaxation processes using a procedure that is independent of any model of the processes involved has been described.[594] A method has been described by which it is possible to measure either component in a mixture of two fluorophores, provided that the components have different emission polarizations.[595] The approach was illustrated by measurements on a mixture of 2′,7′-dichlorofluorescein and rhodamine B. A very interesting paper has described a method capable of measuring very low quantum yields by directly comparing the fluorescence emission intensity with the emission resulting from an internal standard, namely the Raman scattering of the solvent, water.[596] The relatively strong Raman band corresponding to OH, which has a Stokes shift of $3300\,cm^{-1}$ and is relatively broad, $400\,cm^{-1}$, was used in the experiments. The validity of the method was established by preliminary experiments using a dye with known quantum yield and was then applied in a study of

[588] M. E. Wilson, *Lab. Res. Methods Biol. Med.*, 1980, **4**, 171.
[589] I. Tinoco, jun., *NATO Adv. Study Inst. Ser., Ser. C*, 1978, **48** (Opt Act. Chiral Discrim.), 57.
[590] L. B. A. Johansson and G. Lindblom, *Quantum Rev. Biophys.*, 1980, **13**, 63.
[591] T. M. Jovin, M. Bartholdi, W. L. C. Vaz, and R. H. Austin in ref. 568*a*, p. 176.
[592] H. Morawetz, *Pure Appl. Chem.*, 1980, **52**, 277, and papers in ref. 568*a*.
[593] S. R. Meech, D. V. O'Connor, A. J. Roberts, and D. Phillips, *Photochem. Photobiol.*, 1981, **33**, 159.
[594] S. W. Provencher and V. G. Dovi, *J. Biochem. Biophys. Methods*, 1979, **1**, 313.
[595] P. M. Roemelt, A. J. Lapen, and W. R. Seitz, *Anal. Chem.*, 1980, **52**, 769.
[596] P. M. Champion and R. Lange, *J. Chem. Phys.*, 1980, **73**, 5947.

ferrocytochrome *c*. The quantum yield was found to be 3.6×10^{-6}, which implies a singlet-state lifetime of about 0.2 ps. The method must be used with care but is certainly an innovative approach to the measurement of this basic photophysical property. A detailed discussion of the use of quinine sulphate dihydrate as a fluorescence standard material has been published.[597]

Theories of Fluorescence Phenomena.—A wide-ranging discussion of the phenomenon of ultra-weak photon emission (UPE) has appeared.[598] Apparently the UPE of biological systems exhibits a wavelength-dependent equivalent excitation temperature, indicating that the cell populations are far removed from thermal equilibrium. The paper suggests that the role of UPE is as a regulator of biological actions. The concept that electromagnetic fields have an associated entropy has been reviewed, and it has been discussed with particular reference to photosynthesis.[599] Having included earlier references to general articles on bioluminescence and chemiluminescence it should be mentioned that a new mechanism has been proposed for the bacterial luciferase reaction.[600] The key step in the mechanism is a dissociative electron transfer that results in a protonated flavin excited state, a carboxylic acid, and water.

Fluorescence Quenching. The review of fluorescence quenching[569] mentioned earlier discusses a number of points that are fundamental to such studies and that are often overlooked. There is still disagreement about the origins of the upward curvature in the Stern–Volmer plots, *i.e.* plots of the ratio of the unquenched to the quenched fluorescence intensity against the quencher concentration. (Recent work has shown that the transient term in the diffusion equation can account for this behaviour even in low-viscosity solutions.[601]) The results of quenching studies are easier to interpret if the quenching process itself has unit efficiency, *i.e.* every encounter between a quencher molecule and an excited state results in quenching. This is not the case, for example, for Cs^+ and Br^- ions, both of which are used as quenchers. The review also discusses the information that can be obtained about protein structure and dynamics from the quenching of protein fluorescence, particularly the activation energy of the process. The interpretation of these activation energies is also the subject of some disagreement in the literature. Following on from work reported in Volume 12 additional evidence has been put forward to support the idea that the temperature dependence of the penetration of protein structures by quencher molecules is governed by the properties of the surrounding solvent.[602] This idea has been contested and it has been shown that the results can be accommodated within the framework of a model of the quenching process which at one extreme is governed by the properties of the solvent and at the other by the properties of the protein matrix.[603]

A model of fluorescence quenching in systems with cylindrical symmetry has

[597] R. A. Velapoldi and K. D. Mielenz, U.S. Government Printing Office, Washington D.C., 1980.
[598] F. A. Popp in 'Electromagn. Bio-Inf., Proc. Symp.', ed. F. A. Popp, G. Becker, and H. L. Koenig, Urban and Schwarzenberg, Munich, 1979, p. 123.
[599] E. Broda and H. Pietschmann, *Biosystems*, 1979, **11**, 65.
[600] E. M. Kosower, *Biochem. Biophys. Res. Commun.*, 1980, **92**, 356.
[601] G. D. T. Smith and R. Devonshire, *Chem. Phys. Lett.*, submitted for publication.
[602] T. L. Bushueva, E. P. Busel, and E. A. Burstein, *Arch. Biochem. Biophys.*, 1980, **204**, 161.
[603] M. R. Eftink and C. A. Ghiron, *Arch. Biochem. Biophys.*, 1981, **209**, 706.

been published, which may be useful in quenching studies in membrane systems.[604] It includes both static and dynamical quenching processes. A possible explanation for the observation of a sharp fluorescence transition in D-amino-acid oxidase, which normally would be attributed to a conformational change but for which change no calorimetric evidence can be found, has been put forward.[605] It is suggested that it arises from a dynamic quenching process of large activation energy and that it may occur in other systems. The removal of errors in analytical fluorimetry that arise from quencher impurities by measurement of the fluorescent lifetime and not just its intensity has been described.[606] The use of β-cyclodextrin as a model for the hydrophobic regions of biopolymers has been investigated in a fluorescence quenching study,[607] and another interesting paper of relevance to quenching studies has demonstrated the presence of transient effects in the diffusion-controlled recombination of photodissociated myoglobin and haemoglobin with carbon monoxide and oxygen in supercooled solvents.[608]

Singlet Energy Transfer. A number of interesting papers on the theory of energy-transfer processes in model systems of relevance to biological systems have been published. Analytical expressions for the estimation of fluorescence resonance-energy transfer between donors and acceptors on surfaces have been given.[609] The general method is applied to several models of particular interest to membrane studies, namely (i) an infinite plane, (ii) parallel infinite planes, (iii) the surface of a sphere, (iv) the surfaces of concentric spheres, and (v) the surfaces of two separated spheres. Calculations have demonstrated that it is possible to measure the aggregation of cells and lipid vesicles by an energy-transfer technique,[610] and another paper has considered the quenching of fluorescence due to energy transfer between a dilute random array of donors and acceptors in lipid bilayers.[611] In the latter paper a relatively simple expression was obtained for the degree of quenching as a function of quencher concentration, which agreed well with experiment and with a significantly more complex expression derived previously.

The transfer of energy in the pigment system associated with photosynthesis has been modelled in a number of papers. The possibility that a coupling complex is present in such systems to mediate in the energy-transfer processes between the antenna and the reaction centres has been incorporated into a new model.[612] The additional parameters made it possible to explain features in the fluorescence properties of various purple bacteria. The temperature dependence of energy transfer in photosynthesis has been discussed.[613] As the temperature increases, the model used shows that energy transfer takes place between chlorophyll molecules in a process that involves little or no activation energy, and as a consequence the process becomes temperature independent. The saturation of the energy-transfer

[604] J. C. Andre, M. Bouchy, and M. Donner, *React. Kinet. Catal. Lett.*, 1980, **15**, 159.
[605] A. Cooper, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 3551.
[606] G. M. Hieftje and G. R. Haugen, *Anal. Chim. Acta*, 1981, **123**, 255.
[607] A. De Korte, R. Langlois, and C. R. Cantor, *Biopolymers*, 1980, **19**, 1281.
[608] B. B. Hasinoff, *J. Phys. Chem.*, 1981, **85**, 526.
[609] T. G. Dewey and G. G. Hammes, *Biophys. J.*, 1980, **32**, 1023.
[610] G. A. Gibson and L. M. Loew, *Biochem. Biophys. Res. Commun.*, 1979, **88**, 141.
[611] T. N. Estep and T. E. Thompson, *Biophys. J.*, 1979, **26**, 195.
[612] R. Van Grondelle and L. N. Duysens, *Plant Physiol.*, 1980, **65**, 751.
[613] A. Sarai and S. Yomosa, *Photochem. Photobiol.*, 1980, **31**, 579.

chain in photosynthetic systems exposed to high-intensity laser radiation has been observed and discussed in terms of a simple model.[614]

Fluorescence Polarization. A paper that has carefully re-examined the segmental flexibility of immunoglobin G antibody, and which will be mentioned in a later section (see *Covalent Probes*), contains a very useful expression for relating the results of time-resolved and steady-state fluorescence-polarization measurements to each other.[615] A paper has described a Monte Carlo-type simulation of the fluorescence-polarization decay of segmentally flexible molecules.[616] The model used incorporates a linear restoring force opposing any bending motion and the effects of coupling between the translational, rotational, and bending motions. Simulations with myosin exhibited a wide range of behaviour. The major finding was that because of the complex interplay of a number of parameters it appears unlikely that experimental anisotropy decays can be interpreted by any simple method, and this casts doubt on interpretations already in the literature that are based on a dual exponential approximation. A theory of the fluorescence emission anisotropy of a cylindrical probe molecule attached to a macromolecule or embedded in a membrane has been described.[617] An expression is given which relates the effective relaxation time of the anisotropy to the angle of a cone in which the probe executes a wobbling motion and to the diffusion coefficient of this wobbling motion. Equations that represent the fluorescence polarization of cylindrically shaped objects such as muscle fibres have been published,[618] and it has been proposed that the lateral pressures in biomembranes can be related to the steady-state fluorescence polarization of probe molecules, such as diphenylhexatriene, held in the membrane.[619]

Photophysics and Photochemistry of Aromatic Amino-acids.—*Emission Lifetimes.* The disagreements in the literature concerning the fluorescence lifetime(s) of tryptophan in aqueous solution, mentioned in Volume 12, have prompted a number of reinvestigations of this problem. It is now quite clear that one of the earlier measurements was distorted by contributions from a fluorescent photodecomposition product of tryptophan.[620] When these values are ignored, a generally consistent picture does emerge, although additional layers of complexity to the problem have also been revealed. A recent study of the decay kinetics as a function of pH and buffer composition has shown that under certain conditions a triple-exponential decay is observed.[621] In solutions below pH 7.0 a double-exponential decay is observed with the longer-lived component with τ in the range 2.3—3.2 ns contributing about 90% of the total emission and with a short-lived component with τ of 0.4—0.6 ns the remainder of the emission. In the low-pH region below pH 2.0 the longer-lived of these two components is quenched and below a pH $\approx$ 1.2 the decays are single exponential. In the pH region above 7.0 a third longer-lived component appears in the decay whose lifetime, unlike that of the

[614] A. A. Demidov and V. V. Fadeev, *Dokl. Akad. Nauk SSSR*, 1980, **254**, 1262.
[615] D. C. Hanson, J. Yguerabide, and V. N. Schumaker, *Biochemistry*, 1981, **20**, 6842.
[616] S. C. Harvey and H. C. Cheung, *Biopolymers*, 1980, **19**, 913.
[617] G. Lipari and A. Szabo, *Biophys. J.*, 1980, **30**, 489.
[618] Y. M. Rozanov, A. E. Vinogradov, and I. Y. Barskü, *Zh. Prikl. Spektrosk.*, 1980, **33**, 290.
[619] A. C. J. Fulford and W. E. Peel, *Biochem. Biophys. Acta*, 1980, **598**, 237.
[620] K. P. Chiggino, G. R. Mant, D. Phillips, and A. J. Roberts, *J. Photochem.*, 1979, **11**, 297.
[621] E. Gudgin, R. Lopez-Delgado, and W. R. Ware, *Can. J. Chem.*, 1981, **59**, 1037.

other two components, depends on the buffer composition. These new results provide a detailed phenomenological description of the system and can encompass the results of other recent studies where the analysis was restricted to best-fit dual-exponential decays or the earlier studies that reported best-fit single-exponential decays. The origin of the multiple decays is not yet entirely clear, but recent studies in which the fluorescence decay of 3-methylindole was compared to that of tryptophan have indicated that in the pH range 4—8 an additional quenching process is present in tryptophan that arises from the interaction of the ring 2-position with the NH_3^+ group.[622, 623] When no labile proton is available, as in the ester derivatives of indole-3-propionic acid, a weaker quenching mechanism can operate, which involves charge transfer from the excited indole ring to a carbonyl group. These mechanisms also appear to operate in the simple tryptophan-containing dipeptides Trp-Ala and Ala-Trp.[57] Tryptophan fluorescence exhibits a dual-exponential decay in oligopeptides such as Lys-Trp-Lys [624] and polypeptides such as glucagon [623, 625] and ACTH.[626]

Fluorescence Quenching. If there are different tryptophan species giving rise to the separate lifetime components mentioned above (there are other possible explanations that have not, as yet, been fully excluded) and if these species have different spectral characteristics in respect of their relative absorbances or their wavelength distributions, then the results of quenching studies on tryptophan fluorescence will give results that are representative of some averaged lifetime. In the latter case, where the wavelength distributions may be different, this averaged lifetime will itself be wavelength dependent. The recent study of time-resolved tryptophan fluorescence decay, which showed that the decay could be fitted by a dual exponential (work that has recently been extended by the same authors),[627] also showed that the two lifetime components corresponded to emissions with different λ_{max}. The longer-lived component, $\tau = 3.1$ ns, which is dominant in the low-energy region of the emission spectrum, has a λ_{max} of 350 nm, and the short-lived component, $\tau = 0.51$ ns, has a λ_{max} of 335 nm.[627] An interesting quenching study, using acrylamide as a quencher, has demonstrated such a heterogeneity in the emission by use of a steady-state technique.[628] The parameters describing the Stern–Volmer quenching plots were found to be significantly different when measured at 315 nm and 400 nm, the high-energy and low-energy regions of the emission spectrum, respectively. Although in general the observations agree with the lifetime work in that the apparent averaged lifetime was less at 315 nm than at 400 nm, the numerical agreement was not good. The authors make an important point when they suggest that, because of the good accuracy of quenching measurements relative to lifetime measurements, the former should be regarded as a test of the latter. The quenching of indole fluorescence by amide and carboxylic

[622] R. J. Robbins, G. R. Fleming, G. S. Beddard, G. W. Robinson, P. J. Thistlethwaite, and G. J. Woolfe, *J. Am. Chem. Soc.*, 1980, **102**, 6271.
[623] G. S. Beddard, G. R. Fleming, G. Porter, and R. J. Robbins, *Philos. Trans. R. Soc. London, Ser. A*, 1980, **298**, 321.
[624] T. Montenay-Garestier, J. C. Brochon, and C. Helene, *Int. J. Quantum Chem.*, 1981, **20**, 41.
[625] S. A. Cockle and A. G. Szabo, *Photochem. Photobiol.*, 1981, **34**, 23.
[626] L. Brand, J. A. Ross, and W. R. Laws in ref. 568*a*, p. 197.
[627] A. G. Szabo and D. M. Rayner, *J. Am. Chem. Soc.*, 1980, **102**, 554.
[628] M. R. Eftink and C. A. Ghiron, *Photochem. Photobiol.*, 1981, **33**, 749.

acid groups has been investigated in a study in which the quencher groups were held in a rigid structure attached to the fluorophore.[629] The compounds used were *trans*- and *cis*-3-(3′-indolyl)norbornene 2-dimethyl carboxamides or carboxylic acids. The quantum yields were significantly lower in the *cis* compounds compared with the *trans* or open-chain compounds. A further study of tyrosine fluorescence quenching by phosphate ions has been published.[630] The study models the behaviour of protein–nucleic acid complexes using a number of simple compounds. Phosphate monoanions $H_2PO_4^-$ and $(MeO)HPO_3^-$ and bianions HPO_4^{2-} and $MeOPO_3^{2-}$ were found to quench tyrosine fluorescence by a mechanism that resulted in the appearance of weak fluorescence from the tyrosinate anion. Phosphate diesters, used as models of polynucleotide phosphate groups, did not quench tyrosine fluorescence. It was suggested that tyrosine fluorescence is quenched by nucleic acid bases by a Förster-type energy-transfer process.

Photochemistry and Spectroscopy. There have been a number of studies of the photolysis of tryptophan in solution and of proteins containing tryptophan (see section on proteins), in which as a result of the photolysis fluorescent photoproducts are produced. The fluorescence of a number of known photoproducts (a number have still to be identified) has been shown to have λ_{max} values in the range 385—490 nm.[631] The potential problems that these figures present to studies of tryptophan in experiments where significant total u.v. exposures can occur are obvious, and an example of how such a case has been identified has already been mentioned.[54]

The excitation-wavelength dependence of the yields of electron ejection and H-atom release from polar and non-polar solutions of indole, 5-methoxyindole, and *N*-methylindole has been studied.[632] The yields of the two processes were correlated with the fluorescence yield. A valuable paper has presented evidence that there are two sites for exciplex formation in indole compounds.[633] The results from the study of 3-methylindole/n-butanol exciplexes were interpreted by proposing a negative exciplex site at C-3 and a positive exciplex site at N-1. On the formation of each exciplex the emission spectrum is shifted by 15 nm, and this observation clearly has relevance to the interpretation of tryptophan emission in proteins. An investigation of the fluorescence of iodoamino-acids (tyrosine and thyronine derivatives and thyroxine) and iodinated proteins (RNAase A, neurotoxin I, and thyroglobulin, the latter a naturally iodinated protein) showed that the λ_{max} values were in the region 370—385 nm and the quantum yields for fluorescence were very low.[634]

Intrinsic Protein Luminescence.—*General Interest.* An important paper has reported detailed measurements of the efficiency of electronic-energy transfer between tyrosine and tryptophan residues in a number of globular proteins.[635]

[629] J. Royer, M. Beugelmans-Verrier, and J. F. Biellmann, *Photochem. Photobiol.*, 1981, **34**, 667.
[630] T. Alev-Behmoaras, J. J. Toulme, and C. Helene, *Photochem. Photobiol.*, 1979, **30**, 533.
[631] J. Slawinski, M. Elbanowski, and D. Slawinska, *Photochem. Photobiol.*, 1980, **32**, 253.
[632] J. Zechner, G. Köhler, N. Getoff, I. Tatischeff, and R. Klein, *Photochem. Photobiol.*, 1981, **34**, 163.
[633] M. V. Hershberger, R. Lumry, and R. Verrall, *Photochem. Photobiol.*, 1981, **33**, 609.
[634] E. E. Gussakovsky and E. A. Burstein, *Stud. Biophys.*, 1980, **80**, 97 and 57.
[635] Y. Saito, H. Tachibana, H. Hayashi, and A. Wada, *Photochem. Photobiol.*, 1981, **33**, 289.

The efficiencies are calculated from measurements of the relative quantum yield of tryptophan fluorescence as a function of excitation wavelength, made using an instrument capable of simultaneously measuring both the absorbance and the fluorescence of the protein sample. The results from eight proteins, some of which had high tryptophan-to-tyrosine ratios, showed the efficiencies to vary from 0.17 ± 0.12 to 0.69 ± 0.06 in the native state and from -0.04 ± 0.09 to 0.12 ± 0.06 in the denatured state. The results, which are presented in the Table, are the most extensive and accurate results available for this important parameter in protein photophysics. The measurement of the ratios of tryptophan-to-tyrosine residues in proteins using synchronous scanning fluorimetry has been described.[636] In the technique the excitation and emission monochromators are scanned simultaneously with a fixed wavelength interval between them.

A recent paper has reported an interesting study of the pressure-induced dissociation of the protein enolase.[637] Without prior knowledge of the change in the free volume associated with the process it is not possible to predict which of the dissociated or associated forms is favoured by higher pressures. Measurement of the polarization of the tryptophan fluorescence revealed that a dissociation was induced by increasing pressure with a midpoint dissociation pressure of 1.5 kbar and a standard volume change, ΔV_p^0, of -65 ml mol^{-1}. The study also revealed a pressure-induced red shift in the fluorescence spectrum, which, like polarization, was found to be completely reversed on release of the pressure. The volume changes involved originate from the rotations of covalent bonds and not from any changes in bond angles or bond lengths. The first report of fluorescence-detected circular-dichroism (FDCD) measurements on proteins has been published.[638] The measurements were made using an instrument that successfully eliminates large artifacts in FDCD arising from residual linear polarization in the exciting light. The FDCD spectra of a number of single tryptophan-containing polypeptides and proteins (adrenocorticotropic hormone, glucagon, human serum albumin, monellin, and ribonuclease T1) were obtained, and they demonstrated a clear sensitivity to the conformational state of the fluorophore. Interestingly the spectra revealed a possible weak transition in the 230—250 nm region.

The potential of time-resolved fluorescence anisotropy measurements, commented on in my last report, Volume 12, is illustrated by recent studies on a number of protein systems. The single tryptophan residue in adrenocorticotropin(1—24) exhibits two distinct rotational correlation times.[639] The shorter, sub-nanosecond, motion probably relates to a rotational motion of the fluorophore and the slower, nanosecond, motion to more complex chain motions. Less-direct evidence for rapid internal motions of tryptophan residues in proteins has been found in a study where both steady-state and lifetime-resolved fluorescence-anisotropy measurements were made on a number of proteins, mostly containing more than one tryptophan residue.[640] The fluorescence lifetime(s) in each of the systems was systematically reduced by quenching with oxygen. In all the proteins

[636] J. N. Miller and A. F. Fell, *J. Pharm. Pharmacol.*, 1980, **32** (Suppl.), 70P.
[637] A. A. Paladini, jun. and G. Weber, *Biochemistry*, 1981, **20**, 2587.
[638] E. W. Lobenstine, W. C. Schaefer, and D. H. Turner, *J. Am. Chem. Soc.*, 1981, **103**, 4939.
[639] J. B. A. Ross, K. W. Rousslang, and L. Brand, *Biochemistry*, 1981, **20**, 4361.
[640] J. R. Lakowicz and G. Weber, *Biophys. J.*, 1980, **32**, 591.

Table *Molecular weight, contents of tryptophan and tyrosine, and efficiencies of energy transfer*[635]

Protein	*Mol. wt.* ($\times 10^3$)	*No. of residues*			*Efficiency of energy transfer*†			
					Present study		*Previous study*	
		Trp	*Tyr*	*Trp/Tyr**	*Native*	*Denatured*	*Native*	*Denatured*
Lysozyme	14.3	6	3	2‡	0.48 ± 0.28	0.04 ± 0.24		
Trypsin inhibitor	20.1	2	4	0.5[a]	0.38 ± 0.11	−0.04 ± 0.09		
Trypsin	23.2	4	10	0.4‡	0.29 ± 0.07	0.09 ± 0.07	1.07 ± 0.07	0.16 ± 0.05[e]
α-Chymotrypsin	25.1	8	4	2‡	0.17 ± 0.12	0.02 ± 0.16		
Carboxypeptidase A	34.4	7	19	0.37‡	0.55 ± 0.04	0.12 ± 0.06	0.81 ± 0.06	0.43 ± 0.18[e]
Phosphoglycerate kinase	45.8	2	8	0.25[b]	0.22 ± 0.11	0.01 ± 0.08	0.286[f]	
Hexokinase	103	4	15	0.267[c]	0.30 ± 0.06	0.10 ± 0.10		
Alcohol dehydrogenase	150	6	14	0.428[d]	0.69 ± 0.06	0.01 ± 0.09		

* The ratio of the number of Trp residues to that of Tyr residues in a protein. † The amount of error is the standard deviation of $t(\lambda)$ at wavelengths between 275 and 290 nm. The high Trp/Tyr ratio makes the value $1 - A(\lambda)$ very small and consequently will result in a large error in the value of the efficiency, so this deviation is roughly related to the Trp/Tyr ratio. ‡ The mol. wt. and Trp and Tyr contents were derived from amino-acid sequences summarized in the literature.[g]

[a] T. Koide and T. Ikenaka, *Eur. J. Biochem.*, 1973, **32**, 401. [b] W. K. G. Krietsch and T. Bucher, *Eur. J. Biochem.*, 1970, **17**, 568. [c] J. J. Schmidt and S. P. Colowick, *Arch. Biochem. Biophys.*, 1973, **158**, 458. [d] J. I. Harris, *Nature (London)*, 1964, **203**, 30. [e] M. J. Kronman and L. G. Holmes, *Photochem. Photobiol.*, 1971, **14**, 113. [f] H. Nojima, A. Ikai, and H. Noda, *Biochim. Biophys. Acta*, 1976, **427**, 20. [g] M. O. Dayhoff, 'Atlas of Protein Sequence and Structure', National Biomedical Research Foundation, Washington D.C., U.S.A., 1972, Vol. 5.

the correlation times for rotation were found to be significantly shorter than those estimated for rotation of the whole protein. At the opposite end of the time domain accessible to luminescence-decay measurements a study of the time-resolved phosphorescence anisotropy in three globular proteins has been published.[641] The phosphorescence of many proteins is significantly polarized, which can be used to monitor the slower rotational motions of larger proteins in solution or proteins incorporated into larger structures such as membranes. In favourable cases where the tryptophan residue is protected from external quenchers, *etc.*, by the protein structure the emission can be observed at room temperature (*vide infra*). In the time-resolved study, carried out in glycerol–phosphate buffer solutions between −90 and −70 °C, single-exponential anisotropy decays were observed for each of the proteins, and the temperature and molecular-weight dependence of the decays indicated that they originated from the rotation of the entire protein structure. It is possible in time-resolved fluorescence measurements to observe directly the results of dipolar relaxation as a time-dependent growth in the red edge of the emission spectrum. It has recently been shown that the phenomenon can lead to a wavelength-dependent inversion of the phase shift and demodulation lifetimes obtained from the steady-state relaxation lifetime technique.[642] The effect was observed for *N*-acetyl-L-tryptophanamide, a model protein fluorophore, in viscous solutions and in vesicles.

Tryptophan Fluorescence. Several studies of (sodium–potassium ion)- and (calcium ion)-ATPase enzymes isolated from the outer renal medulla membranes of rabbit or pig kidneys have been published. Intrinsic fluorescence studies have revealed differences in the conformational responses of purified (Na^+–K^+)-ATPase and an invalid (Na^+–K^+)-ATPase, obtained by a graded tryptic digestion of the Na^+ form, to catalytic and transport processes [643] and have been used to investigate the sensitivity of the Na^+ and K^+ selectivity of (Na^+–K^+)-ATPase to pH and ATP.[644] A study of energy transfer from Ca^{2+}-ATPase to the fluorescence ionophore X537A has revealed that there are both buried and exposed populations of tryptophan residues.[645] Ca^{2+} titrations of the intrinsic fluorescence of Ca^{2+}-ATPase revealed a co-operative behaviour in the purified ATPase, which was lost when the protein was solubilized in a non-ionic detergent, dodecyloctaethylene glycol monoether.[646] In the latter study the co-operativity could be recovered in the solubilized system by the addition of millimolar concentrations of $CaCl_2$. Stopped-flow measurements have shown that, between pH 5.5 and 8.5, the intrinsic fluorescence level of sarcoplasmic reticulum ATPase is closely correlated with the functional state of the protein,[647] and they have supported the idea that monomeric Ca^{2+}-ATPase is capable of Ca^{2+} transport.[648] This latter conclusion was reached by comparing the properties of sarcoplasmic reticulum, purified ATPase in membrane vesicles, and solubilized, delipidated ATPase.

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Conformational changes in the ADP/ATP carrier protein, which contains several tryptophan residues, induced by ADP, ATP, and specific inhibitory ligands such as carboxyatractyloside, have been followed by monitoring the intrinsic fluorescence.[649]

A comparison of the fluorescence properties of bovine, goat, human, and guinea-pig α-lactalbumin at acid pH has shown that the temperature-induced transition between two partially folded conformers, the U and H forms, changes the interactions of three tryptophan residues, Trp-28, -63, and -109.[650] In the native N form and in the U form Trp-28 and Trp-109 transfer their excitation energy to Trp-63, whose fluorescence is quenched by a pair of vicinal disulphide bridges. This mechanism is absent in the H conformer. The study of changes in the tryptophan fluorescence of goat α-lactalbumin below pH 7 has been shown to be complicated by the binding of an impurity present in the milk mother liquor.[651] The interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles as a function of pH and temperature has been investigated by microcalorimetric and fluorescence techniques,[652] and Ca^{2+} binding to bovine α-lactalbumin has been shown to decrease the tryptophan fluorescence quantum yield and induce a large, 15 nm, spectral shift to shorter wavelengths.[653] Changes in the tryptophan fluorescence of whiting (*Gadus merlangus*) parvalbumin induced by Ca^{2+} binding have been interpreted in terms of three forms of the protein: a form with no bound Ca^{2+}, a form with one Ca^{2+} per protein molecule, and finally a form with two Ca^{2+} mol^{-1}.[654] A disturbingly large range of fluorescence characteristics has been found in a number of commercial samples of human serum albumin.[655] Even after dialysis and charcoal treatment large differences remained and the purification procedure was even found to alter the characteristics of crystalline samples. Stopped-flow studies of the quenching by *N*-bromosuccinimide of the fluorescence of human and bovine serum albumin and of lysozyme have indicated that this quencher provides a useful measure of tryptophan accessibility.[656] The effect on the fluorescence of bovine serum albumin of interactions with phospholipids has been investigated.[657] The fluorescence properties of a chemically modified bovine prothrombin have been compared with those of the native zymogen during the formation of thrombin and, combined with the results of a fluorescence-quenching study, the indications are that during the activation of the zymogen previously buried tryptophan residues become exposed to solvent.[658] The correlation of metal-ion-induced fluorescence transitions with conformational changes in pro-

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thrombin fragment 1 has been reviewed,[659] and the large changes in the fluorescence of human and bovine antithrombin III on binding of the potent anticoagulant heparin have been traced to a number of buried tryptophan residues.[660]

The Ca^{2+}-binding protein calmodulin is known to bind to and activate myosin light-chain kinase. A recent study has shown that the interaction leads to a 30% increase in the tryptophan fluorescence of the kinase, a clear indication of structural changes associated with the activation process.[661] The fluorescence changes that occur when ribose 5-triphosphate binds to myosin subfragment 1 suggest that there are two binding sites.[662] The binding at the second site is of low affinity and the site may be for some other nucleotide. A study of the intrinsic fluorescence of actin has indicated that there is probably very little change in actin structure in the G (monomer) to F (helical aggregate) transition,[663] and the intrinsic tryptophan fluorescence of fibronectin has been shown from quenching studies to originate from buried tryptophan residues.[664] Acrylamide quenching studies have provided evidence of the degree of exposure of tryptophan residues in skeletal muscle and cardiac troponins.[665]

The quantum yield of the tryptophan emission from haem proteins is very low because of an efficient energy-transfer process from excited tryptophans to the haem centre. The yield of such fluorescence is, however, not immeasurably low, and there have been a spate of reports recently giving details of the u.v. emission spectra and lifetimes of haemoglobins. (In part the reports are a result of the continuing improvements in fluorescence measurement techniques and instrumentation.) A study of human adult haemoglobin and its isolated α- and β-subunits found fluorescence quantum efficiencies of about 1% compared with that of tryptophan (280 nm excitation).[666] These values are lower than those of the apoglobins by a factor of $\simeq 40$, and care was taken to show that the emissions did not arise from apoproteins present as impurities. As the authors point out, it is unreasonable to expect the quenching due to energy transfer to be 100% efficient because of the number of tryptophan residues present (six) and the specific orientation requirements of the Förster-type process. The results of a study of the u.v. fluorescence of porphyrin-globin from human haemoglobin (the haem is replaced by the metal-free porphyrin) produced very similar results to the study of the corresponding haem protein.[667] Evidence was found for a slight contribution of tyrosine to the total fluorescence emission, and the fluorescence decay was found to be strongly non-exponential. The very weak tryptophan emission can be

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systematically quenched by increasing pressures of oxygen.[668] The result demonstrates the permeation of the protein matrix by the quencher and poses some interesting questions about the path taken by ligands in reaching, and leaving, the iron in the haem pocket. The very weak tryptophan emission has also been shown to be significantly different in oxyhaemoglobin (T state) from that in deoxyhaemoglobin (R state).[669] The fluorescence lifetime and relative quantum yield were found to be larger in deoxyhaemoglobin. This pattern was found to apply to a number of forms of human haemoglobin.[670] Another research group has also reported measurements on the tryptophan emission from a number of oxyhaemoglobin variants.[671] The fluorescence was collected from the front face of optical cells containing concentrated protein solutions. The emission maximum for 296 nm excitation was found to be somewhat lower (325 nm) than in the studies mentioned above (334 nm). Studies of tryptophan emission from methaemoglobin interacting with phospholipids,[672] from gamma-irradiated human haemoglobin,[673] and from mammalian apomyoglobins[674] have also been reported. In the latter study the spectral emission from two tryptophan residues was resolved by comparison with the emission from an apomyoglobin that contains only one.

A rapid depolarization of the tryptophan fluorescence from bovine rhodopsin and its photobleaching products has been observed in a time-resolved fluorescence study,[675] and the tryptophan content of bovine rhodopsin has been determined to be eight residues per rhodopsin in a careful study involving analysis of the near-u.v. spectrum and the direct analysis of opsin fragments.[675] A method to produce rhodopsin–lipid complexes that can form large vesicles in response to Mg^{2+} has been described.[677] The fluorescence-emission maximum shifted to longer wavelengths on formation of the complexes, indicating that some of the tryptophanyl residues of the protein are at the protein–lipid interface. The u.v. fluorescence of bacteriorhodopsin has features characteristic of buried, surface, and exposed tryptophan.[678] The proportion of exposed tryptophan residues is greater than that for rhodopsin. A very interesting study of the u.v. fluorescence of bacteriorhodopsin during the operation of the photocycle has been published.[679] The experiments were performed by initiating the photocycle with a short pulse of light and after a known delay in the time range 1 μs to 20 ms using a second pulse of light to stimulate the fluorescence emission. No significant changes in the fluorescence were observed at the shortest delays corresponding to the period of

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the cycle when major changes in the retinal–protein interactions are believed to take place, but changes were observed corresponding to the appearance of the L_{550} and the M_{412} intermediates. In another interesting study on this system it has been shown that excitation in the 280 nm absorption band of bacteriorhodopsin results in excitation of the retinyl chromophore and initiation of the usual proton-pumping cycle.[680] The energy-transfer process occurred with 70—80% efficiency and resulted in the total quenching of all the tyrosine residues and 5—6 of the tryptophan residues. Only 1 tryptophan residue is unquenched. Fluorescence-lifetime measurements on ocular lens chromophores have been published.[681] The tryptophan emission lifetime was found to differ in the different parts of the eye that were examined. This was not true for another fluorophore (λ_{exc} = 359 nm and λ_{emis} = 435 nm) present in the lens. More detailed studies have indicated the distribution of the lens proteins α-, β-, and γ-crystallin in the whole lens.[682] The tryptophan emissions from the δ-crystallins isolated from bird and reptile lens have been found to have the same (and very low?) emission maximum of 315 nm.[683]

A study of the fluorescence of two homologous short neurotoxins, erabutoxin *b* and toxin α isolated from snake venoms, has shown that the emission originates from a tryptophan residue common to both,[684] and a study of neurotoxin II from cobra venom has shown that the fluorescence properties of two adjacent tryptophan residues are very similar.[685] The anticoagulant properties of snake-venom phospholipases A_2 have been investigated,[686] and a fluorescence quenching study has indicated that the tryptophan residue in the B protomer of cholera toxin is protected from solute quenching when the toxin forms a complex with a monosialoganglioside or its oligosaccharide moiety.[687] The strong pH dependence of the fluorescence from the tryptophan residues in thioredoxin from *Escherichia coli* has been investigated.[688, 689] One of the two tryptophan residues, Trp-31, is strongly quenched in both the oxidized and reduced forms of thioredoxin, but the other, Trp-28, changes its fluorescence quantum yield by a factor of 6 at neutral pH when the active centre is reduced. The tryptophan and tyrosine emissions from intact, acetylated, and iodinated thyroglobulin have been compared,[690] as have those from intact and iodinated thyroglobulin obtained from normal human thyroid and nodular euthyroid goitre.[691]

An interesting study of the time-resolved tryptophan fluorescence of horse-liver alcohol dehydrogenase has been published.[692] Quenching studies showed that the individual components of the observed dual-exponential decay could be associated

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with particular tryptophan residues. The buried residue, Trp-314, was found to have a lifetime of 3.8 ns and the exposed residue, Trp-15, to have a lifetime of 7.2 ns. The rotational correlation time obtained from the fluorescence-anisotropy decay was compatible with the known size of the protein and thus, unlike several studies reported on above, in this system the tryptophan residues appear to rotate with the protein as a whole and do not have a lot of local rotational freedom. The fluorescence properties of lactate and malate dehydrogenases have been compared,[693] and the binding of the coenzyme NADPH to pig-liver 6-phosphogluconate dehydrogenase has been investigated.[694] The emission from a partially exposed tryptophanyl residue has been shown to dominate the fluorescence from mesentericopeptidase,[695] and a comparison of the fluorescence properties of the apotryptophanase from *E. coli* B/1t7-A in the presence and the absence of the coenzyme pyridoxal phosphate has suggested that a tryptophan residue interacts directly with the coenzyme.[696] The fluorescence properties of neutral protease from *Bacillus subtilis* have been compared with those of the homologous metalloendopeptidase thermolysin,[697] and those of a purified preparation of acidic protease proenzyme from human seminal plasma have been measured.[698] The exposed tryptophanyl residues of leucyl-tRNA synthetase have been shown to be located in tRNA and leucyladenylate binding sites,[699] and the tryptophan and tyrosine fluorescence of thymidylate synthetase from *Lactobacillus casei* is perturbed by the inhibitor 5-fluoro-2′-deoxyuridylate.[700] A kinetic study of the changes in the fluorescence of DNAase A induced by the binding of Hg^{2+} and Ca^{2+} ions has supported the hypothesis that DNAase exists in three conformational states at 25 °C and pH 7.5.[701] The effects of glucose and Mg^{2+} ion on the quenching by acrylamide of the fluorescence from yeast hexokinase isozyme P-II(B) have been interpreted in terms of the degree of accessibility of the four tryptophan residues in the monomeric form of the protein.[702]

A number of discrepancies in the literature regarding the fluorescence properties of the single tryptophan-containing protein holoazurin B may result from the presence of an additional fluorescent component, possibly another protein, which has recently been discovered.[703] The 'impurity' emission spectrum occurs to the low-energy side of the tryptophan emission peak, which appears at 310 nm, and unlike this buried tryptophan emission it can be quenched with acrylamide.

Studies of tryptophan fluorescence in platelet tubulin,[704] *Streptomyces* sub-

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tilisin inhibitor,[705] wheatgerm agglutinin and lysozyme,[706] lectins from the seeds of *Abrus precatorius*,[707] papain,[708] *E. coli* ribosomal protein S1,[709] low-density serum lipoproteins,[710] tetrahymens,[711] and the ACTH-β-lipotropin precursor polypeptide[712] have also been published.

Tryptophan Phosphorescence. There have been a number of interesting studies of tryptophan phosphorescence from proteins, in some cases from solutions at room temperature. The phosphorescence of liver alcohol dehydrogenase (LADH) at room temperature was first reported in 1974. A recent paper has described measurements of the phosphorescence decay rates of LADH solutions in the temperature range 0—30 °C and in the presence of NAD, NADH, and ADPr, each of which forms a complex with LADH.[713] The decay of LADH phosphorescence contained two components, the relative contributions of which were found to depend on the age of the sample preparation. This ageing effect probably results from a slow conformational change of the protein. The decay constants of the phosphorescence for LADH and the LADH complexes were in the range 1.5—20 s^{-1} at 0 °C. Reports of room-temperature tryptophan phosphorescence from the membranes of *Candida utilis* cells,[714] suspensions of erythrocyte membranes and intact animal and microbial cells,[715] and from dehydrated sucrose and glycerol solutions of bovine serum albumin and trypsin[716] have also been published. Discontinuities have been observed in the thermally induced quenching of LADH and alkaline phosphatase tryptophan phosphorescence.[717] These discontinuities were not observed for low-temperature solutions of free tryptophan or of myelin basic protein, which contains only one tryptophan residue. The thermally induced quenching of myelin basic-protein phosphorescence is considerably reduced when the protein interacts with phosphatidyl vesicles.[718] The absence of tyrosine phosphorescence in ternary complexes of LADH is evidence against a Trp-314 or Tyr^+-286 energy-transfer process suggested previously to explain aspects of the fluorescence emission from these systems.[719]

A number of combined phosphorescence and optically detected magnetic resonance (ODMR) studies on protein systems have been published. The optical and magnetic triplet-state parameters of both buried and exposed tryptophan residues in a number of proteins have been compared.[720] Several correlations between the optical (wavelength maximum of the phosphorescence 0,0 vibronic

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band) and the magnetic properties (zero-field splittings) of the residues were tested. None of the correlations was found to be very strong but some general trends were apparent. An interesting feature of new measurements on RNAase T1 and phospholipase A_2 presented in the paper was the clear demonstration of hole burning in the ODMR transitions, which is indicative of heterogeneity in the local interactions of the residues. Phosphorescence-ODMR studies of kinase (C subunit), a protein for which there is no *X*-ray structure available, have revealed that there are two classes of tryptophan residues present.[721] The denaturation of this protein, and several others, was shown to shift the phosphorescence and ODMR parameters to those found in a number of randomly coiled polypeptides. Phosphorescence-ODMR studies of conformational changes in cowpea chlorotic mottle virus[722] and of the identity of the phosphorescent tryptophan residues in lysozyme[723] have also been published.

Tyrosine Fluorescence. A large increase in fluorescence from the single tyrosine residue in histone H1 has been observed as the protein folds from a random coil to a globular structure.[724] This observation and the significantly lower access of the tyrosine residue to external quenchers in the folded state indicate that it is buried and also that it does not belong to any of the groups of tyrosine residues included in a previously published classification of such residues. A number of examples of long-wavelength fluorescence from tyrosyl residues have been reported since the observations on two cytotoxins from cobra venom reported in Volume 12. The fluorescence emission maximum from cattle adrenodoxin, an Fe–S protein that contains no tryptophan but has one tyrosine residue, occurs at 331 nm in the native protein but shifts to 305 nm, the normal position of tyrosine fluorescence, when the protein is denatured.[725] The tyrosyl fluorescence from native human serum albumin, which contains 1 tryptophan and 18 tyrosine residues, has been found to be a mixture of two components, the normal emission with λ_{max} at 305 nm and a component with λ_{max} at 345 nm.[726] The longer-wavelength emission is believed to originate from excited tyrosinate ions formed by excited-state proton transfer from excited-state tyrosine to a local group such as a lysyl residue.

Tyrosine fluorescence from calmodulin, isolated from rabbit skeletal muscle phosphorylase kinase, has been used to monitor conformational changes induced in the protein by Ca^{2+} and Mg^{2+} ions,[727] and tyrosine fluorescence from troponin C has been used to determine the Ca^{2+}-ion binding constants of each of its four binding sites.[728] The tyrosine fluorescence properties of the small protein ubiquitin, found in chromatin, have been investigated.[729] The results indicate that there is a significant interaction of the tyrosine with a proximate carboxylate group. A salt-induced transition in chromatin itself has also been observed by

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monitoring of the anisotropy of the intrinsic tyrosine fluorescence.[730] The anomalous 331 nm tyrosine fluorescence from beef adrenodoxin mentioned above has been used in a study of the binding of this protein with horse-heart cytochrome *c*.[731] A very efficient energy-transfer process between the tyrosine and the cytochrome *c* haem was observed for the 1 : 1 complex formed between the two proteins. The nature of the nucleotide–protein interaction between DNA and the gene 5 protein of bacteriophage f1, which contains a fluorescent tyrosine residue, has been investigated,[732] and the slow-folding form of RNAase A, the U_s form, has been shown to contain two major subspecies from studies of the intrinsic tyrosine fluorescence.[733] Evidence for energy transfer between tyrosine and tryptophan residues in thermolysin has been obtained by comparing the emission spectra of the native and denatured forms of the enzyme,[734] and the tyrosyl fluorescence of human and bovine serum albumins was revealed by selective quenching of the tryptophan fluorescence with bilirubin, which binds to these two proteins.[735]

Other Chromophores. The appearance of visible fluorescence from u.v.-irradiated solutions of tryptophan, which was mentioned in a previous section,[631] has also been noticed in solutions of tryptophan-containing proteins.[736] The fluorescent photoproduct formed from yeast and liver alcohol dehydrogenase was found to have an emission maximum at 400 nm and excitation spectrum maxima at 293 and 350 nm.[737] Fluorescent derivatives were also observed on u.v. irradiation of bovine pancreatic RNAase, trypsin, chymotrypsin, histone, insulin, albumin, and concanavalin. Irradiation of active-site carboxymethylated D-glyceraldehyde-3-phosphate dehydrogenase in the presence of NAD produced a fluorophore with an emission maximum at 410 nm,[738] and an intense blue fluorescence is produced upon irradiation of calf-α-crystallin.[739]

A number of interesting observations of fluorescence from photoreceptor systems have been made recently. The visible fluorescence of metarhodopsin, the photoproduct of rhodopsin in which the retinal part of the chromophore is all *trans*, has been measured for the first time.[740] The quantum yield of the fluorescence, which has an emission maximum at 670 nm, is 1.6×10^{-3}. The fluorescence decay times of bovine rhodopsin and isorhodopsin have been found to be < 12 ps in a laser-excitation study,[741] and the formation of metarhodopsin III_{465} on irradiation of bovine photoreceptor disk membranes has been shown to be associated with an increase in the intrinsic protein fluorescence at 338 nm

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arising from tryptophan residues.[742] The Stokes shift of the red fluorescence emission from light-adapted purple membrane of *Halobacterium halobium* has been shown to depend on the intensity of excitation.[743] A previous value for the Stokes shift that was obtained in a laser-excitation study and was considered to be abnormally large reduces to a normal value when a conventional excitation source is used.

The chlorophyll *a*–protein complex from purified photosystem II has been found to contain two fluorescent components, F-685 and F-695,[744] and four components, each with different emission properties, have been found in chlorophyll–protein complexes isolated from *Euglena gracilis* membranes.[745] Evidence has been obtained for the fact that there is a common chromophore responsible for the emission properties of the green-fluorescent protein obtained from the bioluminescent coelenterates *Renilla reniformis* and *Aequorea aequorea*.[746] A blue-fluorescent peptide with an emission maximum at 430 nm was isolated from both proteins following heat denaturation and pronase digestion. Denaturation of *Aequorea aequorea* with guanidine–HCl or with acid, which results in a loss of the visible fluorescence, has been found to be completely reversible.[747] The photoreceptor chromophore of the large blue-green ciliate *Stentor coeruleus* has been identified as hypericin, the chromophore found in a number of photosensitive plants.[748] A number of papers have appeared detailing the spectral properties of phycobiliproteins isolated from phycobilisomes. Four spectrally distinguishable forms of allophycocyanins, *i.e.* I, II, III, and B, have been isolated from *Nostoc* blue-green algae,[749, 750] and the fluorescence-decay kinetics and quantum yields of these allophycocyanins, and of *c*-phycocyanin and *c*-phycoerythrin, also from *Nostoc*, have been investigated with picosecond laser techniques.[751] The spectral properties of *c*-phycoerythrin, and its α- and β-subunits, from *Pseudabaena* W1173 have been determined.[752, 753] There are five phycoerythrobilin chromophores present in the $\alpha\beta$-monomer, two of which are fluorescent, and the other three are sensitizers. Studies of phycobiliprotein fluorescence from *Fremyella diplosiphon*,[754] *Phomidium luridum*,[755] *Gloeocapsa alpicola* and *Anacystis nidulans*,[756] *Porphyridium cruentum* and *Cyanidium caldarium*,[757] and *Anabaena azollae*[758] have also been published. The picosecond

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fluorescence-decay kinetics and polarization anisotropy of anthocyanin pigments extracted from *Streptocarpus holstii* and *Anthurium andreanum* flower petals have been measured.[759]

Similarities between the luminescence properties of the CO complex of *Neurospora* tyrosinase [760] and the CO complexes of haemocyanins from *Helix pomatia* and *Panulirus interruptus* [761] have indicated a close structural relationship between the active sites of these classes of proteins. The copper metallothionein protein from *Neurospora crassa* displays an orange fluorescence following excitation at 305 nm,[762] and the quantum yield of the emission from the first excited singlet state of ferrocytochrome *c* following excitation into the Soret band was found to be 5.0×10^{-7}.[763, 596] The protein lumazine, obtained from the bioluminescent bacterium *Photobacterium phosphoreum*, has a luminescent prosthetic group 6,7-dimethyl-8-(1′-D-ribityl)lumazine that forms a 1:1 complex with the apoprotein.[764] The changes in the fluorescence properties associated with the binding process provide a convenient means to study the equilibrium. A fluorescent ligand has also been found in association with rat α_1-fetoprotein,[765] and visible fluorescence from the complexes of bilirubin with human and bovine serum albumins has been described.[766]

The proceedings of a symposium on flavins and flavoproteins have been published; they contain several papers dealing with the fluorescence properties of these systems.[767] The excited-state properties of blue-light photoreceptors, such as flavoproteins, have been reviewed.[768] The fluorescence decay of flavin in all pyruvate dehydrogenase complexes has been found to be dual exponential,[769] and the rotational correlation times, which were also measured, indicated that the lipoamide dehydrogenase was able to rotate within the multi-enzyme complex. The fluorescence decay of the coenzyme FAD of D-amino-acid oxidase has been measured in the monomer and dimer forms of the enzyme to be 130 and 40 ps, respectively,[770] and the luminescence properties of flavin coenzymes adsorbed on to BeO, SiO_2, and Al_2O_3 surfaces have been measured.[771] An intense fluorescence from UDP-glucose 4-epimerase from *Saccharomyces fragilis* has been attributed to protein-bound NAD.[772]

Phosphorescence emission from Zn cytochrome *c* has been observed,[773] and

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delayed fluorescence emission from phycobilins of *Grateloupia turuturu* has been measured.[774]

Extrinsic Probe Luminescence.—*Probe Characterization and Development.* A number of new fluorescent probes and fluorescent-probe techniques have been described in the literature. A selection of these are reported in this section; others can be found in the main sections that follow. The absolute diffusion coefficients of proteins can be rapidly determined using the fluorescence photobleaching recovery (FPR) technique in which the dissipation of a sharply defined region of photochemically bleached and previously fluorescent particles is measured by the time-dependent recovery of the fluorescence resulting from the diffusion into the region of unbleached fluorescent particles.[775] The FPR technique has also been used recently to monitor the polymerization of fluorescein-labelled actin induced by the addition of salt or Mg^{2+} to a solution of G-actin of low ionic strength.[776] The results obtained indicated that the actin was present either as a high polymer or as a monomer or low oligomer, and this is consistent with the 'treadmill' model of actin polymerization. Another interesting laser technique developed in recent years, fluorescence intensity fluctuation spectroscopy, a hybrid relaxation technique, has been used to study the action of muscle fibres.[777] Low-frequency (~ 2 Hz) components were observed in the fluorescence from rhodamine-labelled myosin subfragment 1 moieties in steady-state activity but not when they were in static states, such as relaxation or rigor, or when the label was attached to actin. The results show that tension development in muscle is associated with a repetitive rotational motion of the cross-bridges in agreement with the 'cycling cross-bridge' theory of muscle contraction.

Affinity and photoaffinity labelling techniques have achieved some importance in recent years. In the latter technique substrates of interest are attached to aryl azides that decompose on irradiation with near-u.v. light to give highly reactive nitrene intermediates. These can insert into C—H, O—H, and N—H bonds in an almost non-specific manner. Extremely useful probes of surface or active sites can be prepared by incorporating such a photoactivatable group into suitable fluorescent-labelled substrate molecules, and several examples of such probes have been described recently. The synthesis of 3-azido-2,7-naphthalene disulphonate (ANDS) has been described.[778] On photoactivation ANDS, which is itself non-fluorescent, reacts to form fluorescent products, and this behaviour makes it a potentially useful probe of hydrophilic surface sites in proteins and membranes. The use of an active-site label, 8-azido-1,N^6-ethanoadenosine 5′-triphosphate (8-N_3-ε-ATP), has been described in a study of the ATP-binding protein F_1-ATPase, obtained from *Micrococcus luteus*.[779] U.v. irradiation of the enzyme in the presence of the label and Mg^{2+} ions was found to inhibit the enzyme irreversibly, and subsequent analysis showed that the label was bound preferentially to the β-subunit. The preparation of a photoaffinity label based on anthranilic acid has

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been described.[780] The new probe, which becomes highly fluorescent following photoactivation, was evaluated in a study of the active sites of α-chymotrypsin and trypsin. The dipolar relaxation was found to be significantly greater in the deacylated enzyme compared with the crosslinked enzyme (see reference 642). The affinity labelling of rabbit-muscle pyruvate kinase with 5′-[*p*-(fluorosulphonyl)-benzoyl]-1,N^6-ethenoadenosine, a new fluorescent nucleotide alkylating agent, has been described.[781] The label was found to bind covalently at two different sites on the enzyme, and could be released from one of the sites, with the regaining of enzyme activity, by the addition of dithiothreitol or β-mercaptoethylamine, suggesting that the attachment involves a cysteine residue.

The electronic spectrum of the molecule 1-pyrenecarboxaldehyde is known to be very sensitive to environmental effects. A recent detailed investigation of the absorption and emission spectra as a function of solvent polarity, pH, and temperature has provided a basis for its wider use as a non-covalent fluorescence probe.[782] Just as for the carboxaldehyde derivative the fluorescence of pyrene itself is sensitive to the polarity of its environment. A recent study of the fine structure of pyrene fluorescence has shown that the ratio of the emission peaks at 373 and 384 nm is a sensitive measure of solvent polarity and may be used to extend the range of uses of this important membrane probe.[783] The binding properties of four homologous fluorescent carboxylate probes with bovine serum albumin have been investigated.[784] The new probes have spacer groups comprising 1—12 atoms between the carboxylate group and the fluorescent dansyl group. A less positive conclusion was reached in an investigation of the fluorescence-decay kinetics of the probe *N*-phenyl-1-naphthylamine using a phase-modulation technique.[785] In low-viscosity pure solvents the decay was found to be mono-exponential, but in a number of other solvent systems the decay kinetics were more complex and, more importantly, were found to be dependent on the emission wavelength. This change in behaviour is clear evidence for a significant dielectric relaxation process and/or the formation of excited-state complexes (see reference 642). This behaviour may be viewed as extending the usefulness of the probe but, as the authors point out, more characterization is necessary before it can be fully exploited. The antibiotic chlortetracycline is well known to be a fluorescent chelate probe for the divalent metal ions Ca^{2+} and Mg^{2+}. The pH dependence of chlortetracycline fluorescence emission has been investigated and pK_a values for the emission bands at 430 and 530 nm of 7.7 and 3.5, respectively, were found.[786] A detailed investigation of the photophysics of the once ubiquitous probe 1,8-anilinonaphthalene sulphonate (1,8-ANS) has been published.[787] It is proposed that the dominant non-radiative decay process is a one-photon photoionization to a solvent trap.

A number of fluorescent thiocholine esters that appear to have good potential

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for studies of enzyme function have been synthesized.[788] A detailed study of the enzyme-catalysed transfer of the acyl group from one of the esters, [dansyl-(*N*-methyl-β-alanyl)]thiocholine, to a number of amines and alcohols was carried out, and a pH-activity profile for the transfer to methanol suggests that a histidine residue, located in the active site, participates in the process. The fluorescent probe *N*-(3-pyrene)maleimide forms conjugates with bovine serum albumin that display non-exponential fluorescence decays and heterogeneous quenching effects.[789] This complex behaviour arises because the probe not only reacts with SH groups but is physically adsorbed on to hydrophobic areas of the protein. A further complication arises because some of the adsorbed probe is hydrolysed. A number of interesting cryptofluorescent analogues of cobalamin coenzymes have been prepared by reacting cob(I)alamin and alkyl halide derivatives of fluorescent adenosine analogues.[790] Unlike the alkali-halide derivatives the resulting cobalamin analogues do not fluoresce. This intramolecular quenching proves a useful means of following reactions that result in cleavage of the C—Co bond, release of the β-ligand, and the reappearance of the fluorescence. The reactions of a new class of fluorescent labels, bromobimanes, with human haemoglobin have been investigated.[791] The probes were found to react with the thiol site of cysteine-β93. A number of fluorescent pyrenyl organophosphates have been prepared and used to investigate ageing-induced conformational changes in inhibited acetylcholinesterase.[792] The organophosphoryl moiety in the aged conjugate was found to be less accessible to dynamic quenching from the external medium compared with the 'young' conjugate.

Covalent Probes. The addition of Mg^{2+} ions or KCl to solutions of the monomeric form of actin, G-actin, labelled with *N*-iodoactyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine, has been shown to increase the fluorescence emission of the probe.[793] The Mg^{2+}-ion-induced enhancement can be reversed by the addition of Ca^{2+}, and the competition of the two metal ions is evidently associated with reversible conformational changes in the protein. The labelling of actin with *N*-ethylmaleimide occurs predominantly at the cysteine-373 residue.[794] The labelling of *N*-ethylmaleimide actin itself with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole chloride (NBD-Cl), which reacts with lysine residues, was found to occur (60% of total NBD labelling) at the adjacent lysine-372 residue. The cofactor activity of actin for myosin ATPase was not diminished by labelling with NBD. It has been shown that the fluorescence probe 5-iodoacetamidofluorescein, which attaches to the cysteine-373 residue of actin, does not interfere with the polymerization process that leads to F-actin and that the labelled protein retains the ability to activate heavy meromyosin Mg^{2+}-ATPase.[795] An interesting series of

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papers on pig cardiac myosin have been published in which the myosin is labelled with *N*-methyl-2-anilino-6-naphthalenesulphonyl chloride (MNS-Cl). The label, which is preferentially attached to lysyl residues, was shown not to interfere with the Ca^{2+}- and K^{+}-ATPase activities of myosin.[796] The location of the probe in the myosin was found by identifying the fluorescent fragments released on chymotryptic digestion of the myosin.[797] This procedure showed that the labelling site was in peptides in the subfragment 1 (S-1)/subfragment 2 link region of the myosin heavy chains. The binding of actin to labelled myosin in the absence of ATP and in solutions of high ionic strength was found to enhance the fluorescence emission.[798] This effect and the observation that the presence of actin inhibited to some extent the labelling reaction itself suggest that the binding of actin may induce conformational changes in the label-binding region. The labelling was also shown to reduce the actin-activated ATPase activity of the myosin, but interestingly this could be avoided by protecting the reactive residues with Ca^{2+} during the labelling reaction, in which case a different lysyl residue was labelled.[799] The S-1 moiety of bovine cardiac myosin has been shown to have considerable segmental flexibility compared with the rod portion of the molecule in a study in which fast-reacting thiol residues were labelled with *N*-iodoacetylamino-1-naphthylamine-1-sulphonic acid.[800] A good example of the use of energy-transfer studies in protein systems is the successive labelling of skeletal-muscle myosin S-1 with a donor–acceptor pair of thiol-specific probes, *N*-[(iodoacetyl)aminoethyl]-5-naphthylamine-1-sulphonic acid and 5-(iodoacetamido)fluorescein.[801] One of the probes is used to label alkali 1 light chain, which is then incorporated into chymotryptic S-1 by an exchange procedure. The second probe is then introduced at the reactive thiol site on the heavy chain of S-1. Independently of the order in which the probes were introduced, a probe separation of ~ 4.0 nm was estimated from the observed energy-transfer efficiency, and this distance was found to be insensitive to the presence of MgATP and F-actin. Excimer emission has been observed from solutions of rabbit skeletal and cardiac tropomyosin labelled with *N*-(1-pyrene)maleimide,[802] and the binding of Ca^{2+} and Sr^{2+} ions to bovine heart troponin, which was isolated by a new procedure, has been investigated by use of the dimethylaminonaphtheurodine probe.[803] The Ca^{2+}-binding sites of cardiac troponin and troponin C have been investigated in a study in which the proteins are labelled with the thiol-specific probe 2-(4′-iodoacetamidoanilino)naphthalene-6-sulphonic acid,[804] and the kinetics of Ca^{2+} binding of troponin C have been investigated in a fluorescence stopped-flow study of *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide-labelled troponin C.[805] The authors of the last paper have also found, interestingly, that it is possible to monitor separately the effects of Ca^{2+} binding at the high-affinity or the low-affinity sites of troponin C

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by observing changes in the fluorescence from *N*-(1-anilinonaphthyl-4)maleimide- and dansylaziridine-labelled protein, respectively.[806] The effects of ageing on the structure of collagen isolated from rat-tail tendon have been investigated by observing differences in the fluorescence from the probe dansyl hydrazine, which reacts with aldehyde groups in the collagen.[807] The unsaturated aldehydes of aldol-condensation crosslinks in collagen can react with the fluorescent probe pyrenesulfonyl hydrazine.[808] Significant changes in the monomer and excimer fluorescence intensity from the probe(s) are observed during *in vitro* fibril formation of the labelled collagen. The equilibrium between two fibrinolysin fragments of ovine prolactin and the recombinant molecule has been studied by labelling of one of the fragments with fluorescein isothiocyanate and measurement of the resulting fluorescence polarization,[809] and fluorescamine-1 labelled brain-tubulin- and -microtubule-associated proteins have been prepared.[810]

There has been a detailed reinvestigation of the segmental flexibility of immunoglobulin G (IgG) antibody using fluorescence-anisotropy decay techniques.[615] The fluorescent hapten binds to the ends of the arms of the IgG and acts as a convenient monitor of individual-arm or whole-molecule motion. The new interpretation gives a significantly more flexible model for IgG. There has also been a redetermination, using energy-transfer techniques, of the distance between the antibody-binding sites in rabbit IgG.[811] An ambiguity was present in previous studies because the covalent bonds (disulphide) between the chains were broken in the labelling procedure and the minimum separation distance of 9 nm that was found was probably an overestimate. The study was carried out with a hybrid rabbit IgG containing one antilactose site, occupied by derivatives of (*p*-aminophenyl)-β-lactoside, and one anti-5-(dimethylamino)-1-naphthalenesulphonyl site. An almost complete reoxidation of light–heavy-chain interchains and of inter–heavy-chain disulphides was achieved, and the results showed the minimum separation distance to be in the range 5.5—7.0 nm, significantly less than in the earlier study. A related study, using energy-transfer techniques, of the spatial relationship of the C_H2 domains of mildly reduced and alkylated rabbit IgG has also been published.[812] The study demonstrated the importance of the single inter–heavy-chain disulphide bond to the structure of the native, functional F_C segment. The fluorescence properties of the dansylated derivatives of normal rabbit IgG antibody and purified rabbit IgG antibody of anti-2,4-dinitrophenyl specificity have been measured,[813] and a study of the non-covalent association of heavy and light chains of human IgG, in which the light chain was labelled with 5-iodoacetamidofluorescein at the *C*-terminal cysteine residue, has been published.[814] A study of the fluorescence depolarization of dansyl-labelled human-myeloma IgA and IgM has shown that their segmental flexibility is similar to that

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in IgG.[815] The HLA-A2 antigen from lymphoblastoid cell membranes has been purified and then labelled with *N*-[(iodoacetyl)aminoethyl]-6-naphthylamine-1-sulphonic acid (IAEDANS) in its intracellular region.[816] Experiments with the labelled antigen have shown that it is a useful probe of membrane protein–cytoskeletal interactions *in vitro*.

The activity of $(Na^+ + K^+)$-ATPase from *Electrophorus electricus* was progressively reduced as the enzyme was titrated with fluorescein mercuric acetate (FMA), a thiol-specific fluorescent probe.[817] The results are consistent with there being 4—5 reactive SH groups in the catalytic subunit and 1—2 reactive SH groups in the glycoprotein. A small number of groups could be protected from FMA by ATP or ouabain. A similar moderating effect of ouabain was observed for the inhibition of $(Na^+ + K^+)$-ATPase activity by Mg^{2+} ion[818] and for the changes in fluorescence of *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide-labelled $(K^+ + Na^+)$-ATPase (EC 3.6.1.3) from pig kidneys induced by Mg^{2+}, Na^+, and ATP.[819] Studies of conformational changes in $(Na^+ + K^+)$-ATPase induced by ions and ATP where the enzyme is labelled with fluorescein isothiocyanate,[820] and dimethylaminonaphthalene–cysteine–Hg^+,[821] and in Ca^{2+}-ATPase from sarcoplasmic reticulum induced by Mg^{2+} and ATP where the enzyme was labelled with *N*-(1-anilinonaphthyl-4)maleimide[822] have also been reported. The fluorescent AEDANS[823] and dansyl[824] derivatives of the Ca^{2+}-binding protein calmodulin have been prepared, and the application of fluorescent chelate probes to detect free Ca^{2+} has been illustrated in a study of sarcoplasmic reticulum vesicles using chlorotetracycline,[825] and it has been reviewed.[826]

The fluorescence properties of the conjugates formed by cowpea chlorotic mottle virus protein[827] and bovine serum albumin[828] with pyridoxal 5′-phosphate have been measured, and the lysine-binding site of the adenine nucleotide analogue *p*-fluorosulphonylbenzoyl-5′-adenosine with firefly luciferase has been identified.[829] Different forms of the fluorescent NAD derivative of D-glyceraldehyde-3-phosphate dehydrogenase have been compared by use of circular-dichroism spectroscopy.[830] The IAEDANS-RNAase A fluorescent derivative has been found to be enzymatically inactive, and this observation, and other evidence, indicates that the binding takes place at the active site.[831] The binding of yeast and

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E. coli $tRNA^{Phe}$ labelled with dansyl hydrazine or fluorescein thiosemicarbazide to ribosomes has been investigated,[832] and fluorescence-labelling techniques have also been used to investigate the location of the L7 and L12 proteins on the *E. coli* ribosome[833] and the binding of the initiation-factor 3—30S ribosomal subunits[834] and also to label the histone H3 in non-sheared chromatin.[835] The results of fluorescence-polarization studies of dansylated neurophysin have supported the results from other techniques by indicating that the protein has an elongated ellipsoidal shape and that it dimerizes in a side-by-side process.[836]

A temperature-induced intrinsic conformational change in *N*-(1-anilinonaphthyl-4)maleimide-labelled bovine-heart cytochrome oxidase has been observed in lecithin vesicle and solubilized systems,[837] and a study of the fluorescence decay in these systems showed it to be described by a triple exponential.[838] A very interesting paper has described the preparation of fluorescent derivatives of cytochrome *c* by replacing the haem Fe with closed-shell metals such as Zn or Sn.[839] The fluorescent derivatives bind to yeast lactate dehydrogenase (cytochrome b_2), making possible an energy transfer from the haem in the latter to the Fe-free porphyrin, thus providing an estimate of the inter-haem distance.

A new fluorescence-polarization high-pressure technique has been used to study the effects of hydrostatic pressure on lysozyme and chymotrypsinogen[840] (see reference 637). Lysozyme exhibited a stable, reversible, 60% increase in apparent volume as the pressure was raised to 9 kbar, and this can be understood as a solvent penetration of the protein structure. Additional evidence for there being a large distance between the lipoyl group of the pyruvate dehydrogenase complex from *E. coli* and the FAD of the lipoamide dehydrogenase subunit has been obtained in a study in which eosin maleimide was covalently attached to the lipoyl group, where it acts as an acceptor for the excited flavin moiety.[841] The distance between the active site and a peripheral anionic site in acetylcholinesterase has been determined in an energy-transfer study using either of two fluorescent phosphonates to label the active site and propidium to bind to the peripheral site, where it acts as the acceptor.[842] Studies of the effect of pH on the structural changes in pig-heart lactate dehydrogenase labelled with fluorescein isothiocyanate,[843] the photo-induced binding of bilirubin to serum albumin,[844] the absorption and emission spectra of fluorescamine-labelled lysozyme and peroxidase,[845] the temperature-dependent anisotropy of dansyl-labelled bacterio-

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rhodopsin,[846] the aggregation of rat-liver phosphofructokinase labelled with pyrenebutyric acid,[847] and the fluorescence anisotropy of a membrane glycoprotein labelled biologically with 16-(9-anthroyloxy)palmitate[848] have all been published.

Non-covalent Probes. The coverage in this section includes those investigations in which a natural coenzyme, ligand, *etc.* is covalently attached to a fluorescent probe or is itself converted to a fluorescent probe.

Fluorescence techniques have been used to investigate lectin–sugar interactions. The binding of the labelled sugar 3-methylumbelliferyl α-D-galactopyranose to the agglutinins from *Ricinus communis* (castor-bean)[849] and from *Abrus precatorius* (Jequirity beans)[850] results in both cases in a strong polarization of the fluorescence. The excellent agreement that was found between the binding parameters obtained from equilibrium dialysis and fluorescence-polarization measurements showed the potential of fluorescence techniques in this area. The binding kinetics of methyl α-D-mannopyranoside to the lectin concanavalin A have been investigated in a temperature-jump relaxation study in which the fluorescent 4-methylumbelliferyl-labelled sugar acts as an indicator,[851] and the binding kinetics of 4-methylumbelliferyl chito-oligosaccharides to wheatgerm agglutinin have been investigated by use of the same technique.[852] The lipolysis of human very-low-density lipoproteins (VLDL) has been followed by monitoring the fluorescence from dansyl phosphatidylethanolamine (DPE) incorporated into the VLDL.[853, 854] Human low-density lipoprotein (LDL) has been labelled with 3,3′-dioctadecylindocarbocyanine iodide, and the resulting fluorescent derivatives were used to study the binding of LDL–receptor complexes to human fibroplasts.[855] The binding sites of several fluorescent probe molecules on serum lipoproteins have been investigated by use of energy-transfer techniques.[856] A study of the binding of three cholinergic fluorescent derivatives of 1-(5-dimethylaminonaphthalene-1-sulphonamido)-R-trimethylammonium salts (where R is an ethyl, pentyl, or decyl group) to horse-serum cholinesterase has identified two types of binding sites,[857] and a kinetic study of the interaction of a related probe (where R is hexanoic acid) to the membrane-bound acetylcholine receptor from *Torpedo marmorata* has indicated that there are two binding sites for the agonist.[858]

A nice illustration of the chiral discrimination in the primary step of enzyme–ligand interaction is provided in a study of the quenching of the fluorescence of etheno-NAD bound to horse-liver alcohol dehydrogenase by

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methionine.[859] The rate constants for the fluorescence quenching by D- and L-methionine were found to differ by a factor of 3. The polarization of the fluorescence from 1 : N^6-etheno-ATP (ε-ATP) and -ADP (ε-ADP) and from 1 : N^6-ethenoimido-ATP (ε-AMPPNP) bound to the cross-bridges in glycerinated muscle fibre has been interpreted to give the orientation of the bound nucleotides to the fibre axis, and this knowledge has then been applied to give information about the conformation of the cross-bridges under different physiological conditions.[860] The nature of the ATP-binding site of heavy meromyosin has been studied by replacing ATP with its fluorescent analogue ε-ATP and measuring the quenching efficiencies of charged and neutral polar quenchers,[861] and the effect on the activity of membrane-bound adenylate cyclase of replacing the ATP substrate with its fluorescent analogue, formycin 5'-triphosphate, has been measured.[862] The binding of two fluorescent GTP analogues to the exchangeable nucleotide-binding sites of tubulin [863] and the association of gene 32 protein of bacteriophage T4 and the fluorescent polynucleotide poly(1,N^6-ethenoadenylic acid) [864] have also been investigated. It has been observed that the fluorescence from fluorescein bound to high-affinity rabbit and chicken IgG antibodies is significantly different in D_2O compared to normal aqueous solution.[865] The mechanism of the D_2O fluorescence enhancement has been discussed following a kinetic investigation of the effect.[866]

The fluorescence probe 8-anilinonaphthalene-1-sulphonate (ANS) has been used in studies of the heat denaturation of serum albumin,[867] the acid denaturation of bovine serum albumin,[868] the non-polar binding sites on native bovine thyroglobulin,[869] the differences in binding properties of ricin A and B chains,[870] the location of the ANS-binding site in Carlsberg subtilisin,[871] the transition from the low-pH to the high-pH forms of IgG,[872] the exposed hydrophobic regions in histone oligomers and histone H1,[873] the ANS-binding sites in α-fetoprotein,[874] and the hydrophobic binding sites of phytochrome [875] and cytochrome P-450.[876]

The interaction of benzo[*a*]pyrene with bovine serum albumin results in a complicated fluorescence behaviour which in part is derived from a number of

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photoproducts formed on u.v. irradiation of the complex.[877] Evidence from a study of the fluorescence of the anticoagulant drug warfarin bound to human serum albumin has supported the existence of an N–B conformational transition in the protein,[878] and it has been observed that the fluorescence probe 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS) bound to pepsin is displaced by *Streptomyces* peptin inhibitor.[879] The anisotropy decay rate of the fluorescence from ethidium bound to the high-affinity sites of nucleosome DNA was found to be significantly greater for a given fraction of labelling than in the case of ethidium bound to naked DNA.[880] This implies that the high-affinity sites are clustered on a short nucleosomal DNA segment. An interesting study has been published of the c.d. and circularly polarized luminescence of bilirubin bound to human serum albumin.[881] Effects of both pH and NaCl concentration on the ground- or excited-state conformations of bilirubin were observed. The microtubule inhibitor colchicine is non-fluorescent except at very low temperatures, but it forms a fluorescent complex with tubulin. A study of the quenching of the fluorescence from this complex has indicated that there is an anion-binding site in the vicinity of the bound colchicine.[882] The fluorescent probe 5-chloropyrimidin-2-one binds to horse-liver alcohol dehydrogenase as a competitive inhibitor, expelling the coenzyme NAD or NADH.[883] The changes in its fluorescence properties on binding parallel those observed in less polar solvents, suggesting that it attaches to a hydrophobic site in the enzyme. The irradiation of active-site carboxymethylated glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* in the presence of NAD gives a fluorescent product similar to that obtained from muscle enzyme[884] (see reference 738). The kinetics of the binding of anthroyl-ouabain, a fluorescent derivative of the glycoside ouabain, to rabbit kidney and eel electroplax (Na^+,K^+)-ATPase have been studied.[885] The effects of the pH and the concentrations of ATP, phosphate, Mg^{2+}, Mn^{2+}, and Co^{2+} on the kinetics of the binding were also considered. The fluorescence of eosin was found to be practically unchanged when it was bound to ($Na^+ + K^+$)-ATPase in the presence of K^+ but to be significantly altered when it bound to the enzyme in the presence of Na^+, an observation which illustrates the different conformational changes induced by these two ions.[886]

There is a strong current interest in the use of lanthanide-ion luminescence probes. The luminescence can be stimulated either by energy transfer from other excited chromophores or by direct excitation of the luminescent state. The measurement of europium(III) luminescence decay as a function of excitation wavelength has been shown to reveal differences in the environments of the Eu^{3+} ions, and this clearly offers some interesting possibilities for experiments in

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macromolecular systems.[887] The luminescence was excited with a tunable dye laser. The u.v. excitation spectra of a number of aromatic chromophores that transfer their energy to nearby terbium(III) ions, which in turn emit a green phosphorescence, have been measured.[888]

The application of laser-excited lanthanide-ion luminescence to studies of calcium-binding proteins has been briefly discussed.[889] The laser-excited luminescence-decay times of Eu^{3+} and Tb^{3+} ions bound to the Ca^{2+}-binding sites of thermolysin and carp parvalbumin have been measured when other lanthanide(III) ions occupy other Ca^{2+}-binding sites.[890] The results showed that there was efficient energy transfer between Tb^{3+} and Pr^{3+}, Nd^{3+}, Ho^{3+}, and Er^{3+} and between Eu^{3+} and Pr^{3+} and Nd^{3+}. The interionic distances calculated were in good agreement with *X*-ray crystallographic data. The laser-excitation technique has revealed differences in the spectral features of Eu^{3+} in the three Ca^{2+}-binding sites of thermolysin[891] and the two different Ca^{2+}-binding sites in parvalbumin.[892] The energy-transfer efficiencies for tryptophan-sensitized Tb^{3+} and Eu^{3+} emission from labelled cod parvalbumin have been measured.[893] The donor–acceptor separation distances estimated from the energy-transfer efficiencies were again in good agreement with available *X*-ray data. Studies of Tb^{3+} emission from Ca^{2+}-binding sites in parvalbumin,[894] calmodulin,[895,896] actin,[897] and trypsin and α-chymotrypsin[898] have been published.

Energy-transfer experiments between Eu^{3+} and Co^{2+} ions in the two metal-binding sites of bovine UDP–galactose–acetylglucosamine galactosyltransferase have indicated that the distance between the two sites is 1.8 nm.[899] Tb^{3+} luminescence from the single Ca^{2+}-binding site in porcine pancreatic elastase has been shown to be excited by energy transfer from a tryptophan residue[900] and has been used to determine the effect on the Tb^{3+}-binding constant of changes in pH, temperature, and the presence of various inhibitors.[901] The binding of Eu^{3+} to bovine prothrombin fragment 1[902] and to other γ-carboxyglutamate-containing peptides and proteins[903] has been investigated, as has the binding of Tb^{3+}

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to *Limulus polyphemus* haemocyanin,[904] angiotensin II,[905] and bleomycin antibiotics.[906] The excitation spectra of Tb^{3+} luminescence from nucleic acid bases have been measured,[907] and a number of dyes with narrow emission spectra have been prepared that contain lanthanide ions for applications in cytology.[908] The first of these dyes to be prepared were 5-isothiocyano-1,10-phenanthroline tris(trifluoro-2-furfurylacetylacetonato) complexes of Eu^{3+} and Tb^{3+}.

Probe Phosphorescence. An interesting study has described the observation of phosphorescent emission from room-temperature, anaerobic solutions of bovine serum albumin labelled with eosin and erythrosin.[909] The phosphorescence of the dyes was excited with a pulsed dye laser to make it easier to discriminate against the fluorescence emission, although in the case of erythrosin it was even possible to monitor the phosphorescence signal separately whilst using steady-state excitation. A rotational correlation time of 111 μs has been measured for cytochrome P-450 reconstituted into lipid vesicles by measurement of the depolarization of the delayed fluorescence from an eosin label.[910] The lifetimes of the phosphorescent emissions from eosin-labelled haemoglobins and myoglobin have been measured.[911] The slow rotational motions of proteins in viscous media and of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes have been obtained by measurement of the phosphorescence depolarization of an erythrosin probe,[912] and the preparation of the dye–protein conjugates and of the erythrosin isothiocyanate label itself has been described.[913] The erythrosin-label technique has also been used to study the rotational mobility of the membrane-bound acetylcholine receptor of *Torpedo marmorata* electric organ.[914] The use of benzophenone as a room-temperature aqueous-solution phosphorescent probe has been described in a study where it is bound covalently in the acyl linkage at the active site of chymotrypsin.[915]

6 Circular Dichroism

Contributed by T. Brittain

General.—*Reviews.* As in previous years, a wide range of reviews concerned with the general field of application of c.d. methods has appeared.[916–923]

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Theory and Analysis. In a series of papers Rabenold has developed a Green operator technique, used within a susceptibility framework, to describe the c.d. of unordered and helical polypeptides in general and partially and fully oriented helical polymers in particular. In the case of unordered systems ground-state wavefunction corrections have been included, and two limiting cases of the formulation have been identified as the matrix theory of c.d. and a generalization of the rotational strength expression has been derived by Tinoco.[924] The extension of this work to long helical polypeptide c.d. has allowed the correlation of specific bonds with their associated electronic transitions and the interactions between the various transitions.[925] In partially oriented helical polymers the dichroism has been shown to arise from the direction of orientation. For polypeptides a theoretical foundation for the resolution of oriented c.d. into component bands is given.[926] Further application of this theory to the problem of light propagating perpendicular to the helix axes of oriented helical polymers predicts a non-linear mixture of c.d. and linear dichroism, together with birefringence.[927] The c.d. of large molecules has also been presented in relation to the theory of Rosenfeld, and equations have been presented for simple model systems, which illustrate the failure of the dipole approximation.[928] The general aspects of the phenomenon whereby c.d. is induced in the transitions of an achiral species due to dispersive coupling to a chiral species have been discussed. A complete derivation of these contributions has been presented, which includes previously neglected coupled-oscillator terms.[929]

C.d. spectra and oscillator strengths of the π–π^* transition near 190 nm have been calculated for helical $(Gly)_6$ and $(Ala)_6$, using the partially dispersive normal mode treatment of the dipole interaction model. Calculations for $(Ala)_6$ at various torsion angles corresponding to the α-helix, poly(Pro) II helix, a collagen single helix, a poly(Me-Ala) helix, and single β-helix agree well with the available experimental data.[930]

Using c.d. spectra, out to 178 nm, from proteins with known secondary structure as a base set, it is possible to obtain five independent pieces of information, concerning the secondary structure of an unknown protein. Using this information, it has proved possible to predict the secondary structure of an unknown protein in terms of helix, parallel and antiparallel β-strand, and types I, II, and III β-turn and other β-turns combined.[931] The utility of c.d. measurements in the vacuum ultraviolet region (164—255 nm) has also been highlighted in another paper, which compares calculated and experimental spectra for a number

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of proteins.[932] In a number of other studies the secondary structures of proteins predicted by a linear combination of independent structural components have been compared with experimental observations. Satisfactory agreement was obtained for all the proteins studied except concanavalin A.[933-935]

Simple formulae for the frequency difference between absorption and c.d. maxima have been obtained, in terms of electron–vibration interaction parameters, for the case in which one specific vibrational mode is of importance.[936]

Instrumental. Advances in the design of computer-controlled, vacuum ultraviolet c.d. spectrometers, with Fourier digital data smoothing, have been described.[937,938] A new c.d. and m.c.d. system, utilizing an acoustic filter, has appeared. This apparatus performs successfully at a scan rate of 50 nm $10\,ms^{-1}$, with better spectral resolution and higher signal-to-noise ratio than other commercially available spectropolarimeters.[939] Several applications have been given for this machine, and the general field of fast kinetic studies employing c.d. detection has been discussed.[940] C.d. has been used as the method of detection for high-performance liquid chromatography of a number of compounds, including tryptophan, in the microgram range.[941] An apparatus that yields absolute c.d. data directly has been described. The data sometimes differ considerably from those in the literature. The results obtained from this instrument have led to the establishment of a procedure used in the calibration of commercial instruments.[942]

A Fourier-transform i.r. spectrometer, designed around a polarizing Michelson interferometer, appears to offer a previously unobtainable wavelength range. By the use of a metal-grid polarizer of $\sim 0.1\ \mu m$ spacing, on a KBr substrate, the instrument should be capable of covering the spectral range 25 000—$3\,cm^{-1}$.[943]

A technique and appropriate cuvette have been described for c.d. measurements of oriented molecules in a uniaxial system, whereby the light beam propagates parallel to the optical axis. Any defects of the sample, resulting from the anisotropic behaviour of the oriented liquid-crystal matrix, are avoided.[944]

Small Molecules, Model Compounds, and Synthetic Polymers.—*Amino-acids and Derivatives.* The use of dinitrophenyl derivatives of both α- and β-amino-acids has proved to be of value in assigning absolute configuration. The Cotton effects near 400 nm have been measured for a series of derivatives and the results described in terms of exciton chirality theory.[945,946] *N*-Dithioethoxycarbonyl derivatives of L-

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β-homo-Ala, L-β-homo-Leu, and L-β-homo-Glu have been investigated by means of u.v. c.d. It has been shown that a negative Cotton effect in the 330—350 nm region correlates with an L-configuration of the β-amino-acid.[947] In an analogous study of the free *N*-carboxymethyl acids and the dicyclohexylamine salts of the *N*-ethylthiocarbonothioyl derivatives of Ala, Asp, Leu, Phe, Pro, Ser, Thr, tert-Leu, DL-tert-Leu, Tyr, Val, and Lys it was found that a positive Cotton effect at 200—225 nm and one at 335—350 nm correlate with the L-configuration of the α-amino-acids.[948]

As in previous years a great deal of attention has been devoted to metal–amino-acid complexes. It has been shown that the (amino-acidato)bis(ethylenediamine)-chromium(III) complexes of L-Ala and L-Phe exist as two pairs of diastereomeric, not enantiomeric, isomers. The configurational and vicinal contributions to the optical activity, in the region of the *d–d* transitions, have been shown to be additive, and the peaks in the spin-forbidden band have been assigned.[949] The absolute configuration of the first example of an uncharged di-μ-hydroxo dimer complex in solution [Δ(−)546-di-μ-hydroxo-(*SSSS*)-tetrakis-(L-prolinato)-dichromium(III) tetrahydrate] has been reported.[950]

The residual c.d. of complexes, that is the optical activity not accounted for by the additivity of the *d–d* c.d., of tetra-aminecobalt(III) amino-acids has been shown to be a sensitive probe for inter-ligand steric effects.[951] However, in the case of a series of triethylenetetra-aminecobalt(III) amino-acid complexes the observed c.d. appears to be accounted for completely by the additivity of the *d–d* transitions.[952] The c.d. spectra of the resolved isomers of a new complex $[Co(NO_2)(Gly)(NH_3)_3]Cl$ have been measured.[953] The c.d. of over twenty Cu^{II} complexes, with various chiral amino-acids and mixed complexes with amines, has been recorded and discussed in relation to the observed absorption characteristics of these compounds.[954–959]

Band signs in the c.d. spectra of Pt complexes of *cis*- and *trans*-$[Pt(ZH)_2Cl_2]$(ZH = L-Ala, L-Val, L-isoLeu, L-norVal, or D-Leu) in the *d–d* region appear to be determined by both ligand absolute configuration and the geometric configuration of the complexes.[955]

In ternary complexes of the form Pd(L–A)(L–B) (A = L- or D-Asp, L- or D-Glu, or L-Cys; B = Arg or Lys) it has been established that the c.d. magnitudes in the *d–d* region are smaller than those estimated from $Pd(L–A)_2$ and $Pd(L–BH)_2$ but close to those without the possibility of ligand–ligand interactions, such as Pd(L–A)(L-Ala). This supports the absence of such ligand interactions in the former case.[956]

On going from pH 5.5 to pH 0.5 the c.d. maxima of selenocystine move from 320, 272, 232, 211, and 200 nm to 306, 241, 225, and 207 nm, respectively, with the

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two Cotton effects of opposite sign at 320 and 272 nm collapsing into a single band at 306 nm, close to the absorption maximum of the longest-wavelength band in the u.v. The changes in the Cotton effects have been assigned to alterations in dihedral angle, while the $n \rightarrow \pi^*$ transition of the carbonyl function is associated with the 225 nm band. The other bands in the spectrum have therefore been considered to arise from optically active transitions of the Se–Se chromophore.[957]

Dipeptides and Oligopeptides. cyclo(L-Ala-Gly-ε-aminocaproyl) exhibits the c.d. activity expected for a system that is predominantly in a type II β-bend conformation, with smaller quantities of type I and II bend conformations also present. The relative proportions of each type present are dependent on the solvent and temperature employed. This compound thus represents a good model for the β-bend of proteins.[958] In the solid state monodispersed Me_3CO_2C-[Cys(ME)]$_n$-OMe (n = 4—7) takes up a β-conformation.[959] This result has been compared with earlier c.d. studies on related norVal and Met series.

The Fe^{III} complex of Z-Cys-Ala-Ala-Cys-OMe represents a good model for the oxidized centre of rubredoxin, having c.d. characteristics very similar to those obtained for the native protein. The model has a similar electronic structure and is present, probably, as a relatively stable chelate with a hairpin-turn conformation of the tetrapeptide.[960]

Metalloprotein active sites have also been modelled with Pd complexes of tetrapeptides containing two Cys residues. The observed *SS*-chelation depends on the conformational fitness, which is controlled by the steric effects of the two amino-acids present between the two Cys residues.[961]

The ΔH and ΔS values, characterizing the conformational equilibrium of the His side chains of H-Gly-His-OH and H-His-Gly-OH, have been obtained by c.d. measurements in the region 200—320 K. The data obtained in basic solution converged with theoretical energy calculations for the most stable conformation in solution.[962]

The position and intensity of the positive Cotton effects, in the 250—200 nm region, of the c.d. of H-(X)$_n$-OMe (X = Ala, norVal, norLeu, Ile, Leu, Phe, Cys, or Met; n = 3—6) have been measured to determine the effect of lateral chains on the c.d. of protonated peptides. The intensity of the Cotton effect increased with an increase in the number of CH_2 groups in a series having linear, β-branched, and γ-branched alkyl lateral chains and on going from a series of linear to a γ- and β-branched chain. The influence of the chirality of the γ-carbon in a series was minor. It is therefore concluded that both the acidophobic interactions and the inherent conformational rigidity of the main chain play a major role in the c.d. of protonated peptides.[963]

The c.d. spectra of Z-X-OH, Z-Gly-X-OEt, Z-X-Gly-OEt, and Z-X-X-OEt (X = L- or D-Ala, -Val, or -Leu; Z = $PhCH_2O_2C$) have been measured in the

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region 200—260 nm. The dipeptides of Gly and another amino-acid were monodispersed, whilst Z-Gly-L-Ala-OEt and Z-L-Ala-Gly-OEt showed different wavelength maxima and opposite-sign Cotton effects. The diastereomers exhibited characteristically distinguishable c.d.[964]

The similarity of the c.d. spectra of [Cr(L- or D-Asp)(L-His)] to those of [Co(L- or D-Asp)(L-His)] has permitted a tentative structural assignment for three isomers of $[Cr(L\text{-}Asp)_2]$ complexes. The observed isomer distribution suggests that in these complexes the amino groups avoid being *trans* to each other, as previously reported for the Co complexes.[965]

Polypeptides. In solvents promoting β-structure poly(*S*-benzyl-L-Cys), poly(*S*-carbobenzoxy-L-Cys), poly(*O*-acetyl-L-Ser), and poly(*O*-carbobenzoxy-L-Ser) show c.d. troughs in the n–π^* region and similar spectra when cast in films.[966]

H-$[Glu\text{-}Tyr]_n$-OH, H-$[Glu\text{-}Tyr_3]_n$-OH, and H-$[Glu_5\text{-}Tyr]$-OH show hydrolytic activity towards *p*-nitrophenyl acetate only when in a random conformation, as judged by c.d. measurements. The appearance of α-helix and β-structure as the solution pH is changed is mirrored closely by the disappearance of esterase activity.[967] $(Ala\text{-}Orn\text{-}Orn)_n$ and $(Ala\text{-}Orn\text{-}Lys)_n$ possess an extended left spiral conformation, similar to that of the terminal fragments of histones, for which they have been proposed as model systems.[968]

An interesting photoinduced isomerization has been reported for copolymers of H-Asp-(OCH_2Ph)-OH and β-(*m*-phenylazo)benzyl-L-Asp in $ClCH_2CH_2Cl$–$(MeO)_3PO$ solvents. Dark-adapted *trans* copolymers were found to be left-handed helices, which converted to the right-handed form when the amount of $(MeO)_3PO$ in the solvent was increased. Photoisomerization of the azobenzene side chains on the copolymers caused helix reversal, concurrent with the reversal of the azobenzene c.d. band signs in the n–π^* and π–π^* regions.[969]

Isotropic and nematic liquid-crystal solutions of poly(L-Glu) and poly(D-Glu) in aprotic solvents induce c.d. activity in added Acridine Orange at 470 and 500 nm, which is negative for the D-form and positive for the L-form. Solutions of the D-polymer in the cholesteric liquid-crystal phase show higher-intensity c.d. at 490 nm than the bands observed in the other two phases, as a result of dis-symmetric fields. The nature of the c.d. observed in each phase has been associated with a particular helical sense.[970]

Poly(L-Lys) undergoes a coil-to-helix transition at high sodium octyl sulphate concentrations, with a rate of $\sim 2.7 \times 10^{-1}\,s^{-1}$, as determined by stopped-flow c.d. The α-helix conformation is stable at 4—6 M detergent concentration, but shifts to a β-conformation at higher concentrations; the random-coil conformation, however, is stable below this value.[971] The β-coil transition has been followed by c.d. measurements for poly(*S*-carboxyethyl-L-Cys) in the absence of added salts and was found to correspond well with data obtained from viscosity

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[968] R. R. Kamilova, E. I. Ramm, and V. K. Burichenko, *Khim. Prir. Soedin.*, 1981, **2**, 206.
[969] A. Ueno, K. Takahashi, J. Anzai, and T. Osa, *J. Am. Chem. Soc.*, 1981, **103**, 6410.
[970] K. Ohki, Y. Kondo, T. Fujii, and E. Iizuka, *Kobunshi Ronbunshu*, 1981, **38**, 493.
[971] K. Takeda, A. Iba, and K. Shirahama, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 1793.

and titration measurements. In the case of poly(*S*-carboxymethyl-L-Cys), however, marked discrepancies were apparent between the various techniques.[972] The normal α-helical conformation of poly(Glu) in aqueous solution at pH 5.6 is converted completely to the random coil in the presence of oligoamines.[973]

The asymmetric placement of the side-chain phenyl rings in poly(*S*-benzyl-L-Cys), poly(*S*-carbobenzyl-L-Cys), and poly(*O*-carbobenzoxy-L-Ser), when the polypeptides take up an antiparallel β-form, has been established from c.d. data.[974] Long-range ordering of carbazole rings is absent in films of poly{*S*-[2-(9-carbazoyl)ethyl]-L-Cys}, while short-range order seems probable.[975]

Poly(L-Glu), when it is present in the helical form, adsorbs Cu owing to chelation of the metal atom by side-chain CO_2H groups. This complex formation has been compared with that observed with Cu–poly(acrylic acid) complexes.[976] Further quantitation of this process has shown that at pH 5.5 a binuclear complex is formed at a 10 : 1 monomer : Cu ratio. At lower ratios a mononuclear complex is favoured.[977] Vibrational c.d. spectra have been reported for solutions of the L- and D-isomers of poly(γ-benzyl-Glu) in $CHCl_3$ and $CDCl_3$, in the region 1000 cm^{-1} to 2000 cm^{-1}.[978]

Proteins.—*Non-chromophoric Proteins*. Soybean β-amylase exhibits a positive c.d. band at 192 nm, a negative band at 222 nm, and a shoulder at 210 nm. Analysis of the c.d. spectrum indicates the presence of ~ 30% α-helix and 5—10% β-pleated sheet. Positive bands at 292 and 297 nm, which are unaffected by acylation, have been assigned to tryptophan chromophores and have been suggested as being part of the binding site for maltose. 6M guanidine–HCl completely disrupts the protein secondary structure; sodium dodecyl sulphate, however, alters the tertiary but not the secondary structure of the protein. The uniquely folded structure of this enzyme is viewed as containing some mobile domains, together with a rigid core stabilized by hydrophobic interactions.[979]

The c.d. spectrum of trypsin inhibitor, in various states, has highlighted the necessity of correct S—S bond formation for the creation of the fully native conformation of the protein. The fully reduced protein with no S—S bonds exists as a flexible polypeptide chain. In the case of both the S—S intermediates lacking the 14—38 S—S bond and the protein containing the three correct S—S bonds the refolded protein c.d. was slightly different from that of the native molecule. This slight difference was removed on gentle heating. The near-u.v. c.d. has been resolved into Tyr, S—S, and Phe contributions; resolution of the far-u.v. c.d. into contributions from secondary structures, however, has not been successful.[980]

C.d. data have appeared for a number of protein toxins. Pardaxin, the toxin of the Red Sea flatfish *Pardachirus marmoratus*, shows 23% α-helix, 21% β-structure,

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[973] M. K. Pal, V. Boehmer, and W. Kern, *Macromol. Chem.*, 1981, **182**, 1649.
[974] K. Raghavendra and V. S. Ananthanarayanan, *Int. J. Pept. Protein Res.*, 1981, **17**, 420.
[975] R. Oshima and J. Kumanotani, *Polym. Prepr., Am. Chem. Soc., Div. Polym. Chem.*, 1979, **20**, 522.
[976] M. Koide, E. Tsuchida, and Y. Kurimura, *Macromol. Chem. Phys.*, 1981, **182**, 359.
[977] M. Koide, E. Tsuchida, and Y. Kurimura, *Macromol. Chem. Phys.*, 1981, **182**, 367.
[978] R. D. Singh and T. A. Keiderling, *Biopolymers*, 1981, **20**, 237.
[979] E. Mori, B. Mikami, Y. Morita, and B. Jirgensons, *Arch. Biochem. Biophys.*, 1981, **211**, 382.
[980] P. A. Kosen, T. E. Creighton, and E. R. Blout, *Biochemistry*, 1981, **20**, 5744.

and 56% random coil with extensive reversible loss of α-helix at extreme pH. No evidence for salt-induced conformational perturbation was detected.[981]

Although there is evidence that the toxins from the Latin American scorpions *Centruroides noxious*, *Centruroides elegans*, and *Tityus serrulatus* and snake toxins contain extended β-sheet structure, the scorpion toxins show spectra of an overall different type. Amongst the scorpion toxins those obtained from *Tityus*, although globular, are markedly less stable and rigid than those obtained from *Centruroid* species.[982] The bee-venom peptide 401 shows intense c.d. in the 200—230 nm region, associated with the rigid asymmetric ring structure of the S—S bridges. pH, solvent, salts, and temperature were found to have modest, if any, effects on the c.d., although reduction of the S—S bridges shows a marked effect, leading to the formation of a random-coil conformation.[983]

In aqueous solution bovine myelin basic protein has a random-coil conformation, which is partially transformed to α-helix in the presence of lipids. The extent of the transformation decreases in the order phosphatidylglycerol, phosphatidic acid = phosphatidylserine, phosphatidylcholine = phosphatidylethanolamine.[984]

C.d. spectroscopy has provided evidence for differences in heat stability of serum albumin from mice of different ages. The authors suggest no reason for these differences and appear to have overlooked the known glycosylation process of this protein.[985] The enthalpies of interaction of a series of alkylureas with human serum albumin have been measured and have shown that the transfer of the protein from aqueous solution to alkylurea solution is an endothermic process, in contrast to that found for urea. Far-u.v. c.d. measurements indicate that the alkylureas are much less efficient in the denaturation of the protein than is urea.[986]

Spectral observations on human-placental alkaline phosphatase have clearly defined the structural roles played by a number of ligands. The competitive inhibitor, inorganic phosphate, induces a conformational change in the enzyme that alters the active site. The uncompetitive inhibitor, D-Phe, produces a conformational alteration consistent with the proposed prevention of dissociation of the phosphoryl enzyme intermediate, whereas the enzyme activator Mg^{2+} causes a slight separation of the subunits, so exposing the active site.[987]

Trypsin shows no evidence of any conformational change accompanying the binding of either Bowman–Birk soybean inhibitor or chickpea inhibitor. Chymotrypsin–inhibitor complexes, on the other hand, show minor tertiary-structure changes, as evidenced by a c.d. shift between 250 and 320 nm.[988]

Peptide analogues, corresponding to the phosphorylation site of pyruvate kinase, exhibit minima at 225 and 230 nm, indicative of a β-turn conformation.

[981] N. Primor and A. T. Tu, *Biochim. Biophys. Acta*, 1980, **626**, 299.
[982] L. Possani, W. E. Steinmetz, A. R. M. Dent, A. C. Alogon, and K. Wüthrich, *Biochim. Biophys. Acta*, 1981, **669**, 183.
[983] P. Walde, H. Jaeckle, P. L. Luisi, C. J. Dempsey, and B. E. C. Banks, *Biopolymers*, 1981, **20**, 373.
[984] M. A. Keniry and R. Smith, *Biochim. Biophys. Acta*, 1981, **668**, 107.
[985] D. J. Schofield, *Exp. Gerontol.*, 1980, **15**, 533.
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[987] J. B. Orenberg, J. M. Schaffert, and H. H. Sussman, *Arch. Biochem. Biophys.*, 1981, **211**, 327.
[988] M. D. Jibson, Y. Birk, and T. A. Bewley, *Int. J. Pept. Protein Res.*, 1981, **18**, 26.

Phosphoprotein phosphatase-inhibitor peptide analogues showed variable c.d., with no clear correlation between kinetic and spectral properties.[989]

Native egg-white ovomucoid exhibits 26% α-helix, 46% β-structure, 10% β-turn, and 18% random coil, whereas in the reduced protein these figures are 16, 34, 20, and 30%, respectively, and for the re-oxidized protein they are 23, 48, 8, and 21%, respectively. Trypsin-inhibitor activity of the protein was found to be dependent on S—S bond integrity and closely followed alteration in the secondary structure induced by reduction. Almost all of the inhibitory activity of the protein appeared to be located in protease fragment A, which contains 11 Cys.[990] The secondary structure of ovomucoid has been further studied by c.d. and its spectrum decomposed into the contributions from its various structural domains.[991]

The well resolved c.d. spectra obtained for the light-chain derivatives of murine MOPC-315 immunoglobulin A has allowed the assignment of bands above 290 nm to Trp-35L and Trp-91L in the variable domains and Try-148L, Trp-185L, and Cys-214L in the constant domains. Significant differences have been identified between the light-chain derivatives in the absence and presence of heavy chains. Conformational transitions induced by hapten binding have also been demonstrated.[992]

The relative proportions of amino-acid residues in different types of structure were the same in native tryptophanyl-tRNA-synthetase, the Zn-free apoprotein, and the 80000 molecular-weight dimer, whereas the 24500 + 14000 molecular-weight pseudo-dimer showed a decreased amount of α-helix. Near-u.v. studies indicate the requirement of Zn and the presence of all the monomers for long-term stability of the enzyme.[993] C.d. measurements on a synthetic *N*-terminal tridecapeptide sequence of human fibroblast interferon indicate an α-helical conformation in trifluoroethanol.[994]

A set of idealized c.d. spectra have been obtained from a set of proteins of known structure. These spectra, when used in secondary-structure calculations, give better agreement with *X*-ray data than those reference spectra due to Chang.[995]

Perturbation of Kunitz soybean trypsin inhibitor by sodium dodecyl sulphate appears to occur at a low molar ratio of bound detergent. The backbone of the polypeptide moves towards a helical state in a co-operative manner as the detergent concentration increases, being complete before the critical micelle concentration is achieved.[996] Significant individual differences have been observed between various lectins, with regard to their resistance towards alkaline denaturation. C.d. bands at 270—300 nm due to Tyr and Trp, although generally

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[990] T. Matsuda, K. Watanabe, and Y. Sato, *FEBS Lett.*, 1981, **124**, 185.
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[992] R. Zidovestshi, O. Farver, and I. Pecht. *Eur. J. Biochem.*, 1981, **114**, 97.
[993] M. K. Nurbekov, I. Bolotina, V. Jugauskas, and O. O. Favorova, *Dokl. Akad. Nauk SSSR*, 1980, **255**, 482.
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[995] I. A. Bolotina, V. O. Chekhov, V. Lugauskas, and O. B. Ptitsyn, *Mol. Biol. (Engl. Transl.)*, 1981, **15**, 130.
[996] E. Mori and B. Jirgensons, *Biochemistry*, 1981, **20**, 1630.

diminished by alkaline conditions, in some cases actually increase in intensity.[997] A novel transient trough near 230 nm, observed during the detergent denaturation of pea lectin and concanavalin A, has been tentatively assigned to β-turns, which are retained in the intermediate product.[998]

Comparison of the peptide c.d. of antifreeze glycoprotein with model peptides suggests the presence of a three-fold left-handed helix of the collagen type as the most likely conformation of the glycoprotein. In this model, which accommodates the Pro residues present, the disaccharide groups are attached to every third residue and are present in identical environments.[999] Salmine A-I, a small basic protein obtained from salmon, is predominantly unordered in aqueous solution, but the addition of perchlorate and 2-chloroethanol to the solution induces a right-handed α-helix. These results are discussed in comparison to those previously reported for other protamines.[1000]

The difficulty encountered in assigning the secondary structure of pancreatic trypsin inhibitor has prompted a study of the c.d. of iodinated proteins. This has shown that in some cases in which abnormal contributions of Tyr affect the protein c.d. iodination with KI_3 removes this complication and allows the analysis to be made by conventional procedures.[1001]

Chromophoric Proteins. Both human holo- and apo-caeruloplasmins show a low α-helical content but a relatively high β- and unordered content. A conformational transition leading to a decrease in β-structure occurs at pH 11 and probably involves ionization of buried Tyr. The apoprotein undergoes this transition at pH 10.5 and shows a band at 292 nm, which has been taken as indicative of Trp close to the Cu-binding site. Between 320 and 700 nm six Gaussian type I Cu bands are observed, of which only two show pH dependence, confirming the non-equivalence of the Cu-binding sites within the protein.[1002]

Both reduced and oxidized thioredoxins show remarkable stability towards pH and temperature changes. Secondary structure, obtained by conventional analysis of the c.d. spectra, differs markedly from that obtained from *X*-ray data. A fragment of the molecule (1—73) assumes a largely aperiodic structure at neutral or alkaline pH but folds into a globular structure below pH 3.0. The mechanism of this folding and its implications have been discussed.[1003]

The c.d. spectra of the general acyl-CoA dehydrogenase from pig liver reflect specific flavin–ligand interactions. Different c.d. spectra of the hydroquinone form of the enzyme are obtained, depending on whether reduction is photochemical or substrate induced, and presumably reflect differences in the flavin environment in each case. Similar studies on the electron-transfer flavoprotein also highlight differences in flavin environments in the various reduced forms.[1004]

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[1001] A. G. Walton, D. D. Solomon, S. P. Rao, and F. Hurwitz, *Polym. Prepr., Am. Chem. Soc., Div. Polym. Chem.*, 1979, **20**, 82.
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[1004] H. E. Aver and F. E. Frerman, *J. Biol. Chem.*, 1980, **255**, 8157.

The c.d. spectrum of retinol bound to β-lactoglobulin has been found to be of the same structure as the fluorescence-excitation spectrum.[1005] Spectra have been reported for large and small phytochromes in native and denatured states, under a variety of conditions of pH and urea concentration.[1006]

Extrinsic Chromophores. α-Chymotrypsin, in complexes with type I dyes such as proflavine, Acridine Yellow G, and acriflavine, gives a single, positive c.d. band due to 1 : 1 complex formation. Type II dyes such as Pyronin β and Rhodamine 6G, on the other hand, give 1 : 2 complex formation and both a positive and a negative c.d. band. C.d. appears to be induced in type I complexes when a single dye molecule is bound in an asymmetric environment, whereas in type II complexes the two dye molecules are bound at a specific site exerting a dipole–dipole interaction with one another.[1007]

The negative C band at 445 nm in the induced c.d. spectrum of 2-(4′-hydroxyphenylazo)benzoic acid–bovine serum albumin complex has been used as a marker in displacement studies with tolbutamide. Tolbutamide enhances the binding of the azo form, in spite of the fact that it displaces the hydrazone form.[1008] C.d. has been used to monitor the interaction of FMN with human serum albumin in model studies of binding in native flavoproteins. Specific binding sites exist, with well defined environments.[1009]

Titrations of the β_2-subunits of tryptophan synthase with pyridoxal phosphate have been made, using the induced c.d. signals in the 260—285 nm region. The generation of c.d. signals was found to occur at a rate intermediate between the rate of internal aldimine formation and the rate of enzymic activation. Binding to the α_2-apo-β_2 enzyme was hyperbolic with an associated dissociation constant of 9×10^{-7} M.[1010]

When colchicine binds to purified bovine-brain tubulin $\alpha\beta$ dimers the colchicine negative c.d. band at 340 nm is abolished. The association constant for complex formation has been estimated as $0.9\,\mu M^{-1}$. This effect is not observed when colchicine binds to a variety of other acidic proteins, and it is considered to arise from a specific conformational change. In the 250—300 nm region the c.d. spectrum is the sum of the separate contributions expected from each component.[1011]

A novel c.d. signal has been observed in complexes of lutein with proteins that arises from mutual interactions between lutein molecules. Similar changes have been observed in β-cryptoxanthin complexes but not with those of canthaxanthin or Et-β-apo-8′-carotenoate. The presence of more than one asymmetric carbon atom seems essential for these novel changes to occur, and a possible correlation of the *trans–cis* conformational change in the conjugated-double-bond system has been discussed.[1012]

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[1006] T. Brandlmeier, H. Lehner, and W. Ruediger, *Photochem. Photobiol.*, 1981, **34**, 69.
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[1010] H. Balk, I. Merkl, and P. Bartholmes, *Biochemistry*, 1981, **20**, 6391.
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[1012] S. Takagi, M. Shiroishi, and T. Takagi, *Agric. Biol. Chem.*, 1981, **45**, 1159.

p-Amidinophenyl esters of an enantiomeric pair of *N*-(2,4-dinitrophenyl)Ala has sufficiently low rates of reaction with trypsin to allow isolation of the acyl-enzyme intermediates. The spectra of these intermediates show that the two extrinsic chromophores are somewhat differently oriented in the chiral environment of the active site, although both chromophores couple intermolecularly. Significant differences between the two enzyme derivatives are apparent in the transient c.d. spectra, produced by deacylation with *p*-amidinophenol. The acyl groups are in different microenvironments in each case.[1013]

The c.d. spectra of D-glyceraldehyde-3-phosphate dehydrogenase in the native form, when carboxymethylated and when irradiated in the presence of an NAD-derived fluorophore, are quite similar in the 200–250 nm region and are little affected by the addition of sodium dodecyl sulphate. In the 250—300 nm region the c.d. of the irradiated sample resembles that of the native protein with two NAD bound per tetramer, and sodium dodecyl sulphate abolishes activity in this region. In the 300—400 nm region the irradiated sample spectrum is similar to that of the NADH-bound enzyme except that sodium dodecyl sulphate, rather than abolishing activity in this region as it does in the native enzyme, reduces the optical activity by 50%.[1014]

Hormones.—Addition of methanol to a solution of β-endorphin, and a number of its fragments, leads to a non-co-operative formation of α-helix.[1015] In cerebroside sulphate solution the fragments of β-endorphin showed partial helical structures but α-, γ-, and β-endorphins remain unordered. The helical-forming segment of β-endorphin has been identified as the middle segment of the protein and probably involves 2—3 helical turns between residues 13 and 24. This conformation in lipid may be responsible for biological activity.[1016]

Bradykinin analogues, in which dipeptide sequences were replaced by one 5-aminovaleric acid residue, show 3 → 1 and 4 → 1 hydrogen bonds and a salt bridge between the guanidino group of Arg 1 and the terminal COOH group. The backbone structure of bradykinin has a more flexible structure than gramicidin S, whereas [D-Ala-4]bradykinin contains a β-turn.[1017] The c.d. spectra of bradykinin homologues (L-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro), the *C*-terminal octapeptide Trp^2–Pro^9, the *N*-terminal heptapeptide L-Glu^1–Ile^7, and the internal hexapeptide Trp^2–Ile^7 show the presence of similar conformations in all cases. The *N*-terminal octapeptide L-Glu^1–Pro^8 is rather different, particularly in the Trp region. The results indicate an important orienting role for the *C*-terminal Pro of the aromatic side chain in position 2. The integrity of this structure is correlated with the inhibitory activity towards kininase II.[1018]

Near-u.v. c.d. of somatostatin-28 and -14 and of the *N*-terminal tetradecapeptide of somatostatin-28 shows almost identical characteristics, suggesting the retention of S—S linkages in both cases. Far-u.v. c.d. spectra are consistent

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[1014] Y. N. Lian and C. L. Zou, *Sci. Sin.*, 1981, **24**, 1146.
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[1017] C. Hashimoto, M. Tamaki, H. Uchida, S. Nozaki, S. Sofuhu, and I. Muramatsu, *Pept. Chem.*, 1978, **16**, 115.
[1018] D. I. Marlborough, G. H. Fisher, and J. W. Ryan, *Arch. Biochem. Biophys.*, 1981, **210**, 43.

with an essentially aperiodic structure in all cases.[1019] The spatial arrangement of oxytocin and its analogues in the region of residues 1, 2, and 6 has been reported.[1020]

Addition of SDS, dodecylammonium chloride, or dodecyltrimethylammonium chloride to insulin slightly alters its c.d. spectrum but does not affect the des(B23—30) octapeptide. Insulin in SDS resembles the octapeptide in the absence of detergent, suggesting that the *C*-terminal antiparallel β-form of insulin oligomers dissociates in the presence of SDS. Reduced insulin and the octapeptide lose their ordered structure in water, but their helical conformation is restored by detergent. Oxidized A- and B-chains of insulin are unordered in water solution, and the A-chain lacking cationic side groups remains unordered even in SDS. The B-chain is partially helical in all three detergents, whereas the A-chain shows small amounts of helix in dodecylammonium chloride and the β-form in SDS.[1021]

High pH increases the amount of β-turn conformation in tuftsin.[1022] In aqueous solution human big gastrin, human little gastrin, and Nle-11-HG-13 all assume a random-coil structure in the pH 3—7 range. In 2,2,2-trifluoroethanol the three hormones fold into partially ordered structures. Comparison with tetragastrin, pentagastrin, and heptagastrin shows that the biologically important *C*-terminal sequence does not maintain the same geometry on chain elongation at the *N*-terminal from 4 to 34. It is proposed that the *C*-terminal tetrapeptide folds into an active structure only on interaction with its receptor.[1023]

The first reported use of fluorescence-detected c.d. for protein measurements has been applied to ACTH, glucagon, human serum albumin, monellin, and RNase T1 and demonstrates its high sensitivity for detection of Trp environments in proteins.[1024] The vibrational c.d. of Met-enkephalin shows a large signal near 3300 cm^{-1}, which represents the stretching of the terminal OH group bonded to the S of Met, to give a seven-membered ring.[1025]

Nuclear Proteins.—The presence of headpieces, covalently attached to the core of the *lac* repressor, stabilizes it against urea denaturation. When the inducer iso-Pr-β-D-thiogalactoside is bound to the core, it is stabilized to a greater extent than the repressor itself. The isolated headpiece is more stable to urea than the tryptic core or the *lac* repressor and is unfolded in 0.9 M urea with a free energy of activation of ~ 2.5 kcal mol^{-1}. It appears that relatively weak interdomain contacts are responsible for the core stabilization by covalently linked headpieces but are weakened upon binding to the inducer.[1026]

The σ-subunit of *E. coli* DNA-dependent RNA polymerase, whether free or combined in the holoenzyme, is a highly structured protein with ~ 75% of its residues present in an α-helical conformation. Although subunit assembly does not affect secondary structure, interaction with DNA does, leading to a 10—20%

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[1020] K. Blaha, *Perspect. Pept. Chem.*, 1981, 272.
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[1026] M. Schnarr and J. C. Maurizot, *Biochemistry*, 1981, **20**, 6164.

reduction in α-structure. Glycerol is also found to have significant effects on both far- and near-u.v. c.d., associated with a higher affinity binding of the core and σ-subunit.[1027]

The tumour promoters 12-*O*-tetradecanoylphorbol 13-acetate, SDS, cholic acid, and polyoxyethylene sorbitan monostearate all increase the asymmetry of the aromatic side-chain environments in histones H2B and H4 and H2A–H4 mixtures. The promoters were active at molar ratios as low as 0.03, and their effects mirror quite closely their tumour-promoting activity.[1028] C.d. measurements suggest that high-mobility-group non-histone chromosomal proteins HMG1 and HMG2 do not affect the conformation and stability of nucleosomes.[1029]

7 Magnetic Circular Dichroism

Contributed by T. Brittain

General.—The modification of an electromagnet for use in a conventional c.d. spectrometer, at cryogenic temperatures, has been described.[1030] A general model of ligand-polarization transition probabilities in transition-metal complexes, as applied to m.c.d., has appeared.[1031]

Model Compounds.—Solvent and pH effects on Phe and some of its derivatives have been studied. In alkaline pH Phe shows an m.c.d. spectrum of the toluene type, whereas, remarkably, at low pH the intensity of the spectrum is drastically reduced.[1032]

In the 500—700 nm region it has been found that m.c.d. is an extremely sensitive technique for distinguishing between metal and porphyrin π-ring oxidation in various metalloporphyrins.[1033] Zinc metalloporphyrins have also been extensively characterized and may well be used in future in clinical studies.[1034, 1035]

Proteins.—The m.c.d. spectra of metmyoglobin cyanide and oxidized horse-heart cytochrome *c* show the magnetization characteristics of an isolated Kramers ground state with $S = \frac{1}{2}$, whereas reduced cytochrome *c* oxidase exhibits a curve associated with an $S = 2$ state. Further analysis of the $S = \frac{1}{2}$ states allowed an estimate of the ground-state g-factors, even in the presence of rhombic anisotropy The difference between the magnetization curves of a simple $S = \frac{1}{2}$ paramagnet and a magnetically complex ground state should prove useful when probing metal centres in proteins. The experimental analysis presented in this paper will prove a powerful technique in this field, as it will allow the first unequivocal association of optical absorption bands with known e.p.r. signals for a number of metalloproteins.[1036]

Extension of m.c.d. studies on cytochrome *c* oxidase out to 2000 nm has allowed the location of low-energy electronic transitions, which have been assigned to each

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[1034] K. J. Reimer, M. M. Reimer, and M. J. Stillman, *Can. J. Chem.*, 1981, **59**, 1388.
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of the four metal centres of this protein.[1037] M.c.d. spectra of cytochrome P450 11β and P450 scc have been found to be very similar to those of P450 from rabbit-liver microsomes and *Pseudomonas putida* in their high-spin ferric, high-spin ferrous, and ferrous CO complexes. Low-spin ferric P450 scc, free of substrate, is very similar to that in the presence of 20-α-hydroxycholesterol but distinct from low-spin ferric P450 11β and P450 scc, produced in the presence of external nitrogenous ligands. M.c.d. parameters for these proteins have been compared with those of other haemoproteins and discussed in relation to the haem environment of P450.[1038]

Horseradish peroxidase compounds II and III exhibit Faraday A terms in both the Soret and Q regions. Myoglobin compound II shows large C-term contributions in the Soret region. Comparison of the m.c.d. data for the O_2 complexes of these haem proteins shows that the trough depth of the Q O—O band is directly correlated to the electronic states of the O_2 complexes of the haemproteins. The implications of these findings are discussed in terms of the iron structures perturbed by the axial ligation.[1039]

Oxidized aconitase presents a m.c.d. spectrum similar to that of 2Fe–2S ferredoxins but distinct from those of 4Fe–4S proteins. Reduction of aconitase produces spectra different from any previously reported iron–sulphur centre. It is assumed the active site of the protein is a 2Fe–2S centre or possibly a cyclic 3Fe–3S centre.[1040] The m.c.d. spectra of ascorbate oxidase contain one major band at 14 000 cm^{-1} and a weak feature at 13 200 cm^{-1} consistent with the presence of 2—3 type I copper centres in each enzyme molecule.[1041]

The spectrum of the reduced molybdenum–iron protein of *Azotobacter vinelandii* nitrogenase is unaffected by 30% inactivation by O_2. The similarity of the spectra of the reduced molybdenum–iron protein of *A. vinelandii* to that of *Klebsiella pneumoniae* suggests the presence of metal clusters of identical number and structure in both proteins. Although the recorded spectra are similar to those reported for model $[Fe_4S_4(SR)_4]^{3-}$ clusters, significant differences exist between the two systems.[1042]

The reduced form of the high-potential Fe–S protein from *Chromatium* exhibits diamagnetic properties at temperatures down to 1.5 K. Oxidized 8Fe ferredoxin from *Clostridium pasteurianum* shows the presence of low concentrations of a paramagnetic species, which is active over the wavelength range of 300—800 nm and so has been assigned to another form of Fe–S centre. Oxidized high-potential protein shows a large number of highly structured bands across the visible region. The striking differences observed for the two oxidation states should provide a means of establishing the identity of these states of 4Fe clusters in more complex proteins. The magnetization plots obtained for the paramagnetic states of these

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[1040] D. Piszkiewicz, O. Gawron, and J. C. Sutherland, *Biochemistry*, 1981, **20**, 363.

[1041] D. M. Dooley, J. H. Dawson, P. J. Stephens, and H. B. Gray, *Biochemistry*, 1981, **20**, 2024.

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two proteins, together with that obtained for the reduced form of the 2Fe–2S ferredoxin from *Spirulina maxima*, were all satisfactorily fitted to the theoretically computed curves for $S = \frac{1}{2}$ systems.[1043]

8 Mössbauer Spectroscopy

Contributed by D. P. E. Dickson

During 1981 work has been reported on haem proteins, iron–sulphur proteins and enzymes, and iron-transport and -storage compounds. There have also been a number of studies involving the use of Mössbauer spectroscopy to investigate protein dynamics. All of the above work has been of iron-containing systems and has used the ^{57}Fe Mössbauer nuclide. In addition there have been two investigations using other isotopes.

Molloy *et al.*[1044] have reported a temperature-dependent ^{119}Sn Mössbauer study of the L-cysteine monohydrate and DL-penicillamine derivatives of chlorodimethyltin(IV). The data obtained on the Mössbauer recoil-free fraction suggest a lattice structure in which small molecular or oligomeric units are associated by a network of hydrogen bonds.

In a study of tobacco mosaic virus Andl *et al.*[1045] used the ^{129}I Mössbauer isotope. Measurements of the quadrupole splitting associated with tyrosine-139 iodinated with two ^{129}I atoms, as a function of the pH of the virus-containing solution, give information on the stabilizing effect of a hydrogen bond associated with the hydroxyl group of this residue. The data indicate that there is no correlation between hydrogen bonds on tyrosine-139 residues in different subunits of the virus.

Haem Proteins.—The electronic structure of the iron atoms in haem proteins and related model compounds has been extensively studied by Mössbauer spectroscopy. Interpretation of the Mössbauer data, including comparison with theoretical calculations, can improve our understanding of the binding of various ligands to the haem group.

The nature of the bond between the iron and the oxygen molecule in oxyhaemoglobin has been the subject of considerable debate, which has involved uncertainty over the valence state of the iron. Tsai *et al.*[1046] have made a comprehensive study of the temperature dependence of the Mössbauer spectra of oxyhaemoglobin and its isolated α- and β-subunits. They propose a model in which the ground state of the iron ion is a quantum mixture of ferrous and ferric states and for which the probability of finding the iron ion in each state can be calculated. The large temperature dependence of the Mössbauer quadrupole splitting is then explained as resulting from anharmonic vibration of the oxygen molecule in the binding potential well of the iron ion.

Maeda *et al.*[1047] have derived information on the electric-field gradient (e.f.g.) at the iron atom in oxymyoglobin and carboxymyoglobin from Mössbauer data

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obtained from single crystals and frozen solutions in high magnetic fields. It was found that in oxymyoglobin the quadrupole interaction is negative, the asymmetry parameter is between 0.3 and 0.4, and the principal axis of the e.f.g. tensor is nearly parallel to the iron–pyrrole bond. In carboxymyoglobin it was found that the quadrupole interaction is positive, the asymmetry parameter is between 0.2 and 0.3, and the principal axis of the e.f.g. tensor is probably perpendicular to the haem plane.

In order to investigate haem proteins in non-equilibrium states Prusakov *et al.*[1048] have carried out a Mössbauer study of the reduction of water–glycerol solutions of methaemoglobin by thermolytic electrons at 77 K. The spectra indicate the presence of two non-equivalent low-spin ferrous states, presumably corresponding to different protein conformations, which may imply the presence of two different low-spin ferric states in the original methaemoglobin before reduction. On thawing the frozen solutions only one high-spin ferrous state is observed, indicating a relaxation between the different conformational states that occur in the frozen solutions.

Although not a haem protein, haemerythrin is another oxygen-binding protein found in several marine invertebrates. A study of this protein by Mössbauer spectroscopy has been carried out in order to elucidate the nature of the dioxygen binding state.[1049] The results indicate that when the protein is oxygenated there are two inequivalent high-spin ferric ions that are sufficiently close to be antiferromagnetically coupled. When the protein is deoxygenated there appear to be two identical ferrous ions. Mössbauer spectra obtained from azidohaemerythrin are similar to those of oxyhaemerythrin but show that one of the iron ions has a significantly different quadrupole interaction.

Because of the wide range of possibilities for the state of the iron atom in the haem proteins there has been a considerable amount of work on model compounds. These are simpler molecules in which, by a suitable choice of ligands, it may be possible to reproduce the unusual spin and valence states of the iron atom that can occur in the actual proteins.

Sano *et al.*[1050] have used Mössbauer spectroscopy, together with magnetic resonance techniques, to study the electronic structure of the iron ion in oxyporphyrin and verdohaemochrome. They report that the state of the iron in these two compounds is most likely to be low-spin Fe^{I} and low-spin Fe^{II}, respectively.

Berry *et al.*[1051] have synthesized a number of heterobinuclear complexes of iron porphyrins that involve halide bridges between the iron atom and other metal atoms such as copper. These complexes are proposed as possible models for cytochrome *c* oxidase, for which there are indications that the iron may be in an unusual spin state. The Mössbauer data are interpreted in terms of a spin equilibrium between high-spin ($S = \frac{5}{2}$) and intermediate-spin ($S = \frac{3}{2}$) forms of Fe^{III}. Any spin coupling between the Fe^{III} and Cu^{II} atoms appears to be very small.

[1048] V. E. Prusakov, R. M. Davydov, and R. A. Stukan, *Biofizika*, 1981, **26**, 27.
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[1051] K. J. Berry, P. E. Clark, M. J. Gunter, and K. S. Murray, *Nouv. J. Chim.*, 1980, **4**, 581.

Ogoshi *et al.*[1052] have used Mössbauer spectroscopy, together with magnetic susceptibility and electron paramagnetic resonance, in a study of several model compounds for ferricytochrome *c'*. They found that octaethylporphyrinato-iron(III) perchlorate and its hexa-co-ordinated bis(tetrahydrofuran) complex are in the intermediate-spin ($S = \frac{3}{2}$) state with a short spin-relaxation time, but that the mono(puridine) adducts are in a mixed, low-spin ($S = \frac{1}{2}$)–intermediate-spin ($S = \frac{3}{2}$) state, which gives a positive quadrupole splitting.

Because of the relative simplicity of model compounds, calculations of their Mössbauer parameters may be made more readily than in the case of the actual proteins. A general review of the calculations of the Mössbauer parameters of iron–porphyrins has been given by Ionov and Gavrilov.[1053] Veilard *et al.*[1054] have made calculations of the Mössbauer quadrupole splitting and other data for three very simple haem model compounds: $FePO_3NH_3$ (for oxyhaemoglobin), $FePNH_3$ (for deoxyhaemoglobin), and FeP (P = porphyrin). From calculations of the temperature dependence of the Mössbauer spectra of $Fe(S_2CNMe_2)_2$ and $Fe(S_2CNPh_2)_3$ Sasaki[1055] has suggested that the high-spin–low-spin transitions in haem compounds may be induced by a Jahn–Teller interaction.

Iron–Sulphur Proteins and Enzymes.—Iron–sulphur clusters containing one, two, three, or four iron atoms are found in many proteins and enzymes. Investigations of these systems by Mössbauer spectroscopy can yield valuable information on the equivalence and electronic structure of the iron atoms within the cluster. Mössbauer spectroscopy has played an important role in the recent discovery of iron–sulphur clusters containing three iron atoms and is now providing evidence for the possible interconversion of the different types of cluster in certain circumstances.

The ferredoxin found in mammalian adrenal cortex, previously known as adrenodoxin, contains an iron–sulphur cluster of the two-iron type. Gukasian *et al.*[1056] have studied the effect of metopirone, the inhibitor of steroid-hormone biosynthesis, on this protein. Their data show that the interaction of metopirone, as well as of pyridine and *o*-phenanthroline, with adrenal ferredoxin leads to the removal of iron atoms from the iron–sulphur cluster, with the reaction of the two iron atoms being unequal.

The benzene dioxygenase system from *Pseudomonas putida* consists of a flavoprotein, an intermediate electron-transfer protein, similar to a two-iron ferredoxin, and a terminal dioxygenase protein containing four atoms each of iron and inorganic sulphur. Mössbauer spectra obtained from the terminal dioxygenase protein show that it contains [2Fe–2S] clusters, similar to those found in the two-iron plant-type ferredoxins.[1057] The two high-spin ferric iron atoms in the

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oxidized protein show a greater degree of inequivalence than is usually found in the two-iron ferredoxins. In the reduced protein each two-iron cluster contains an extra electron localized on one of the iron atoms, which becomes high-spin ferrous.

Putidamono-oxin, the oxygenase of the 4-methoxybenzoate mono-oxygenase enzyme system of *P. putida,* appears to be a similar protein to the terminal dioxygenase protein discussed above. Mössbauer data on putidamono-oxin clearly indicate that it contains [2Fe–2S] clusters similar to those of the two-iron plant-type ferredoxins.[1058] An additional interesting feature of both putidamono-oxin and the terminal dioxygenase protein is that they both appear to need additional non-haem cofactor iron in order to achieve enzymatic activity. In the Mössbauer study of Bill *et al.*[1058] selective enrichment of both cluster and cofactor iron with ^{57}Fe provided useful additional data and enabled them to establish that one cofactor iron atom is required for each [2Fe–2S] cluster.

Further Mössbauer data on the recently discovered three-iron clusters in *Azotobacter vinelandii* and *Thermus thermophilus* ferredoxins (which also contain a four-iron cluster) and ferredoxin II from *Desulfovibrio gigas* have been presented by Münck and co-workers.[1059–1061] Also presented is evidence for the presence of three-iron clusters in aconitase from beef heart and in glutamate synthetase from *Escherichia coli.* The Mössbauer spectrum of the reduced cluster at low temperatures, which shows two quadrupole-split doublet components with an intensity ratio of 2:1, gives the most unequivocal evidence for the presence of three iron atoms. This evidence is confirmed by the spectra of both redox states of the clusters taken in applied magnetic fields.

D. gigas ferredoxin exists in different oligomeric forms, containing clusters of either the three- or the four-iron type. Electron paramagnetic resonance and magnetic c.d. data suggest that the interconversion of these two types of cluster may be possible. Mössbauer data have recently been presented that clearly indicate that both in *D. gigas* ferredoxin [1062] and in beef-heart aconitase [1063] [3Fe–3S] clusters can be converted into [4Fe–4S] clusters. Another Mössbauer investigation by Bell *et al.*[1064] has shown that following treatment of the ferredoxin from *Bacillus stearothermophilus* with the oxidizing agent potassium ferricyanide the single [4Fe–4S] cluster that normally occurs in this protein is converted into a cluster of the [3Fe–3S] type.

Mössbauer data on the thermal denaturation of cells and chromatophores of *Chromatium minutissimum* suggest that [2Fe–2S] clusters may be formed from the

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original [4Fe–4S] clusters, with the excess iron being precipitated in an inorganic form corresponding to ferrous sulphate.[1065]

The enzyme nitrogenase consists of a number of proteins, some of which contain clusters that involve molybdenum as well as iron and sulphur atoms. Smith *et al.*[1066] have made a Mössbauer study of the molybdenum–iron protein of the nitrogenase from *Klebsiella pneumoniae* in order to investigate the redox properties of the various metal clusters. They have associated various clusters within the protein with particular spectral species and have also observed magnetic interactions between some of these clusters. The structural and mechanistic implications of their data are discussed in relation to other published results.

Christner *et al.*[1067] have carried out a Mössbauer study of the enzyme sulphite reductase from *E. coli*. Their data are interpreted to give information on the haem group and iron–sulphur clusters found in this enzyme.

There is interest in the possibility that iron–sulphur clusters may be associated with the primary electron acceptor in photosynthetic reaction centres. Boso *et al.*[1068] have investigated reaction centres from *Rhodopseudomonas spheroides* using Mössbauer spectroscopy. Their results indicate that the iron present is in the high-spin Fe^{2+} state, has substantial 3*d*-electron delocalization on to the neighbouring quinone group, but is essentially unchanged following reduction of the sample.

The cyanobacteria (blue-green algae) contain two types of reaction centre, Photosystem-I and Photosystem-II. Evans *et al.*[1069] have obtained computer fits to high applied magnetic-field spectra of the Photosystem-I reaction centres of the cyanobacterium *Chlorogloea fritschii*. These data indicate that all of the three redox components of this system, which had previously been identified by electron paramagnetic resonance, contain iron–sulphur clusters of the four-iron type.

There has been considerable previous work on model compounds containing cubane clusters with the general formula $[Fe_4S_4(SR)_4]^{n-}$.[1070] These represent good analogues for the [4Fe–4S] clusters found in many iron–sulphur proteins. Mössbauer data on two more examples of this type have been presented by Rzaigni *et al.*[1071]

A cubane cluster containing three iron atoms and one molybdenum atom has been proposed as a possible model for one of the active centres of nitrogenase. Wolff *et al.*[1072] have reported Mössbauer data on four examples of double cubane complexes containing $MoFe_3S_4$ and WFe_3S_4 cores. The measurements suggest formal valences of $1[Mo^{4+}, 2Fe^{3+}, Fe^{2+}, 4S^{2-}]^{4+}$, $1[Mo^{3+}, 2Fe^{3+}, Fe^{2+}, 4S^{2-}]^{3+}$, and $2[Mo^{3+}, 2Fe^{3+}, Fe^{2+}, 4S^{2-}]^{3+}$. The data also indicate the involvement of both ferrous and ferric ions in the bridging between the two cubane clusters.

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Iron-transport and -storage Compounds.—Siderophores are protein-based iron-transport molecules found in fungi. Matzanke and Winkelmann [1073] have used Mössbauer spectroscopy in a study of siderophore iron uptake, storage, and release in fungal cells. They conclude that siderophores are involved in iron storage as well as transport.

A Mössbauer investigation of the dermal granules of *Molpadia intermedia,* which are known to have iron-containing micelles, shows that the iron component undergoes a transition to an antiferromagnetically ordered state below 10 K, with a saturation hyperfine field of 44 T.[1074] The iron-storage material in this system therefore appears to be more similar to that found in bacteria than to ferritin, which is the iron-storage protein found in higher organisms.

The ability to detect ferritin and other iron-containing proteins has enabled Mössbauer spectroscopy to be applied to a number of medical investigations. An investigation of normal and pathological red blood cells by Jacobs *et al.*[1075] has shown that the amount of ferritin-like iron measured by Mössbauer spectroscopy is generally higher than that measured by immunoradiometric assays. This is interpreted as indicating that part of the iron detected by Mössbauer spectroscopy may be in partly denatured ferritin polymers and haemosiderin (which is often regarded as a denaturation product of ferritin with similar iron-containing cores).

The kinetics of iron uptake from ferritin during haemoglobin synthesis are of considerable medical interest. Ofer *et al.*[1076] have measured ferritin, oxyhaemoglobin, and deoxyhaemoglobin concentrations in differentiating erythroid cells, using Mössbauer spectroscopy.

Protein Dynamics.—The Mössbauer effect is very sensitive to any motion of the Mössbauer nucleus. For this reason Mössbauer spectroscopy can yield important information concerning protein dynamics. The parameters of the Mössbauer spectrum that are of interest in this context are the spectral area and the linewidth.

Cohen *et al.*[1077] have carried out Mössbauer measurements on ferritin crystals. The spectra obtained above 256 K show, in addition to the normal quadrupole-split doublet, very wide Lorentzian wings, which are interpreted as arising from a bounded diffusive motion of the iron-containing core of the protein. Very similar results have been obtained from iron-storage material in other systems, including those corresponding to nearly *in vivo* situations.

Parak *et al.*[1078, 1079] have obtained Mössbauer spectra from polycrystalline deoxymyoglobin and metmyoglobin between 4.2 K and room temperature. The results are interpreted in terms of the dynamics of the molecules, and their motional behaviour at higher temperatures is described as fluctuations between slightly different conformational states. A similar study has been carried out on

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carbon monoxy-liganded human haemoglobin crystals, selectively enriched with ^{57}Fe in the α-chains.[1080] The spectra show an additional broad component at 260 K, which is attributed to quasi-elastic processes associated with fluctuations between conformational substates of the quarternary 'R' structure. Microwave measurements by Singh *et al.*[1081] emphasize the role of adsorbed water on the dynamics of metmyoglobin, and this can be related to the Mössbauer data discussed above. Shaitan *et al.*[1082] have interpreted the bounded diffusion and segmental phase-transition models for the motion of the Mössbauer atom in proteins as an aspect of the protein conformational model. They also discuss the influence of the thermodynamics of the water content on protein dynamics.

A somewhat different technique for monitoring protein dynamics involves Rayleigh scattering of Mössbauer radiation. An investigation of myoglobin crystals using this technique has been reported by Krupyanskii *et al.*[1083, 1084] The authors report that the value of the mean-square displacement of the myoglobin molecules is more accurately determined by this method than by *X*-ray analysis.

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9 Protein-association Reactions

Contributed by P. D. Jeffrey, L. W. Nichol, and D. J. Winzor

The study of protein-association reactions has continued to prove an active research field during the period covered by this Report. A notable development has been the emergence of a series of studies concerned with the effect of inert compounds on the self-association of proteins. Also of note is the appearance of a book devoted entirely to the study of protein interactions,[1085] which comprises a series of authoritative review articles on structural, methodological, and theoretical aspects of their study. In keeping with the format adopted in last year's Report, this discussion of protein-association reactions is arranged according to the type of interacting system.

Intramolecular Interactions.—*Conformational Considerations.* A new approach to the problem of calculating hydrodynamic properties of different arrays of subunits has been presented.[1086] Each subunit in an array was taken as a sphere and preliminary calculations indicated that an excellent approximation, hydrodynamically, to such a sphere was a cubic array of eight smaller spheres. Using this model the authors calculated and tabulated translational and rotational frictional coefficients and intrinsic viscosities for rigid regular structures composed of up to

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[1086] J. M. G. Bernal and J. G. de la Torre, *Biopolymers*, 1981, **20**, 129.

eight identical spherical subunits. Harding,[1087] in considering the description of protein conformation in terms of ellipsoids of revolution, formed a new function by combining the molecular covolume with the viscosity increment. Although it is insensitive to axial ratio for oblate ellipsoids, the function is a useful addition to those already available for defining prolate shapes.

The large number of spatially different forms that can be generated by the binding of ligands to oligomeric proteins and the potential effects on their properties were emphasized by Gust and Dirks.[1088] Rigorous methods for enumerating all possible species were described and illustrated for aspartate transcarbamoylase. The enthalpy change of the substrate-promoted conformational change of this enzyme from the T to the R state was measured calorimetrically.[1089] Combination of the value so obtained with the free-energy change previously measured revealed that the transition is entropically controlled at pH 7.

Stopped-flow light scattering and spectral measurements were utilized[1090] to show that in the dimer–tetramer transition of concanavalin A the association or dissociation precedes a conformational change, while in the calcium-mediated reaction in *Limulus* haemocyanin the reverse is apparently true. Spectral methods in conjunction with sulphydryl reactivity and trypsin susceptibility were used to study the interplay of calcium and magnesium binding and their effect on structural changes in sarcoplasmic calcium-binding protein from crayfish.[1091] It was concluded that the changes can be attributed to the binding of calcium to calcium-specific sites rather than to replacement of magnesium on Ca–Mg sites. The pH dependence of the isomerization of ferricytochrome *c* was studied[1092] by the circular-dichroism stopped-flow method. In the pH range 7—13 the existence of three different forms was indicated. Between pH 6 and 1.6 the high-spin form appears. At pH 1.6 the transition from the low-spin to the high-spin form occurs within about 2 ms and is followed by unfolding of the polypeptide chain.

The response to proton binding by human serum albumin and the influence on it of calcium ions and warfarin were interpreted in terms of a two-state model for the protein.[1093] According to this picture an allosteric constant governs the equilibrium between the N state (low pH) and the B state (high pH). The proton is considered to be the ligand and calcium and warfarin the effectors. A number of features of the conformational change such as the co-operativity of proton binding and the fraction of protein found in one of the two conformations are described in terms of this model. The sensitivity of intrinsic fluorescence in haemoglobin to the R–T conformational transition was utilized to reveal differences in conformation between these two states in different haemoglobins.[1094]

Subunit–Subunit Interactions. In an interesting predictive paper it has been shown that βB_p, the principal component of the β-crystallins, should have a three-

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dimensional structure with homologies to that of γ-crystallin.[1095] However, extensions of the polypeptide chain at the *N*- and *C*-termini to form flexible arms, which may be involved in the self-association to dimers and possibly higher oligomers, are predicted in β-crystallin. Studies of the effects of pH, ionic strength, temperature, and calcium ions on the stability of the quaternary structure of α-crystallin[1096] gave additional support to the three-layer model previously proposed.

Several reports were concerned with stoicheiometries and arrangements of subunits in oligomeric proteins. The overall configuration of pyruvate carboxylase appears to be a tetrahedron-like structure consisting of two pairs of subunits in two different planes orthogonal to each other.[1097] A crustacean erythrocruorin was modelled as two eclipsed regular pentagons each formed of spherical subunits.[1098] Gel filtration in SDS followed by quantitation of subunit peaks led Verheul and his colleagues[1099] to a 12-subunit model with minimum molecular weight of about 170 000 for the cytochrome *c* oxidase from bovine heart. Zinc ions were found to play a structural role in stabilizing subunit interactions in the 7S nerve growth factor, probably an oligomer with composition $\alpha_2\beta\gamma_2$.[1100] It was also found that symmetrical or asymmetrical dissociation of the oligomer could occur, depending upon conditions.[1100] Work continued on the communication between subunits in aspartate transcarbamoylase:[1101] the propagation of gross conformational changes in a derivative of the enzyme with modified regulatory chains was found to be consistent with the concerted model for ligand-promoted transitions of the enzyme. Studies of other proteins containing heterologous subunits have involved γ-glutamyl transpeptidase,[1102] transcarboxylase,[1103] ferritin,[1104] and the phosphorylase–phosphatase complex from skeletal muscle.[1105] In reconstitution studies it was found for the first-mentioned enzyme that the active HL dimer could be formed from denatured subunits only if they were mixed before denaturation. In transcarboxylase, anaerobic conditions are necessary to prevent dimer formation if reconstitution to an active hexamer is to be successful. Gilbert and Gilbert,[1106] in drawing attention to the feasibility of computer modelling of the sedimenting boundary shape for oligomeric proteins as a check of homogeneity, produced evidence that the pyruvate dehydrogenase multienzyme complex may be quite heterogeneous.

Wood and co-workers[1107] took advantage of changes in intrinsic fluorescence of mitochondrial malate dehydrogenase to follow the kinetics of regain of specific

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activity between pH 5 and 7.5 and its connection with the dimerization of the enzyme. A model with dimerization preceding a conformational change was proposed for the transition to the high-activity form. Fluorescence measurements using labelled derivatives were also employed to study the mobility of subunits within the haemoglobin molecule.[1108] It was suggested that in solution haemoglobin molecules may be visualized as spheres with a fluid periphery and a more rigid interior.

Haemocyanins. The arthropodan, xiphosuran, and molluscan proteins continue to attract much interest as model examples of the relationship between structure and function in multi-subunit proteins. In the first two groups the isopod *Bathynomus giganteus*,[1109] the crab *Cancer magister*,[1110] the freshwater crayfish *Cherax destructor*,[1111] the lobster *Homarus americanus*,[1112] the scorpion *Androctonus australis*,[1113] and the horseshoe crab *Limulus polyphemus*[1114] were studied, while *Helix pomatia*[1114] and *Nautilus pompilius*[1115] represented the molluscs. Some findings which emerged from these investigations are that physiological requirements may be more important than phylogenetic relationships in determining properties,[1109] that different relative rates of aggregation of subunits may explain differences between *in vitro* and *in vivo* oligomer assembly patterns,[1111] and that *Cancer magister*[1110] and molluscan[1115] subunit heterogeneity may be greater than previously thought. Interesting results related to the aggregation states were the estimate of about twenty-five as the number of amino-acids in the contact areas of *Homarus* hexamers[1112] and the effect of rapid conformational changes accompanying oxygenation in *Limulus* and *Helix* haemocyanins.[1114] A very neat application of immunological labelling and electron microscopy led to considerable progress in the definition of the positions of eight different subunits in *Androctonus* oligomers.[1113] It was confirmed that two dimeric subunits constitute a central ring to which all twenty monomeric subunits are linked in the 34S species.[1113]

Self-associating Proteins.—*Discrete Reactions.* The temperature and pH dependence of the dimer–tetramer equilibrium of concanavalin A was analysed[1116] in terms of a Wyman linked function. It was shown that a histidine residue was involved in the association-linked ionization reaction and that the association is characterized by large entropy and heat-capacity changes consistent with the interaction of hydrophobic surfaces, as in the crystal. A calculation of the free-energy change associated with the interaction of hydrophobic groups in the dimer formation of insulin yielded a value in good agreement with that measured

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experimentally.[1117] The triphosphopyridine-specific isocitrate dehydrogenase from pig heart has been shown to dimerize in response to magnesium DL-isocitrate, NADPH, or EDTA,[1118] the dimer being the active species.[1118, 1119] The dimerization of neurophysin was studied by sedimentation velocity, viscometry, and fluorescence-polarization methods, and the results were found to be consistent with a side-by-side aggregation of prolate ellipsoids.[1120]

Several association reactions involving tetramers as stable species were recorded. Thus: an IgG heteropolymer containing rheumatoid factor activity isolated from the serum of a patient with polyclonal hyperviscosity syndrome is apparently a cyclic tetramer;[1121] bovine β_2-microglobulin apparently undergoes a monomer-to-tetramer reversible self-association under certain conditions;[1122] the lysosomal acid β-galactosidase from the porcine adrenal cortex, existing *in vivo* as a dimer, may further associate into the active tetramer;[1123] chorismate mutase–prephenate dehydratase from *E. coli* undergoes a dimer–tetramer–octamer conversion connected with its allosteric kinetics;[1124] and rabbit-muscle phosphofructokinase associates in a monomer–tetramer–16mer or monomer–dimer–tetramer–16mer scheme depending upon temperature.[1125] Examples of proteins involved in more complex, but still discrete, self-association reactions are the reported two-stage association of ornithine aminotransferase to form a trimer and then higher aggregation states[1126] and the polymerization of 8S clathrin protomers into 30S basket structures.[1127]

It was shown by Tellam and Winzor[1128] that allowance for the osmotic shrinkage of polyacrylamide gels could permit prediction of the concentration dependence of elution volume and that in some systems such as the monomer–dimer equilibrium in bacterial α-amylase it is necessary to take this effect into account to obtain even a qualitative indication of the association. Computer simulation was employed by Stevens and Schiffer[1129] to describe the small-zone gel-filtration behaviour of a reversibly associating protein. Good agreement was obtained for an equilibrium constant determined in studies using this procedure and those determined by other methods for a Bence-Jones protein. A model predicting that oligomeric proteins can generally be expected to dissociate under pressure was discussed by Paladini and Weber,[1130] who showed by fluorescence-polarization measurements that pressure promotes the dissociation of enolase, in agreement with the prediction. A two-step model of protein

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association was presented by Ross and Subramanian.[1131] The first step, mutual penetration of hydration layers causing disordering of the solvent structure and thus hydrophobic in nature, is followed by short-range interactions. The net free-energy change for association is primarily the resultant of the positive entropy change accompanying the first step and the negative enthalpy change accompanying the second. Evidence was presented that the strengthening of hydrogen bonds and van der Waals interactions introduced as a consequence of the hydrophobic effect are important factors in the stability of protein-association complexes.

Indefinite and Macro Assemblies. It appears that the deoxy forms of the haemoglobins HbA, HbS, and HbC aggregate according to the nucleation-controlled mechanism and that two types of nuclei are formed that are specific for the formation of gels or crystals.[1132] Oxy and carbonmonoxy HbA and HbS aggregate by a simple linear mechanism. Studies of the polymerization of haemoglobin in sickle-trait erythrocytes by magnetic double-resonance methods[1133] revealed oxygen-saturation-related differences from that in sickle erythrocytes. It was also shown that the thermodynamic theory that successfully describes gelation in the latter case requires modification for application to gelation in sickle-trait erythrocytes. In an *X*-ray diffraction study of tobacco mosaic virus (TMV) protein it was shown that the two helical aggregates with $16\frac{1}{3}$ and $17\frac{1}{3}$ subunits per turn have structures very similar to each other and to the protein part of the virus.[1134] In another study[1135] the use of scanning calorimetry showed that the two types of polymerization of TMV protein leading to helical rods and double discs, respectively, involve quite different subunit bonds. Other structural proteins to receive attention were the mammalian neurofilament triplet proteins[1136] and a hollow cylinder protein from the cytoplasm of human erythrocytes.[1137] In the former case it was shown that, of the three individual triplet proteins isolated from porcine spinal cord, only the 68 000 molecular-weight protein is readily reconstituted into morphologically normal intermediate-sized filaments. The results with the erythrocyte cytosol protein indicated that it is a hollow cylinder assembled from multiple copies of five non-identical subunits.

Effect of Inert Compounds. The effect of small molecules, such as sucrose and glycerol,[1138-1140] and of macromolecules[1141, 1142] on the stability and preferential hydration of protein structures has received detailed attention. In addition, the important and related concept has been developed that non-binding macromolecular entities acting as space-fillers may significantly affect the extent of protein self-association[1142, 1143] and the kinetics of enzyme action.[1142] The

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theoretical approach[1142,1143] has been to express the activity coefficients of species as functions of total solution composition and to assess the magnitudes of constants in the expansions in terms of excluded volume effects. Expressions derived on this basis demonstrate explicitly, for example, the possibility of greatly increasing the apparent self-association constant of a protein by inclusion of a relatively high concentration of volume-occupying polymer.[1143] This effect has been demonstrated experimentally with several self-associating systems, including a fluorescent derivative of myoglobin,[1144] rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase,[1145] and pyruvate dehydrogenase complex.[1143] In the same area the effect of non-binding molecules on the gelation of HbS[1146] and the precipitation of various proteins by polyethylene glycols[1147] have been examined. It is noteworthy, however, that polyethylene glycol 6000 and 20000 were reported[1144] to be without effect on the self-association of myoglobin (which is influenced by the presence of other proteins) and of haemoglobin, whose ability to self-associate[1148] has been questioned.[1149]

Mixed Associations.—*Protein–Ligand Binding.* Developments in chromatographic procedures to determine thermodynamic parameters governing protein–ligand (small molecule) interactions included the description of a recycling gel-partition technique as a batch equilibrium process aimed at reducing the large volumes of reaction mixture normally required in frontal analysis.[1150] In addition, theory was presented describing the competitive interaction of a multi-valent protein with both a univalent ligand and an affinity matrix; the derived expressions are useful in determining interaction parameters from either batch partition equilibrium or frontal-affinity chromatographic studies, particularly when the binding of ligand to protein is weak, as in the aldolase–phosphate interaction governed by a site-binding constant of $350 \pm 60\,M^{-1}$.[1151] Spectral methods were also employed to evaluate thermodynamic parameters, as exemplified by the development of a difference spectroscopy technique to study haemerythrin–thiocyanate ion reactions,[1152] and by the use of fluorescence quenching to establish that malate and lactate dehydrogenases bind NAD dimers specifically and with high affinity.[1153] Other thermodynamic studies with systems of this type included the use of solubility and birefringence measurements to investigate the effects of oxygen and butylurea (an anti-sickling agent) on the gel–solution phase equilibrium of haemoglobin S[1154] and the use of a protein–ligand system (bovine serum albumin and 2-nonanone) to probe the changes in hydrophobic interactions within the protein induced by addition of various salts.[1155]

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Kinetic studies on ligand binding were also in evidence, an example being the use of flash photolysis at low temperatures to postulate that carbon monoxide must surmount four barriers in migrating from the solvent to the haem iron of myoglobin.[1156] More conventional enzyme kinetic studies have been employed to investigate the effects of binding selected inhibitors on hysteresis phenomena observed as slow attainment of steady states in enzymic assays involving sucrase[1157] and dihydrofolate reductase.[1158] With respect to detailed structural considerations of enzyme action, crystallographic work has continued as typified by studies on the binding of substrate, product, and inhibitor molecules to carboxypeptidase A,[1159] one noteworthy result being that competitive and non-competitive inhibitors can bind to the same site on the enzyme.

The development and application of binding theory have also progressed from the use of binding functions expressed conventionally as ratios of polynomials of free-ligand concentration to interpret precise results of oxygen–carp haemoglobin binding,[1160] through further consideration of steric hindrance of neighbouring sites caused by bound ligand,[1161] to consideration of the competitive binding of different ligands to sites on a macromolecular acceptor.[1162] A variation on the latter theme, also of particular biological relevance, arises when the ligand itself exists as an equilibrium mixture of different states, exemplified by a monomer $\rightleftarrows$ micelle self-association, with either or both states capable of binding to a particular site on the acceptor.[1163] Sculley and co-workers[1163] have explored the forms of binding curves that might result and showed, for example, that the Scatchard plot exhibiting two critical points, found previously for the chlorpromazine–brain tubulin system, could be quantitatively explained by preferential binding of the micellar state of the tranquillizer to one site on the brain protein. This type of analysis may well be of relevance to other similar studies, such as the binding to tubulin of colchicine,[1164] a ligand capable of self-association,[1165] or the binding to pancreatic phospholipase A_2 of micellar substrate analogues.[1166, 1167] In this connection, methods for estimating micelle size and the equilibrium constant for micelle formation have become important.[1168]

Dissimilar Protein Associations. The interactions between serine proteases and their protein inhibitors provide clear examples of mixed protein associations with physiological significance. Work in this area has continued, preliminary crystallographic data now being available on the structure of the complex between Kazal

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trypsin inhibitor and trypsinogen[1169] and of the ternary complex of α-chymotrypsin, β-trypsin, and the Bowman–Kirk inhibitor.[1170] Stable complex formation between α_1-antitrypsin and (separately) trypsin, chymotrypsin, and elastase has also been revealed by end-group analysis.[1171] Mixed associations of this type are not restricted to serine proteases, as is evident from kinetic and gel-filtration studies on inhibitors from wheat-flour and human α-amylases.[1172] There has been considerable interest in the association of calmodulin with enzymes[1173–1175] such as myosin light-chain kinase and with non-enzymic proteins.[1176] In one paper[1173] it was suggested that exposure of hydrophobic regions of calmodulin, induced by the binding of calcium ions, is related to the activation of calmodulin-dependent enzymes. In another,[1176] calmodulin-binding proteins were implicated in the particulate distribution of calmodulin in brain extracts.

The reported incidence of enzyme–enzyme interactions is also growing: association between pyrimidine dimer DNA glycosylase and apurinic/apyrimidine DNA endonuclease has been reported,[1177] as has complex formation between citrate synthase and malate dehydrogenase,[1178] pyruvate dehydrogenase complex,[1179] and fumarase.[1178] Indeed, in the latter study[1178] affinity chromatography was used to demonstrate a wide variety of interactions between consecutive enzymes of the citric acid cycle and the aspartate–malate shuttle.

Mixed associations have been detected by a variety of chemical, kinetic, migration, and spectral methods, including fluorescence polarization, where a warning was sounded that complexing may be effected by bridging through the fluorescein moiety.[1180] There have, however, been relatively few attempts to characterize such systems in terms of equilibrium constants determining solution composition, notable exceptions being the use of equilibrium sedimentation to describe quantitatively the equilibrium between the S5 and S8 proteins from *E. coli* 30S ribosomal subunit[1181] and between derivatives of apolipoproteins C-I and A-II.[1182] Quantitative methods have also been devised to study interactions between proteins and other macromolecules, such as DNA[1183] and heparin.[1184]

Insulin-receptor Interactions. A great deal of effort continues to be invested in the attempt to purify the insulin receptor from membranes of various cell types and to characterize it, especially with respect to subunit composition. Thus, Harrison and Itin,[1185] utilizing immunoprecipitation with autoantibodies, claim to have sub-

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stantially improved the purification factor with a preparation from human placenta and observed subunit bands of molecular weights 126 000, 90 000, and 42 000 on polyacrylamide-gel electrophoresis. Biosynthetic labelling with [^{35}S]methionine followed by precipitation with antibodies has yielded evidence for two major glycoprotein subunits in the receptor from human lymphocytes, the molecular weights being about 130 000 and 90 000.[1186] In another study of receptors from the same source, bands (on acrylamide gels) with molecular weights corresponding to 134 000, 98 000, and 206 000 were found;[1187] it was demonstrated that the major subunits were complex glycoproteins with differences in carbohydrate composition. It has also been reported that the insulin receptor contains *N*-acetylglucosamine, mannose, and galactose but that these are neither receptor nor tissue specific.[1188] From characterization of two forms of a photolabelled insulin receptor it was found that one, R1, has a molecular weight of about 250 000 and the other, R2, a molecular weight of about 80 000.[1189] The latter, the insulin-binding component, is thought to represent the receptor subunit usually ascribed a molecular weight near 130 000. The insulin-binding receptor component appears to be bound to other receptor components by hydrophobic interactions.

It has been suggested that the binding properties of the hepatic insulin receptor are influenced by the polar head groups of phosphoglycerides and sphingolipids within the plasma membrane.[1190] Evidence has also been presented that implicates the use of Tris buffer in artifacts simulating down-regulation of adipocyte insulin receptors *in vitro*.[1191] Experiments with rat-liver plasma membranes demonstrated an up-regulation phenomenon, believed to be mediated by exposure of cryptic binding sites.[1192] The binding of insulin to erythrocytes was shown to be a function of cell age, its decrease with cell age apparently being due to a change in receptor concentration per cell rather than in receptor affinity.[1193] Olefsky and co-workers[1194] contributed to the speculations about the nature of 'negative co-operativity' in insulin-receptor binding with the finding that in adipocytes the enhancement of the insulin dissociation rate at high levels of receptor occupancy (identified with negative co-operativity) does not reflect a phenomenon inherent in the action of insulin in augmenting glucose metabolism. It is reassuring to see evidence that the binding behaviour and the biological activity of insulin produced by recombinant DNA techniques appear to be indistinguishable from those of native human insulin.[1195]

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Crosslinking Reactions.—*Chemically Induced Crosslinking.* Photoinduced crosslinking of ATP and NAD to Fraction II of diphtheria toxin has been achieved, the results suggesting that the B chain of the toxin influences nucleotide binding to a single site located on Fragment A.[1196] This example of the use of crosslinking reactions in the elucidation of relatively simple protein–ligand interactions finds extension in other crosslinking studies aimed at exploring the chemical topology[1197] and subunit–subunit interactions[1198] of oligomeric protein entities. Thus, the self-association pattern of the monomeric subunit of lactate dehydrogenase from porcine muscle has been examined in reconstitution experiments by analysing electrophoretically the distribution of monomers, dimers, and tetramers, the polymeric forms being rendered stable by a fast crosslinking reaction with the bifunctional reagent glutaraldehyde; it appears that the pattern comprises an equilibrium reaction between monomers and dimers followed by a rate-limiting association of dimers to tetramers.[1198] Moreover, bifunctional reagents are available to crosslink dissimilar proteins, an example being provided by a study in which troponin C was modified at cysteine-98 with a specific photoaffinity probe and then allowed to react with troponin I to form a covalently bridged complex.[1199] The potential of chemical crosslinking in determining the likely association of macromolecules in a cellular structure was well displayed by the use of rotational-diffusion measurements, made before and after crosslinking with cupric phenanthroline, to provide evidence that cytochrome P450 can form aggregates in microsomal membranes.[1200]

Self-association and Mixed Association. Reversible interactions in solution between species exhibiting multivalency also fall into the domain of crosslinking reactions. In the simplest context a single solute is involved, as in the self-association of fibrin monomer where work has continued on the localization of the polymerization sites[1201] and on the development of the network.[1202] Where two reactants are involved, the simplest situation arises where one is bivalent and the other univalent, as in the ternary-complex formation of soybean trypsin inhibitor (Kunitz) and α-chymotrypsin.[1203] In the latter study the question was also addressed of forming an inhibitor-bridged complex between two different proteolytic enzymes. Consideration of systems involving three reactants was extended in the theoretical framework of forms of binding curves to be expected when a bivalent bridging ligand reversibly crosslinks two different multivalent acceptors to form an array of complexes with the three reactants in alternating sequence.[1204] Correlation was made in this work[1204] with formulations of binding theory relevant to the crosslinking of two reactants governed by a single site-binding constant, exemplified by a multivalent antigen and bivalent antibody system. The advent of monoclonal antibody preparations[1205] may well be relevant to the

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application of this type of theory, but attention has also been given to the reality that naturally occurring antibody systems are heterogeneous. Thus, in one theoretical study antibody affinity distribution was discussed in terms of alternative graphical representations of binding results,[1206] and in another[1207] the equilibrium binding of a bivalent ligand (synthetic hapten) to a heterogeneous population of bivalent cell-surface receptors (surface antibodies) was considered. A bivalent ligand hypothesis has also been formulated to provide a model for hormone action on cell surfaces.[1208]

Interplay of Ligand Binding.—*Theory and Examples.* The concept of linked functions has been used[1209] to extend again the Monod–Wyman–Changeux model of allostery to include effects of subunit dissociation, the theory being illustrated with recent experimental data on the oxygenation-linked dimer–tetramer equilibrium in human haemoglobins. This equilibrium has also been the subject of a more extensive study[1210] in which the association constants for oxy- and deoxy-haemoglobin were measured as a function of pH and salt concentration. The associative effect of deoxygenation on the dimer–tetramer equilibrium[1210] also seems to apply to the further self-association of tetramer ($\alpha_2\beta_2$) that occurs at higher, and physiologically more pertinent, haemoglobin concentrations;[1148,1211] the evidence, however, is necessarily less conclusive because of uncertainty about the magnitudes of the covolume radii of tetrameric oxy- and deoxy-haemoglobins to be used in allowance for thermodynamic non-ideality.[1149] Removal of oxygen certainly favours association of the $\alpha_2\beta_2$ haemoglobin HbII from the blood clam.[1212]

Systems for which qualitative indications of an interplay between protein association and ligand binding have been obtained include the calcium-dependent polymerization of lactoferrin[1213] and the chaotropic salt-dependent dissociation of erythrocyte ADP-ribosyltransferase.[1214] The effect of metal ions on the self-association of bovine prothrombin and its fragment 1 has also been investigated.[1215] In studies of associations involving dissimilar macromolecular entities, malate is reported to decrease the extent of interaction between mitochondrial aspartate aminotransferase and glutamate dehydrogenase,[1216] and calcium has been shown[1217] to promote the binding of an additional calmodulin molecule to phosphorylase kinase, which already contains one such molecule (the δ-subunit).

In a quantitative study of the effects of the binding of α-methylmannoside and α-methylglucoside upon the dimer–tetramer equilibrium for concanavalin A, Senear and Teller[1218] obtained no evidence of preferential binding with fragment-free

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protein preparations but evidence of preferential binding by dimer in studies of commercial concanavalin A. The presence of dimers with hydrolysed subunits is invoked as the source of the preferential binding of methylglycosides observed with the commercial preparation of concanavalin A.

Tubulin and Microtubules. This system has continued to be the subject of considerable research activity. Theoretical studies have appeared in relation to: microtubule formation, with emphasis on the role of subunit entropy in co-operative assembly;[1219] microtubule depolymerization, where thermal denaturation is treated in the framework of the Ising model;[1220] and the problem of reversible tubulin–microtubule exchange reactions [1221, 1222] that are encountered in head-to-tail polymerization, the process whereby polymer growth and depolymerization occur simultaneously at opposite ends of a microtubule.

Experimental studies have been concerned with the conformational requirements for efficient assembly of tubulin into microtubules,[1223] and also with the induced polymerization of tubulin by compounds such as nucleoside triphosphates,[1224, 1225] glutamate,[1226] the drug taxol,[1227] and glycerol,[1228] an inert solute, to which reference has already been made. There have also been studies of the induction of microtubule depolymerization by heat [1229] and by calcium,[1230, 1231] which promotes dissociation of subunits from the microtubule ends. It has been found [1232] that the presence of the calcium-binding protein calmodulin is required for this calcium-induced depolymerization, a finding that is evidently related to the identification [1233] of one of the microtubule-associated proteins (tau factor) as a calmodulin-binding protein. Other reports have described the inhibition of microtubule assembly by colchicine [1234] and griseofulvin.[1235] For the latter compound an apparent dissociation constant of 12.5 μM was found to describe its inhibition of both microtubule polymerization and depolymerization rates. Colchicine inhibition is believed to occur by the binding of one colchicine–tubulin dimer complex to a microtubule assembly end, the results being interpreted further to indicate that, in assembly, addition of tubulin occurs in a helical manner on each of eight independent helical starting points at the microtubule assembly end. Evidence for an interaction between microtubules and neurofilaments has recently been obtained [1236] from viscosity and sedimentation

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velocity studies, which demonstrate the requirement of ATP for complex formation. This finding is of particular interest because of the possible physiological relevance of such an interaction.

Muscle-filament Proteins. To conclude this Report we discuss studies of thin (*i.e.* actin-containing) filaments, of the thick filament (myosin), and of the interaction between the two filament systems. Fluorescence studies of the interaction of terbium ions with actin have been used [1237] to detect structural differences between actin filaments formed under various conditions. The finding of only four binding sites for terbium on actin polymerized by $MgCl_2$ (2 mM) and six on actin polymerized by KCl (0.1 M) was considered to indicate a looser structure for the latter polymer.

There have been several investigations of interactions responsible for the formation of the regulated thin filament (*i.e.* the F-actin–tropomyosin–troponin complex). Johnson and Stockmal [1238] have concluded that, at physiological ionic strength, the tropomyosin-binding subunit of troponin (TN-T) interacts with actin and thereby facilitates the binding of tropomyosin. Côté and Smillie [1239] observed that equine platelet tropomyosin binds to muscle F-actin much less strongly than does skeletal muscle tropomyosin, the weaker binding being attributed to the relatively poor polymerization of platelet tropomyosin and its fewer actin-binding sites. From a light-scattering study of the assembly of actin filaments with tropomyosin–troponin it has been shown [1240] that tropomyosin–troponin binds 200—300 times more tightly to binding sites with adjacent tropomyosin–troponin units than to isolated sites on actin filaments. This evidence of co-operative binding is of interest in that the concept of nearest-neighbour interactions between filament repeat units was invoked by Hill and co-workers [1241] to account for the sigmoidal binding of the ADP complex of myosin subfragment 1 to regulated filaments.

The interaction of muscle glycolytic enzymes with thin filament proteins has been investigated by affinity chromatography on Sepharose to which F-actin–tropomyosin had been covalently linked.[1242] Aldolase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, and phosphoglycerate kinase were all found to interact with the F-actin–tropomyosin, but glucose phosphate isomerase, triosephosphate isomerase, phosphoglycerate phosphomutase, and enolase did not. However, phosphogycerate phosphomutase interacted with the matrix if the column was first pre-equilibrated with lactate dehydrogenase, a result that implicates an interaction between the two enzymes. Conventional partition experiments have been employed [1243] to investigate the effect of ionic strength on the adsorption of aldolase to synthetic thin filaments, a case being made for the probable occurrence of the calcium-sensitive aldolase–filament interaction as a physiologically significant phenomenon in skeletal muscle.

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Studies of myosin included the finding[1244] that this protein isolated from smooth muscle of the calf aorta is similar to rabbit skeletal-muscle myosin in size, shape, and amino-acid composition but that the self-association reactions differ from those for skeletal muscle myosin; a smaller polymeric species is formed, and the effect of varying protein concentration upon the sedimentation velocity pattern for aorta protein does not comply with the Gilbert theory for a simple two-state monomer–polymer equilibrium. There have also been studies of subunit interactions within the myosin molecule, Burke and Sivaramakrishnan[1245] having observed the formation of hybrids by incubating myosin with free alkali light chains under conditions physiological with respect to ionic strength and temperature; a similar exchange was detected on incubation of light chains with the single-headed myosin subfragment 1.[1246] Such exchange phenomena indicate that the assembly of myosin from its constituent subunits is to be regarded as an equilibrium rather than a stoicheiometric process. The association of myosin light chains with heavy chains to form the intact myosin molecule has a pronounced effect on the calcium-binding properties of the light chains, whose affinity is several magnitudes higher in the presence of heavy chains than in their absence.[1247]

The sigmoidal nature of the binding of myosin subfragment 1 to regulate actin[1248] has been confirmed[1249] by employing light scattering to follow the transient-state kinetics of binding. Results obtained in the absence of calcium ions were considered to reflect positive co-operativity, a phenomenon that has now been incorporated into theoretical models of the regulated actin–subfragment 1 system[1250, 1251] and of the regulated actin–heavy meromyosin system,[1252] which is complicated by the fact that the ligand is bivalent. Some consequences of the presence of the second globular head on heavy meromyosin are evident from a comparative study[1253] of the binding of it and subfragment 1 to unregulated F-actin.

Studies of the binding of subfragment 1 (S-1) to regulated actin have also been presented to question the validity of the steric blocking model of muscle relaxation, which postulates that tropomyosin blocks the myosin-binding sites on the actin in relaxed muscle. An alternative model has been discussed in which the binding sites for tropomyosin and S-1 are separate but partially overlapping,[1254] whereas results for S-1 : ATP binding have been reported[1255] that imply complete independence of the S-1- and tropomyosin-binding sites on the F-actin. Not only is the binding of S-1 : ATP to F-actin unaffected by the presence of tropomyosin–troponin but also there is no calcium-sensitivity or sigmoidality associated with the interaction. Such seeming negation of the concepts of the steric

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model must, however, also be viewed in the light (i) that it apparently repudiates the earlier studies with S-1 : ADP [1248] and (ii) that Taylor and Amos [1256] have employed *X*-ray diffraction and electron microscopy to reaffirm the basic tenets of the steric blocking model of muscle relaxation.

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3
Peptide Synthesis

BY I. J. GALPIN

1 Introduction

The published literature has continued to produce many examples of the synthesis of relatively large polypeptides. It appears that the efforts of peptide chemists have remained steadfast even in the present climate where genetic engineering might have created several inroads into the domain of workers in this area.

Proceedings have been published for the 18th Japanese Peptide Symposium,[1] the 16th European Peptide Symposium in Helsingør,[2] and the 7th American Peptide Symposium.[3] Also, the third volume of 'The Peptides: Analysis, Synthesis and Biology' has appeared.[4] This volume covers amino, carboxy, sulphydryl, and hydroxy protecting groups and discusses minimal side-chain protection.

A series of papers arising from a meeting in Osaka to celebrate the 80th birthday of Professor Shiro Akabori has appeared in *Biopolymers*, Vol. 20 (9). Many of the papers are of a review nature, and the first by Meienhofer [5] asks some interesting questions as to why peptide synthesis continues to be a formidable challenge. Particular emphasis is placed on aspects of coupling, protection and deprotection, measurement of racemization, and purification techniques. The problems of protection and new methods of deprotection are also examined in detail in a subsequent paper.[6] A series of reviews concerned with many aspects of peptide synthesis has also been published.[7] A comprehensive review with over 400 references [8] examines the variety of side reactions that may be encountered with both functionalized and non-functionalized amino-acids. Particular attention is paid to the ease of racemization, oxidation, and attack by electrophiles on various centres in peptides. Interestingly, no side reactions were observed with alanine or leucine.

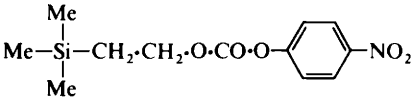
The general format of this chapter follows that established previously, and the majority of routine syntheses are presented in the Appendices at the end of the chapter.

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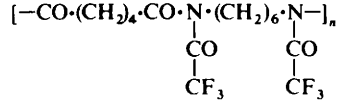
2 Methods

Protective Groups.—*Established Methods of Amino-group Protection.* The 2,2,2-trichloroethoxycarbonyl (Troc) derivatives have not seen wide usage for amino protection; however, the group, which may be removed by treatment with zinc dust in a suitable solvent, is occasionally difficult to introduce. This is due to the fact that the corresponding chloroformate is less reactive than, for example, benzyl chloroformate and therefore the symmetrical carbonate is always produced as a by-product. If, however, t-butylammonium salts of the amino-acids are used in the place of the standard Schotten–Baumann conditions, much improved yields of the corresponding Troc derivatives are obtained.[9] This paper also cautions the use of this protecting group, as the removal by zinc was found to be highly dependent on the solvent chosen. Also, it was found that the protecting group showed some instability towards the conditions of the catalytic hydrogenolysis normally used for removal of the benzyloxycarbonyl group.

The 2-trimethylsilylethoxycarbonyl protecting group introduced by Carpino[10] has also seen little usage; an improved method of introducing the protecting group that uses the mixed nitrophenylcarbonate (1) has, however, now been developed.[11] Thus, by direct acylation of the amino-acid sodium salt, the protected derivative is obtained in high yield, the method being claimed to be superior to that originally proposed using the corresponding chloro- or azido-formate.



(1)



(2)

A new method of preparing trifluoroacetyl amino-acids has been reported,[12] which employs the trifluoroacetyl derivative of polyamide '66' (2). When stirred with this polymer, amino-acid methyl esters or protected peptides are rapidly trifluoroacetylated. The method is claimed to be particularly useful for trifluoroacetylation prior to analysis of the corresponding ester by gas chromatographic methods. Further studies on the removal of the dithiosuccinoyl (Dts) group have been carried out.[13] Kinetic analysis showed the thiolysis to be a complex series of consecutive pseudo-first-order reactions.

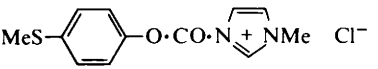
Considerable interest has also been shown in the methods available for the removal of the benzyloxycarbonyl protecting group. A new catalyst, $K_3[Co(CN)_5]$, that can be used for cleavage of benzyloxycarbonyl groups and benzyl esters has been developed.[14] Hydrogenolysis in the presence of this catalyst leaves benzyl ethers unaffected. The use of catalytic transfer hydrogenation for the removal of benzyloxycarbonyl has increased. For example, in a liquid-phase

[8] M. Bodanszky and J. Martinez, *Synthesis*, 1981, 333.
[9] J. F. Carson, *Synthesis*, 1981, 268.
[10] J. H. Tsao, H. Ringsdorf, E. Fell, and G. Hettisch, *J. Chem. Soc., Chem. Commun.*, 1978, 358.
[11] E. Wünsch, L. Moroder, and O. Keller, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1289.
[12] H. W. Tesch and R. C. Schulz, *Makromol. Chem.*, 1981, **182**, 2981.
[13] G. Barany, *Anal. Biochem.*, 1980, **109**, 114.
[14] G. Losse and H.-U. Stiehl, *Z. Chem.*, 1981, **21**, 188.

synthesis of wasp venoms, benzyloxycarbonyl was removed by transfer hydrogenation, using cyclohexa-1,4-diene as hydrogen donor and palladium as catalyst.[15] Palladium Black with formic acid as the hydrogen donor [16] has also been used in a dodecapeptide synthesis, but the yield appeared to be rather variable. Other workers [17, 18] have turned to the use of ammonium formate as the hydrogen donor; in this case 10% palladium on charcoal may be used as a catalyst with methanol or DMF as solvent. This method is satisfactory for removing benzyloxycarbonyl and benzyl ethers and for removing the nitro function from nitroarginine. In the latter case the cleavage is achieved in less than five minutes, but benzyl ethers on the other hand require 30—90 min for complete removal from serine and threonine. Although the majority of applications have been in solution, the method may also be used to remove peptides from solid-phase resins.[17] Surprisingly, 70% cleavage was achieved in ten minutes and more than 95% within two hours – in all cases [17, 18] acid-labile t-butyl-based protection was unaffected by these cleavage conditions.

Removal of 2-halogenoalkyl protecting groups, for example the TCBoc group, has been achieved by use of the redox catalyst cobalt phthalocyanin in the presence of sodium borohydride.[19] Under these conditions the TCBoc group is removed from peptides at 0 °C in under 90 min.

New Methods of Amino-group Protection. The 1-methyl-3-(*p*-methylthiophenyloxycarbonyl) protecting group has been proposed for the protection of α-amino functions.[20] The group, which is introduced using the imidazolium chloride derivative (3), is stable to Tfa, although not completely to 1M sodium hydroxide or ammonium hydroxide. Cleavage of the protecting group is achieved by oxidation with hydrogen peroxide in methanol, followed by treatment with ammonium hydroxide.

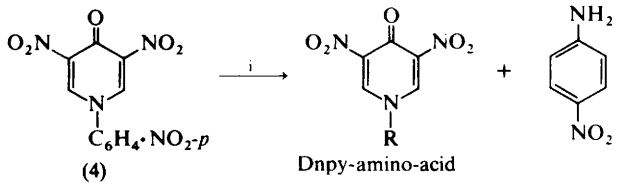


(3)

The use of 3,5-dinitro-1-(*p*-nitrophenyl)-4-pyridone as a novel protecting group for α-amino functions has been reported.[21] The group is introduced by reaction with the activated intermediate (4) as shown in Scheme 1. The Dnpy protecting group is unusual in that both amino hydrogens are blocked and the free amino function may be reliberated from the amino-acid or from peptides by treatment of the Dnpy derivatives with aniline or propylamine in pyridine. The utility of this protecting group was demonstrated by the synthesis of several small model peptides.

Chromone-derived protecting groups for both amine and carboxy functions have

[15] R. Colombo, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1393.
[16] S. A. Khan, G. J. Merkel, J. M. Bekcer, and F. Naider, *Int. J. Pept. Protein Res.*, 1981, **17**, 219.
[17] M. K. Anwer and A. F. Spatola, *Tetrahedron Lett.*, 1981, **22**, 4369.
[18] M. K. Anwer and A. F. Spatola, *Synthesis*, 1980, 929.
[19] H. Eckert and Y. Kiesel, *Synthesis*, 1980, 947.
[20] E. Guibe-Jumpel and M. Wakselman, *Bull. Soc. Chim. Belg.*, 1981, **90**, 517.
[21] E. Matsumura, M. Ariga, Y. Tohda, and T. Kawashima, *Tetrahedron Lett.*, 1981, **22**, 757.

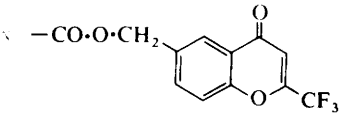


(4) Dnpy-amino-acid

Reagent: i, $R \cdot NH_2$(amino-acid)

Scheme 1

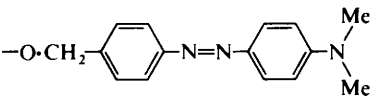
been developed.[22, 23] The 2-trifluoromethyl-6-chromonylmethylenecarbonyl (Tcroc) (5) derivatives may easily be prepared and may be rapidly removed by treatment with neat propylamine or a dilute solution of hydrazine in ethanol. The group is not cleaved by amino-acid esters or by acidic reagents, and it is particularly useful as it has a strong u.v. absorbance at 246 and 311 nm. The group was used in the preparation of methionine enkephalin [22] and bisacetamidomethyl dihydrosomatostatin,[23] and in these syntheses related protecting groups were used for the protection of other functional groups.



(5)

Protection of Terminal Carboxy Groups. The 2-trifluoromethyl-6-chromonylmethyl ester has been used for carboxy protection,[22] and 2-methyleneanthraquinone has also been used in related syntheses.[23] Whereas the chromonylmethyl ester was cleaved by propylamine or hydrazine, the 2-methyleneanthraquinone ester was removed reductively by treatment with sodium dithionite in bicarbonate, the cleavage being complete in DMF/water after 5—8 min at 25 °C.

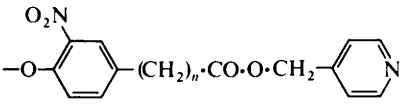
Esters containing groups that facilitate purification have also been studied. The chromogenic *p*-[(*p*-dimethylamino)phenylazo]benzyl ester (6) [24] was used in a synthesis of leucine-enkephalin. The intense colour of this protecting group facilitates purification on silica gel, and deprotection by catalytic hydrogenolysis over 10% palladium on charcoal can be easily monitored. The group withstands treatment with trifluoroacetic acid and dichloromethane and hence can be used in conjunction with acid-labile t-butyl-based protecting groups.



(6)

[22] D. S. Kemp and G. Hanson, *J. Org. Chem.*, 1981, **46**, 4971.
[23] D. S. Kemp, D. R. Bolin, and M. E. Parham, *Tetrahedron Lett.*, 1981, **22**, 4575.
[24] G. D. Reynolds, D. R. K. Harding, and W. S. Hancock, *Int. J. Pept. Protein Res.*, 1981, **17**, 231.

A group of active esters based on the structure (7)[25] in which the picolyl-solubilizing group is used in combination with a nitrophenyl-active ester has been used in the preparation of leucine-enkephalin. Such esters appear to be slow to couple owing to the considerable bulk of the carboxy protecting group.



(7) $n = 0$—2

2,4,5-Trimethylbenzyl esters[26] have been used in the synthesis of tryptophan-containing peptides. The esters that may be removed by treatment with HBr in acetic acid, Tfa, catalytic hydrogenolysis, or base hydrolysis are stable to 98% formic acid and thus may be used in conjunction with Boc for amino-terminal protection as under these conditions no butylation is observed on Boc removal. Ester cleavage by hydrogenolysis over 10% palladium charcoal catalyst in methanol gives the free tryptophanyl peptide in 1—2 h.

An alkaline protease from *B. subtilus* strain DY has been used for the hydrolysis of methyl, ethyl, and benzyl esters.[27] This enzymic hydrolysis proceeded to give products of high optical purity in 90% yield. The enzyme appears to give satisfactory results when benzyloxycarbonyl is used for α-amino protection, and minimal side-chain protection based on t-butyl is used. However, with a free α-amino function cleavage did not proceed satisfactorily. This enzymic method is advantageous in that it alleviates the possibility of racemization during ester cleavage.

A useful method of converting peptide hydrazides to the corresponding carboxylic acids has been reported.[28] Treatment of the hydrazide with three equivalents of *N*-bromosuccinimide in the dark gave a 90% recovery of the carboxylic acid after $1\frac{1}{2}$ hours at room temperature. The method appears to be compatible with t-butyl- and benzyl-based protection, although it is essential to protect tyrosine during this conversion. The possibility of side reactions of peptides containing arginine, cysteine, methionine, histidine, and tryptophan may also be anticipated, although so far none has been observed. This preparative method of converting the hydrazide into the corresponding carboxylic acid allows considerable flexibility during synthesis as previously it has been necessary to resynthesize a peptide fragment where an azide coupling has not given satisfactory results.

Side-chain Protection. The 2-trifluoromethyl-6-chromonylmethylenecarbonyl (Tcroc) protecting group mentioned above[22, 23] has been used for side-chain amino protection of lysine in a synthesis of bisacetamidomethyl dihydrosomatostatin. Careful control of the pH is required when introducing the *N*-α-Boc function

[25] F. H. C. Stewart, *Aust. J. Chem.*, 1981, **34**, 2013.
[26] D. E. Rivett and F. H. C. Stewart, *Aust. J. Chem.*, 1980, **33**, 2693.
[27] B. Aleksiev, P. Schamlian, G. Widenov, S. Stoev, S. Zachariev, and E. Golovinsky, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1323.
[28] J. Meienhofer, M. Dzieduszycka, M. Smulkowski, R. C. Makofske, and W. Danho, *Int. J. Pept. Protein Res.*, 1981, **18**, 6.

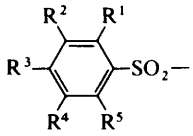
to this compound as the Tcroc group is readily removed at high pH in aqueous media. Large-scale preparations of Lys(Boc) and Z-Lys(Boc)-OH have also been examined,[29] and it has been found that treatment of the lysine–copper complex with t-butyl pyrocarbonate gave an excellent yield of the side-chain-protected amino-acid. Direct preparation of the N^{α}-benzyloxycarbonyl derivative from the side-chain-protected material gave a low yield of the protected derivative. A better yield was obtained when the N^{α}-Z derivative was initially prepared *via* its benzylidine complex, the reaction with benzyl chloroformate being carried out at low temperature. Then, after removal of the benzylidine group, the t-butyloxycarbonyl group was introduced to the side chain by means of the corresponding phenyl mixed carbonate and TMG in dimethylsulphoxide.

Frequently, in semisynthesis the acetimidoyl group is used for the protection of side-chain amino function of lysine. An improved method for liberating the free amino groups from the acetimidoyl derivative has been developed [30] that utilizes methylamine buffers at pH 11.5 at room temperature. This method of removal had a $t_{\frac{1}{2}}$ of 26 min, whereas with the more usual ammonium chloride cleavage occurred 29 times more slowly. Thus a 4 h treatment with the methylamine buffer system gave 95% removal of the protecting group with no deleterious effects.

N^{ε}-Phenylacetyl protection has been used for the side chain of lysine.[31] The derivative is prepared by direct acylation of the lysine–copper complex and is stable to a variety of conditions, including HBr in acetic acid and sodium in liquid ammonia. The protecting group is, however, particularly easily removed by the enzyme penicillin amidohydrolase (EC 3.5.1.11), which is better known for cleaving the phenylacetic acid moiety from penicillin G to give 6-aminopenicillanic acid. Thus, the unusual enzyme specificity is used in this case to provide a selectively cleaved protecing group for the side chain of lysine.

A wide range of sulphonyl-based protecting groups have been used to protect the guanidine function of arginine, the most well established being the mesitylene-sulphonyl (Mts) protecting group. This group remains mostly intact during careful Tfa treatment but is readily removed by the action of trifluoromethane sulphonic acid in the presence of thioanisole in Tfa. Several syntheses [32–35] have used the Mts protecting group and it seems to provide a reasonable degree of protection for guanidine. The use and preparation of the 4-methoxy-2,6-dimethylbenzenesulphonyl (Mds) (8a) group have been re-examined.[36, 37] Although a synthesis of dynorphin (1—13) [36] had been used to confirm the usefulness of the Mds protecting group, further investigations [37] threw some doubt on the exact nature of the protecting group. This arose from the initial

[29] J. W. Scott, D. Parker, D. R. Parrish, and J. W. Scott, *Synth. Commun.*, 1981, **11**, 303.
[30] G. C. Dubois, E. A. Robinson, J. K. Inman, R. N. Perham, and E. Apella, *Biochem. J.*, 1981, **199**, 335.
[31] F. Brtnik, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 1983.
[32] H. Yajima, Y. Minamitake, S. Funakoshi, K. Akaji, M. Oishi, Y. Akazawa, T. Segawa, Y. Nakata, and A. Inoue, *Chem. Pharm. Bull.*, 1981, **29**, 2587.
[33] K. Yasumura, K. Okamoto, S. Shimamura, and H. Yajima, *Chem. Pharm. Bull.*, 1981, **29**, 1088.
[34] K. Akaji, N. Fujii, H. Yajima, M. Moriga, M. Murakami, K. Mizuta, and A. Takagi, *Chem. Pharm. Bull.*, 1981, **29**, 3080.
[35] K. Akaji, N. Fujii, H. Yajima, M. Moriga, M. Aono, and A. Takagi, *Int. J. Pept. Protein Res.*, 1981, **18**, 180.
[36] M. Wakimasu, C. Kitada, and M. Fujino, *Chem. Pharm. Bull.*, 1981, **29**, 2592.
[37] C. W. Smith, G. Skala, and J. R. Boal, *J. Chem. Soc., Chem. Commun.*, 1981, 1243.

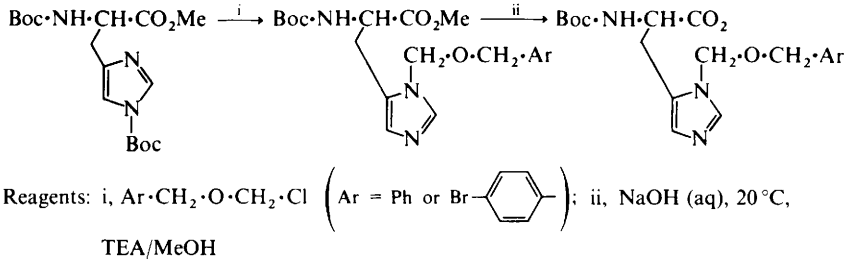


(8) a; Mds: R^1 = Me, R^3 = OMe, R^5 = Me
b; Pme: $R^1 = R^2 = R^3 = R^4 = R^5$ = Me
c; Mtr: $R^1 = R^2 = R^5$ = Me, R^3 = OMe

chlorosulphonation of 3,5-dimethylanisole, which is used to prepare the starting sulphonyl chloride. Thus the presence of some 3-chloro-2,4-dimethyl-6-methoxysulphonyl chloride along with the required Mds chloride caused some difficulties in the isolation of the protected amino-acid. Detailed investigation [38] of the substituted benzenesulphonyl groups (8a), (8b), and (8c) has been carried out. The Mds and Pme derivatives are both resistant to treatment with Tfa and may, therefore, be used where intermediate protection of other groups is by treatment with Tfa; on the other hand, the Mtr protecting group (8c) is most useful when Tfa treatment is not required. In fact, the $t_{\frac{1}{2}}$ values of the protecting groups on treatment with Tfa/thioanisole are Mts 30 min, Mds 1—2 h, and Pme >4 h. Thus, the Pme group is the only one that is sufficiently stable to withstand the conditions of solid-phase peptide synthesis where there is repeated Tfa treatment.

The diphenyl-4-pyridylmethyl group has been proposed for the protection of the imidazole nitrogen of histidine.[39] In contrast to trityl, the group is stable to acid and is cleaved by hydrogenolysis over palladium; the group may also be removed reductively by treatment with zinc in 80% acetic acid or by electrolysis. As with trityl, it is assumed that the substituent is in the τ position on the imidazole ring.

The π-benzyloxymethyl or π-bromobenzyloxymethyl group has been shown to be highly successful for the protection of the π-nitrogen of histidine,[40] being introduced by the method shown in Scheme 2. The protecting group overcomes the problems of racemization of histidine and may be used in conjunction with other benzyl-based protecting groups in solution or solid-phase synthesis. The group is unaffected by exposure to nucleophiles, bases, or Tfa but is rapidly cleaved by treatment with saturated HBr in acetic acid. Cleavage also occurs on hydrogenolysis over palladium/charcoal in 80% acetic acid. The utility of this



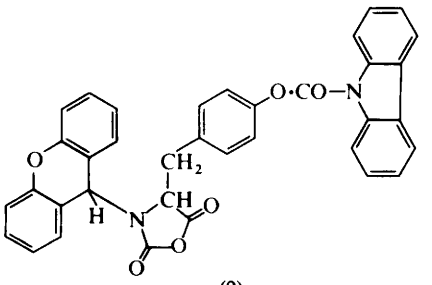
Scheme 2

[38] M. Fujino, M. Wakimasu, and C. Kitada, *Chem. Pharm. Bull.*, 1981, **29**, 2825.
[39] S. Coyle, A. Hallett, M. S. Munns, and G. T. Young, *J. Chem. Soc., Perkin Trans. 1*, 1981, 522.
[40] T. Brown and J. H. Jones, *J. Chem. Soc., Chem. Commun.*, 1981, 648.

protecting group has been proven in test cases that include a solid-phase synthesis of [Ile^5]angiotensin II.

Little work has been carried out on side-chain carboxy protection. In a study of deprotecting systems [38] it was found that treatment with Tfa led to less than 10% of the β-peptide when peptides containing an asparagine/alanine linkage were exposed to trifluoroacetic acid, frequently only 1—2% of the β-peptide being observed. However, treatment with anhydrous HF, trifluoromethanesulphonic acid in Tfa, or methanesulphonic acid led to much higher levels of the β-peptide. This very low tendency of Tfa to promote formation of the β-peptide was also corroborated by other workers who found 0.1% or less of the β-isomer.[41] The same workers found that the best yield of peptide containing the pure α-linkage was obtained by debenzylation of the β-benzyl aspartate peptides by HBr in acetic acid. In this study the nature of the linkage was characterized by ^{13}C n.m.r. as it has been shown that there are marked differences between the ^{13}C signal of the α- and β-linked peptide.[42]

Protection of the phenolic hydroxy group of tyrosine has received some attention. The 9-carbazolylcarbonyl protecting group has been used to mask the phenolic group of the 9-xanthenyl-NCA derivative of tyrosine.[43] This intermediate (9) was used along with other 9-xanthenyl-NCA derivatives for the preparation of



(9)

a cyclic leucine-enkephalin analogue. The carbazolylcarbonyl protection is stable to the mild acid conditions used to cleave the xanthenyl protection, but it is smoothly removed by treatment with hydrazine (10 equivalents) over two days at room temperature. The use of unprotected 3′-iodotyrosine in the synthesis of the peptides containing this amino-acid has been described.[44] The simple model peptides were synthesized with Boc for amino protection and coupling by the DCCI/HOBt method; at the end of the synthesis after alkaline hydrolysis it was clear that the 3′-iodotyrosine had survived the synthesis without protection.

The preparation of sulphated tyrosine derivatives has for some time presented problems; however, the use of tyrosine sulphate as its hemi-barium salt has alleviated the difficulties of sulphating the peptide after synthesis. Two syntheses utilizing the tyrosine sulphate hemi-barium salt have been recorded. In the first,

[41] V. Saudek, H. Pivcova, and J. Drobnik, *Biopolymers*, 1981, **20**, 1615.
[42] H. Pivcova, V. Saudek, J. Drobnik, and J. Vlasak, *Biopolymers*, 1981, **20**, 1605.
[43] J. Halstroem, M. A. Qasim, K. Brunfeldt, and E. Nebelin, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **326**, 593.
[44] B. Rzeszotarska, B. Nadolska, and J. Tarnawski, *Liebigs. Ann. Chem.*, 1981, 1294.

CCK-PZ (25—33)[45] analogues were synthesized; in this case the salt was used in combination with benzyloxycarbonyl for amino-group protection, and the sulphated tyrosine showed no side reactions during hydrogenolysis or acidolysis. A related synthesis of CCK-PZ (24—33)[46] used the Fmoc group for amino protection; on this occasion the sulphated tyrosine was again found to be satisfactory during both DCCI/HOBt coupling and deprotection cycles with piperidine. These two syntheses serve to confirm the fact that tyrosine sulphate hemi-barium salt can be used satisfactorily for the synthesis of sulphated peptides.

The choice of protection for the thiol group of cysteine has always been difficult. The favoured acetamidomethyl group has been successfully used on several occasions, including a synthesis of bovine prothrombin precursor (18—23).[47] In this synthesis, iodine oxidation in methanol gave a 62—69% yield of the required cyclic analogue. The use of a tritiated acetamidomethyl protecting group has been demonstrated in the synthesis of apamin,[48,49] the radioactive protecting group being used to facilitate chromatography and to monitor the progress of reactions.

The diphenyl-4-pyridylmethyl group[39] has also been applied to cysteine protection. The protecting group is readily introduced by reaction of the corresponding alcohol with cysteine hydrochloride using $BF_3.Et_2O$ in acetic acid. The protecting group is stable to HBr in acetic acid and Tfa over 48 hours but may easily be cleaved by treatment with zinc in acetic acid, mercury(II) acetate, iodine, or electrolysis. The group possesses the same advantages that have been demonstrated for the picolyl esters when used for carboxy protection.

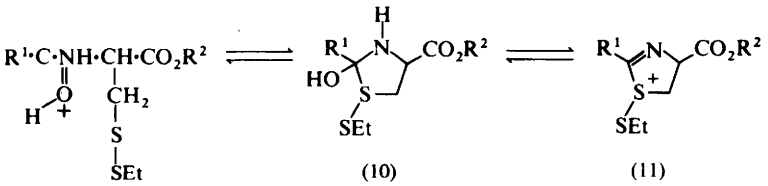
The 2,4,6-trimethylbenzyl group has been used for the protection of cysteine sulphur in syntheses of oxytocin and arginine vasopressin.[50] The protecting group resists the action of Tfa and HBr in acetic acid, although some cleavage is observed with the latter system at 40 °C. Removal of the protection is achieved by treatment with anhydrous hydrogen fluoride at 0 °C, in the presence of anisole, requiring 30 min for complete cleavage. Alternatively, removal may be effected by treatment with trifluoromethanesulphonic acid.

Protection of the thiol function as a t-butylthioether[51] has been investigated in detail. The protecting group is best introduced by reaction of cysteine with t-butanol in the presence of anhydrous HCl. The thioether is stable to acid and alkali and to iodine oxidation; however, it may be readily removed by treatment with *o*-nitrophenylsulphenyl chloride, giving the relatively activated Cys(Nps) derivative that may then be used in the formation of disulphides. Thus, specific disulphide linkages may be generated in peptides containing up to three cysteine residues by the correct combination of t-butyl, acetamidomethyl, and benzyl protecting groups.[52]

[45] L. Moroder, L. Wilschowitz, M. Gemeiner, W. Goehring, S. Knof, R. Scharf, P. Thamm, J. D. Gardner, T. E. Solomon, and E. Wünsch, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 929.
[46] E. Wünsch, L. Moroder, L. Wilschowitz, W. Goehring, R. Scharf, and J. D. Gardner, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 143.
[47] D. H. Rich, M. Kawai, H. L. Goodman, and J. W. Suttie, *Int. J. Pept. Protein Res.*, 1981, **18**, 41.
[48] A. R. Nuriddinov, V. V. Okhanov, V. I. Tsetlin, and V. T. Ivanov, *Bioorg. Khim.*, 1981, **7**, 5.
[49] A. R. Nuriddinov, V. I. Tsetlin, and V. T. Ivanov, *Bioorg. Khim.*, 1981, **7**, 165.
[50] F. Brtnik, M. Krojidlo, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 286.
[51] J. J. Pastuszak and A. Chimiak, *J. Org. Chem.*, 1981, **46**, 1868.
[52] J. J. Pastuszak and A. Chimiak, *Rocz. Chem.*, 1977, **51**, 1567.

Protection of the cysteine thiol using the non-symmetrical disulphide *S*-t-butylthiocysteine has been demonstrated in a synthesis of somatostatin.[53, 54] The total sequence was assembled from four fragments by DCCI/HONSu fragment coupling with the cysteine residues being protected as their *S*-t-butylthio derivatives. After Tfa deprotection, the butylthio protecting group was removed by the reaction with either triphenyl or tri-n-butyl phosphine in trifluoroethanol. Obviously scavengers containing thiol groups need to be avoided in the Tfa deprotection in order to prevent disulphide interchange. Removal of the protecting group by tri-n-butyl phosphine in 80% acetic acid gave the dihydro derivative, which was subsequently oxidized to somatostatin.

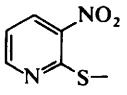
The *S*-alkylthio derivatives of cysteine suffer from the disadvantage that *N*-acyl and *N*-urethane derivatives undergo disulphide interchange in acidic media.[55] Thus, the Boc group cannot be removed from Boc-Cys(SEt) derivatives without disulphide interchange. The *S*-ethyl and *S*-isopropyl groups both suffer from this problem, but the *S*-t-butyl derivatives mentioned above only undergo disulphide formation to a limited extent. The mechanism proposed for such a disulphide interchange involves the intermediates (10) and (11) shown in Scheme 3. Such acid-catalysed disulphide interchange has, however, been used to advantage.[56] In this case the non-symmetrical disulphide of insulin A(19—21)–B(15—27) was formed from the two components containing *S*-ethyl protection for the thiol groups of cysteine.



(10) (11)

Scheme 3

The 3-nitro-2-pyridylsulphenyl group (12) has also been used to protect cysteine in the form of a non-symmetrical disulphide.[57] The protecting group may be introduced by reaction of the thiol (cysteine) with the corresponding sulphenyl chloride. The disulphide produced is stable to Tfa, HCl, and HF but is readily cleaved by aqueous tri-n-butyl phosphine or by the action of other thiols; in the latter case a new non-symmetrical disulphide is produced. Thus, the Npys



(12)

[53] L. Moroder, M. Gemeiner, W. Goehring, E. Jaeger, J. Musiol, R. Scharf, H. Stocker, E. Wünsch, L. Pradayrol, N. Vaysse, and A. Ribet, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 697.

[54] L. Moroder, M. Gemeiner, M. Goehring, E. Jaeger, P. Thamm, and E. Wünsch, *Biopolymers*, 1981, **20**, 17.

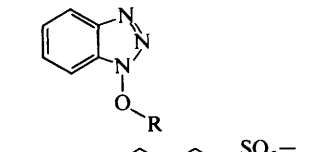
[55] H. Kunzek, R. Haupt, and G. Huschert, *J. Prakt. Chem.*, 1980, **322**, 381.

[56] G. Losse, H. Stange, and W. Naumann, *Liebigs Ann. Chem.*, 1981, 143.

[57] R. Matsueda, T. Kimura, E. T. Kaiser, and G. R. Matsueda, *Chem. Lett.*, 1981, 737.

protecting group has considerable potential for the activation of thiols, as well as protection. This ability to form a symmetrical disulphide probably means that the group will be of considerable use in semisynthesis.

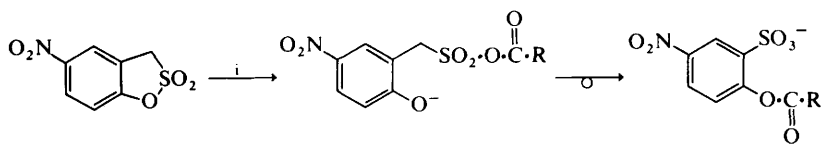
Formation of the Peptide Bond.—A wide variety of new coupling methods have been reported, but many of these are only extensions of previously reported coupling methods. For example, the hydroxybenzotriazole derivatives (13a) and (13b) containing the electron-withdrawing β-naphthalenesulphonyl [58] and trifluoroacetyl [59] groups have little to recommend them over their predecessors.



(13) a; R = SO_2—

b; R = CF_3·CO—

5-Nitro-(3*H*)-1,2-benzoxathiole *SS*-dioxide (14) has been used as a coupling reagent,[60] the compound (14) reacting according to the mechanism shown in Scheme 4. Using the triethylammonium or tetra-n-butyl ammonium salts of an *N*-protected amino-acid, the mixed anhydride (15) is initially obtained. However, evidence is produced to show that this mixed anhydride rearranges to the active ester (16) prior to attack by the incoming amino component. The method showed an acceptable level of racemization in the Anderson test (0.7%), but unfortunately side reactions were observed when asparagine or serine was coupled.



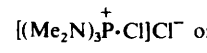
(14) (15) (16)

Reagent: i, $RCO_2^-Bu^n{}_4N^+$

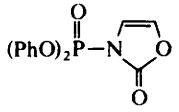
Scheme 4

Many phosphorous-containing coupling reagents are again in evidence. The intermediate (17) may be generated by a reaction of HMPA with hexachloroethane [61] or by a reaction of HMPA with thionyl chloride.[62] The intermediate (17) in conjunction with hydroxybenzotriazole may be used as a one-pot coupling reagent; examples are given of the syntheses of a dozen or so dipeptides. A variety of phosphonates and phosphinates have been used for the formation of peptide

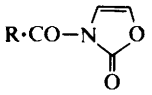
[58] S. D. Sharma and K. B. Mathur, *Indian J. Chem., Sect. B*, 1981, **20**, 227.
[59] L. A. Pavlova, Y. A. Davidovich, and S. V. Rogozhin, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1981, 1413.
[60] M. Wakselman and F. Acher, *J. Chem. Soc., Chem. Commun.*, 1981, 632.
[61] R. Appel and E. Hiester, *Chem. Ber.*, 1981, **114**, 2649.
[62] R. Appel, U. Glaesel, and V. I. Glaesel, *Chem. Ber.*, 1981, **114**, 1542.



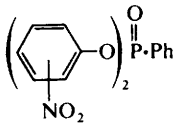
(17)



(18)



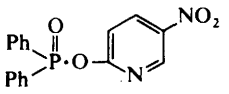
(19)



(20)

bonds. Diphenyl-2-oxo-3-oxazolinyl phosphonate (18)[63] has been used to form active esters of type (19), which couple directly with an amino component with virtually no racemization. Similarly, bis-(*o*- or *p*-nitrophenyl) phenylphosphonate (20) has been used as a coupling reagent with the preparation of leucine-enkephalin acting as an example.[64, 65] This work demonstrates the benefit of using tetra-n-butylammonium salts in preference to the more usual alternatives. For example, $Z(Gly)_4OH$ was found to be only sparingly soluble in DMF after the addition of triethylamine; however, the tetra-n-butylammonium salt was readily soluble and gave an 82% yield using a reagent of the type (20) when coupled to glycylglycine ethyl ester. In contrast, the triethylammonium salt gave only a 25% yield; thus the use of the n-butylammonium salt is advocated to increase both solubility and product yield.

The related 5-nitropyridyldiphenylphosphinate (21) appears to be particularly useful as a condensing reagent in cyclization.[66] In a synthesis of gramicidin S very little racemization was observed and a coupling yield of 80% was recorded. Various other dialkyl and mixed aryl/alkyl nitropyridylphosphinates were investigated, but compound (21) proved to be the most satisfactory.

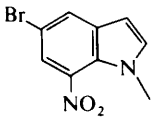


(21)

Relatively few new active esters have been reported, although the use of hydroxyiminocyano-α-picoline esters has been described,[67] as have new methods of preparing hydroxysuccinimide active esters.[68] The latter paper claims that disuccinimido-carbonate and succinimidodiphenyl phosphate are superior reagents for the preparation of *N*-protected amino-acid hydroxysuccinimide active esters.

[63] T. Kunieda, Y. Abe, T. Higuchi, and M. Hirobe, *Tetrahedron Lett.*, 1981, **22**, 1257.
[64] Y. Watanabe and T. Mukaiyama, *Chem. Lett.*, 1981, 285.
[65] Y. Watanabe, N. Morito, K. Kamekawa, and T. Mukaiyama, *Chem. Lett.*, 1981, 65.
[66] T. Mukaiyama, K. Kamekawa, and Y. Watanabe, *Chem. Lett.*, 1981, 1367.
[67] T. Kubiak and S. Drabarek, *Pol. J. Chem.*, 1981, **55**, 73.
[68] H. Ogura and K. Takeda, *Nippon Kyata Kaishi*, 1981, 836.

Symmetrical anhydrides have become increasingly popular in solid-phase synthesis, particularly as they are not as susceptible to hydrolysis as might have been expected and may even be used in aqueous DMF.[69]



(22)

The 5-bromo-7-nitroindolyl protecting group (22) provides an interesting approach in that the group normally provides protection of the carboxy group, but when irradiated at 420 nm the carboxy is activated and may be coupled with an amino component.[70] The Anderson, Bodanszky, Izumiya, and Weygand racemization tests showed less than 1% racemization when the irradiation was carried out at −25 °C.

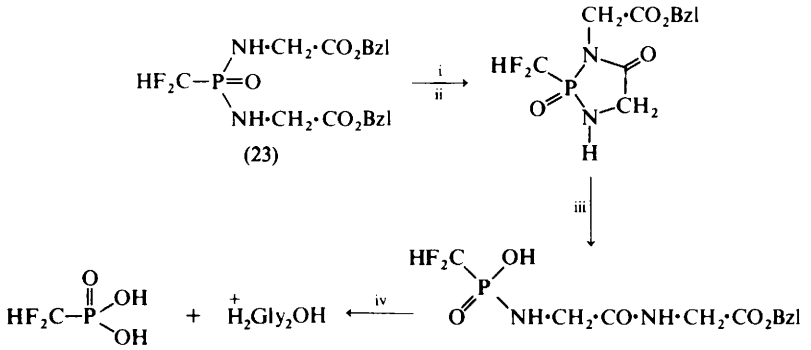
A rather novel new approach is provided by the activation of thiolcarboxy groups by silver.[71,72] The technique, which has so far been applied mainly to solid-phase work, utilizes thiol acids that are prepared from the corresponding nitrophenyl or hydroxysuccinimide active esters by treatment with sodium hydrosulphide or ethyldi-isopropyl ammonium hydrosulphide. A thiol hexapeptide acid that was synthesized on a solid-phase support was coupled to a free zwitterionic tetrapeptide[71] by treatment with 2.2 equivalents of silver nitrate at pH 7, a 40% coupling being observed in one hour. The product was identical to the normal peptide and precipitation of silver sulphide indicated that the reaction was over in a few minutes. Mercury(II) and copper(II) may also be used to initiate coupling of thiol acids. More conventional activation of the thiol carboxylic acids may be achieved in non-aqueous solvents by treatment with 2,4-dinitrofluorobenzene, *N*-bromosuccinimide,[72] or by treatment with DCCI. Racemization under Izumiya conditions could be reduced to a very low level using this approach, and in general racemization was less than 1%. In competitive coupling the thiol carboxylic acid couples much more efficiently than the normal carboxylic acid; thus hydrolysis does not cause serious problems.

A synthesis of [Gly[17]]-β-endorphin has been carried out utilizing this silver-mediated coupling approach;[73] the 1—17 and 18—31 fragments were assembled by conventional solid-phase methods using the Fmoc group for α-amino protection. In this case the [thiol-Gly[17]]endorphin (1—17) was activated by treatment with silver nitrate in the presence of hydroxysuccinimide, which gave yields of between 30 and 40% of the coupled 1—31 endorphin analogue. Thus, it appears from the preceding papers that synthesis of a thiol peptide fragment on solid phase followed by cleavage from the resin and subsequent coupling with silver salts provides a totally new method of fragment condensation.

[69] N. L. Benoiton and F. M. F. Chen, *FEBS Lett.*, 1981, **125**, 104.
[70] S. Pass, B. Amit, and A. Patchornik, *J. Am. Chem. Soc.*, 1981, **103**, 7674.
[71] J. Blake, *Int. J. Pept. Protein Res.*, 1981, **17**, 273.
[72] D. Yamashiro and J. Blake, *Int. J. Pept. Protein Res.*, 1981, **18**, 383.
[73] J. Blake and C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4055.

Peptide-bond formation mediated by tin(IV) compounds has also been recently reported.[74, 75] However, the examples given only involved the coupling of glycine residues and thus the method has not been proven to any extent.

Peptide-bond formation by intramolecular reaction has been demonstrated using the compound (23) by the routes shown in Scheme 5.[76] Although this method is ingenious, it does not seem capable of extension to more complex examples.

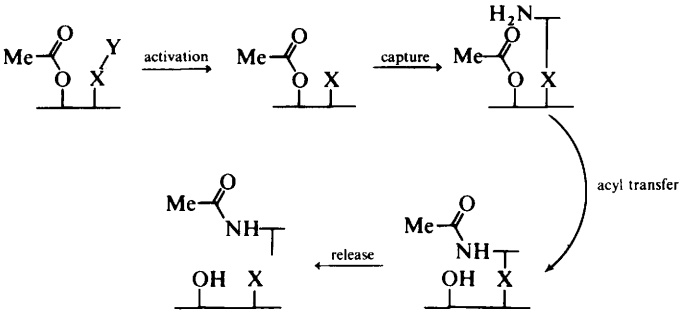


Reagents: i, DBU; ii, NaOH; iii, H_2O; iv, NaOH, H^+

Scheme 5

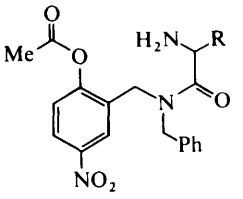
A much more general approach to intramolecular amide-bond formation has been put forward by Kemp, and the present state of the method has been reviewed.[77] A variety of approaches have been tried, including thiol capture of *N*-terminal cysteine by mercury and disulphide systems.

The overall approach to intramolecular peptide-bond formation is outlined in Scheme 6.[78] The method indicated in this scheme involves *O*- to *N*-acyl transfer and has been demonstrated by transfer in 9- and 12-membered ring compounds.

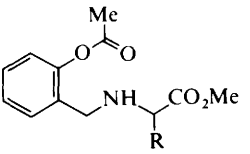


Scheme 6

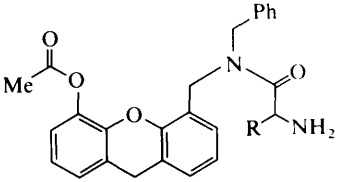
[74] N. M. Oleinik, I. P. Garkusha-Bozhko, and L. M. Litvinenko, *Zh. Org. Khim.*, 1980, **16**, 1094.
[75] L. M. Litvinenko, I. P. Garkushabozhko, N. M. Oleinik, M. S. Klebanov, and Y. A. Nesterenko, *Zh. Org. Khim.*, 1981, **17**, 307.
[76] M. Mulliez, *Tetrahedron*, 1981, **37**, 2027.
[77] D. S. Kemp, *Biopolymers*, 1981, **20**, 1793.
[78] D. S. Kemp, D. J. Kerkman, S.-L. Leung, and G. Hanson, *J. Org. Chem.*, 1981, **46**, 490.



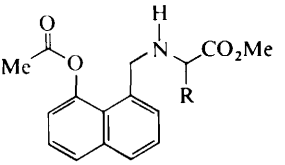
(24)



(25)



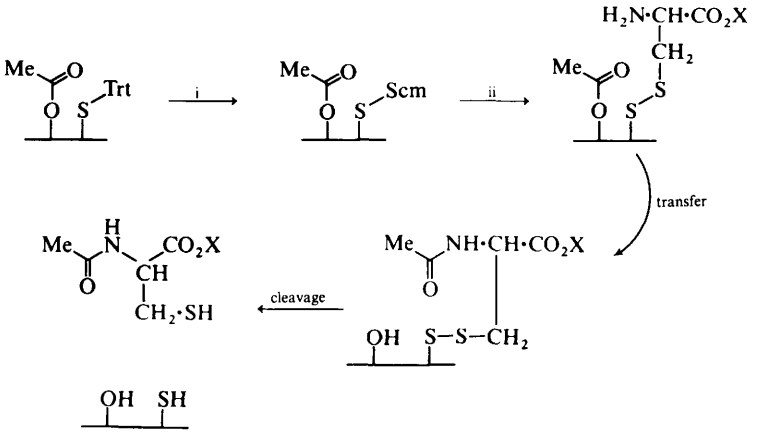
(26)



(27)

Acyl transfer has been demonstrated and the kinetics have been elucidated in many cases; compounds (24)—(27) inclusive represent the range of compounds studied. Compounds (24) and (26) showed the greatest rates of acyl transfer, although (26) was rather less activated than (24); in fact without the nitro group compound (24) did not undergo acyl transfer. The compounds (24) and (26) tolerate bulk at the amine site rather more than (25) and (27), and in all cases the solvent is particularly important.

Model systems that demonstrate peptide-bond formation by prior thiol capture have also been examined.[79] The general approach is that shown in Scheme 7, and in fact compounds (28)—(30) have been used to demonstrate acyl transfer. Yields

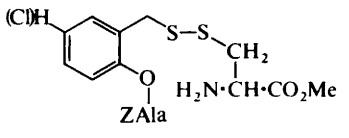


Reagents: i, Scm·Cl; ii, H-Cys-OX

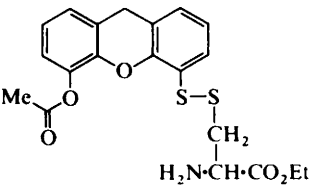
Scheme 7

[79] D. S. Kemp, S.-L. Leung, and D. J. Kerkman, *Tetrahedron Lett.*, 1981, **22**, 181.

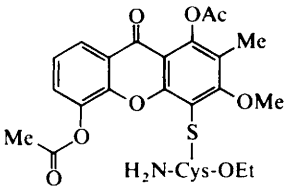
of up to 60% were recorded for the reaction, although the amide-bond-forming step was relatively slow and led to complications in that disulphide interchange became competitive. The reactions by which (28)—(30) are formed from the corresponding Scm derivatives and cysteine give poor yields and would require improvement before the method could be generally applied.



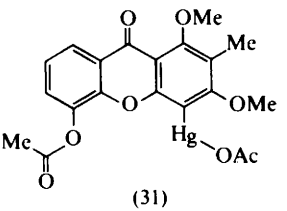
(28)



(29)



(30)



(31)

Thiol capture by organomercury derivatives has also been investigated.[80] The compound (31) was found to capture cysteine ethyl ester in a highly efficient manner at concentrations of 10^{-2}—10^{-3} M using DMF or DMSO as solvent. Intramolecular transfer of the acyl group then occurred with a $t_{\frac{1}{2}}$ of 24 h in DMF and 8 h in DMSO. After transfer the thio compound can be liberated by treatment with iodine. The methoxy groups present in this compound are an artefact of availability and in fact are undesirable as they increase the lability of the carbon–mercury bond.

The four-component condensation method has seen little application, although one paper[81] has described the use of 9-aminomethylfluorene as the amine component. The base-labile protecting group was removed after formation of the peptide by treatment with pyridine and 1,8-diazabicyclo(5,4,0)undec-7-ene (DBU).

Racemization.—Reaction of *N*-protected amino-acids with di-imides has once again been the subject of considerable interest. The formation of 2-alkoxy-5(4H)oxazolones from *N*-alkoxycarbonyl amino-acids is now well established.[82-84] Reaction of Z- or Boc-valine (1 equivalent) with *N*-ethyl-γ-dimethylaminopropylcarbodi-imide hydrochloride (2 equivalents in dichloromethane) rapidly gives 70—80% of the symmetrical anhydride. In the presence of excess di-imide a second product may be isolated which, after removal of the

[80] D. S. Kemp and D. J. Kerkman, *Tetrahedron Lett.*, 1981, **22**, 185.
[81] C. F. Hoyng and A. D. Patel, *J. Chem. Soc., Chem. Commun.*, 1981, 491.
[82] N. L. Benoiton and F. M. F. Chen, *J. Chem. Soc., Chem. Commun.*, 1981, 1225.
[83] E. Atherton, N. L. Benoiton, E. Brown, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1981, 336.
[84] N. L. Benoiton and F. M. F. Chen, *Can. J. Chem.*, 1981, **59**, 384.

anhydride, shows the characteristics of the corresponding oxazolone.[84] The presence of a tertiary amine (for example triethylamine) leads to the conversion of the symmetrical anhydride to the corresponding oxazolone. In fact after 15 min 80% of the symmetrical anhydride and 3% of the corresponding oxazolone are present. However, after 24 h only 12% of the anhydride remained and the concentration of the oxazolone had risen to 38%. During this period the *N*-acylurea concentration had also increased from 6 to 40%.[82] Racemization of the intermediate oxazolone has always been assumed to be low, and this was confirmed. However, in the presence of excess (0.2 equivalents) triethylamine 15% racemization was observed, and with 1 equivalent excess triethylamine 50% racemization was found. Also, the acylation catalyst dimethylaminopyridine was found to cause pronounced racemization. The observation of a stable *O*-acylisourea from the reaction of Z-valine with di-isopropylcarbodi-imide reported previously[85] has been shown to be in error; in fact under the conditions of the experiment the symmetrical anhydride and the corresponding urea are the major products and no *O*-acylisourea is observed.[86] The effects of temperature and solvent on the rate of racemization have been investigated by studying the coupling between Z-Gly-X-OH and H-Lys(Z)-OBzl.[87] In apolar solvents the order of racemization is Phe > Leu $\simeq$ Ala > Val $\simeq$ Ile, but in polar solvents the order becomes Ile > Val > Phe > Leu $\simeq$ Ala. Coupling by the DCCI/HOBt method showed that racemization was least in dichloromethane followed by DMF/pyridine and DMF/water, also that the racemization increased with temperature. The additive HOOBt was found to be better than both HOBt and HONSu when polar solvents were used as the coupling medium.

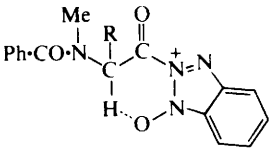
Asymmetric induction during peptide-bond formation has also been observed.[88, 89] When an activated D residue in a protected dipeptide is coupled, it racemizes at twice the rate of the corresponding dipeptide containing an L-amino-acid as the second residue. This finding, which was demonstrated by couplings in DMF, clearly shows that the chance of racemization is greater when residues of differing chirality are coupled. It is also interesting that when racemic 2,4-dialkyl-5(4H)oxazolones are reacted with L-amino-acid esters an excess of the DL isomer is produced in apolar solvents and that in polar solvents the LL isomer predominates.[89] Thus, the asymmetric induction observed in such a coupling is highly dependent on the nature of the solvent, and indeed it has also been shown to be dependent on temperature. The proceeding experiments used proton n.m.r. to distinguish between the diastereoisomers, basing the concentrations on the signals observed for the methyl ester protons. Asymmetric induction was also observed when benzoyl dipeptide methyl esters were formed from benzoyl valine and valine methyl ester or proline methyl ester.[90] In this paper it is pointed out that in order to maximize the separation of the methyl ester signals in the proton n.m.r. it is necessary for the aromatic ring of the benzoyl group to be present as this increases the separation of the methyl ester signals by 0.05 p.p.m.

[85] H. J. Bates, J. H. Jones, and M. J. Witty, *J. Chem. Soc., Chem. Commun.*, 1980, 773.
[86] N. L. Benoiton and F. M. F. Chen, *J. Chem. Soc., Chem. Commun.*, 1981, 543.
[87] N. L. Benoiton and K. Kuroda, *Int. J. Pept. Protein Res.*, 1981, **17**, 197.
[88] N. L. Benoiton, K. Kuroda, and F. M. F. Chen, *Tetrahedron Lett.*, 1981, **22**, 3359.
[89] N. L. Benoiton, K. Kuroda, and F. M. F. Chen, *Tetahedron Lett.*, 1981, **22**, 3361.
[90] J. S. Davies and R. J. Thomas, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1639.

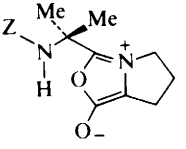
The racemization encountered on coupling *N*-methylated amino-acids has also been assessed by a similar n.m.r. method.[91] As expected, it was found that the *N*-methyl amino-acids were more susceptible to racemization on coupling. Reaction of DCCI with benzoyl-*N*-methyl amino-acids produces an oxazalonium ion that is bright yellow in colour, and this pseudo-aromatic oxazalonium ion (32) is clearly detectable by n.m.r. The racemization of *N*-methyl amino-acids is clearly dependent on temperature; by coupling at $-5\,^{\circ}C$ in the presence of HONSu or HOBt racemization can be virtually eliminated. If, however, a pre-activation technique is used with hydroxybenzotriazole, considerable racemization ensues. It is proposed that an activated intermediate (33) exists in which the α-hydrogen is rendered particularly labile by the proximity of the oxygen of the hydroxybenzotriazole.



(32)



(33)



(34)

From n.m.r. experiments it is clear that the α-hydrogen in benzoyl-*N*-methyl amino-acid hydroxybenzotriazole esters is exchanged very rapidly with D_2O, whereas the corresponding benzyloxycarbonyl derivatives show no exchange of the α-CH whatsoever. This finding has particularly serious implications for fragment couplings, and it is emphasized that, when couplings of this type are carried out, DCCI/HOBt should not be used and HONSu may safely be used as the additive, particularly if the temperature is maintained at about $-5\,^{\circ}C$. Racemization of proline residues during peptide-bond formation has also been observed by proton n.m.r. in the synthesis of alamethicin fragments.[92] In this case a related oxazolonium ion (34) is implicated, but racemization was suppressed by the addition of either HONSu or HOBt.

The use of zinc chloride as an additive in DCCI couplings has been reported,[93] and it is claimed that racemization during coupling is considerably reduced by it. In a synthesis of methionine-enkephalin, which was used to exemplify the technique, racemization was inhibited and coupling yields were maintained at about 70%.

The racemization during 6M acid hydrolysis of free amino-acids and protein-bound amino-acids has been studied by g.c.–m.s.[94] The electronic and steric effects of the side chain of the amino-acids on racemization are studied by separation of enantiomers on chirasil-Val prior to mass spectrometry. A more sophisticated approach using hydrolysis with 6M DCl in place of HCl has also been reported.[95–97] Using this technique amino-acids that are racemized during

[91] J. S. Davies and A. K. Mohammed, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2982.
[92] R. Nagaraj and P. Balaram, *Tetrahedron*, 1981, **37**, 2001.
[93] E. Berger, K. Neubert, H.-D. Jakubke, and G. L. Kovacs, *Z. Chem.*, 1981, **21**, 37.
[94] R. Liardon and R. Jost, *Int. J. Pept. Protein Res.*, 1981, **18**, 500.
[95] S. Kusumoto, M. Matsukura, and T. Shiba, *Chem. Lett.*, 1981, 1017.
[96] S. Kusumoto, M. Matsukura, and T. Shiba, *Biopolymers*, 1981, **20**, 1869.
[97] R. Liardon, S. Ledermann, and U. Ott, *J. Chromatogr.*, 1981, **203**, 385.

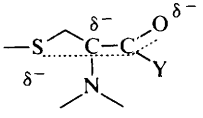
hydrolysis are labelled with deuterium, then after conversion to the trifluoroacetyl amino-acid isopropyl ester g.c. is carried out on a chiral phase to separate the enantiomers. The extent of racemization can then be estimated by monitoring a specific peak in the mass spectrum. Thus, for example, the peak at *m/e* 140 may be monitored to check racemization of alanine, and if the peak corresponding to the D enantiomer from the chiral-phase g.c. is examined then the extent of racemization during hydrolysis can be checked by comparing the peak at 140 with that at 141, which will have been produced by deuterium labelling during the hydrolysis. An interesting finding from this work is that deuterium exchange readily occurs at some other positions in the amino-acid side chain. For example, the hydrogens at the β-CH_2 of the aspartic acid are particularly labile under the normal conditions of hydrolysis.

Racemization under specific conditions has also been examined. In the high-dilution cyclization of Pro-Val-Orn(Z)-Leu-Phe to produce gramicidin S [98] considerable variations in racemization were observed (see Table). The Table shows that racemization is particularly high when preformed active esters are used, particularly when pyridine is used as the solvent. On the other hand, a very good coupling yield and particularly low racemization were observed for the azide method.

Table *Racemization during high-dilution cyclization of* H-Pro-Val-Orn(Z)-Leu-Phe-OX

Method	*Solvent*	*Yield*	*Racemization* (%)
DCC/HONSu (2/4)	DMF	5	8.7
DCC/HONSu (30/2)	DMF	18	1·2
ONSu (active ester)	DMF	36	10.9
ONSu	Pyridine	58	18.6
Azide	Pyridine	65	0.6
Azide	DMF	58	0.5

During the preparation of analogues of angiotensin II, containing hexafluorovaline,[99] it was found that this fluorinated amino-acid showed a very high tendency to racemization. In fact, when coupled using either EEDQ or DCCI/HOBt, extensive racemization was observed. Proof of the delocalization–stabilization that has been proposed [100] to account for the racemization of sulphur-containing amino-acids has been provided by kinetic data.[101] This invokes overlap either of the *p*-orbital of the α-carbanion or of the π-orbital of the delocalized enolate with the *d*-orbital of sulphur as indicated in (35). This *dp* or $d\pi$ overlap thus facilitates racemization at, for example, the α-carbon of cysteine.



(35)

[98] N. Izumiya, T. Kato, and M. Waki, *Biopolymers*, 1981, **20**, 1785.
[99] W. H. Vine, K. Hsieh, and G. R. Marshall, *J. Med. Chem.*, 1981, **24**, 1043.
[100] M. Barber, J. H. Jones, and M. J. Witty, *J. Chem. Soc., Perkin Trans. 1*, 1979, 2425.
[101] M. J. O. Anteunis, F. Borremans, D. Wante, and R. Schrooten, *Tetrahedron Lett.*, 1981, **22**, 3101.

General Deprotection and Side Reactions During Synthesis.—The final deprotection of a synthetic peptide remains one of the major problems in the synthesis of peptides. Concern over the available deprotection methods has been apparent,[5,6] and a general increase in the usage of base-labile protecting groups, particularly the Fmoc group, has been observed. The use of HF is still the method of choice for many research groups, and in a recent synthesis of human parathyroid hormone (1—84)[102] the HF deprotection technique was chosen as the preferred method for the removal of the protecting groups, the deprotection procedure being carefully monitored using h.p.l.c. The use of HOBt in conjunction with water-soluble carbodi-imides is cautioned in this paper as it is possible that the water-soluble carbodi-imides are sufficiently basic to induce racemization. For this reason the addition of HOBt prior to the water-soluble carbodi-imides is recommended.

Many syntheses now seem to use deprotection using the trifluoromethanesulphonic acid/thioanisole/Tfa method.[33—35,50,103,104] The method appears to be particularly useful for the removal of sulphonyl-based arginine protection, and on some occasions the addition of *m*-cresol was also required when mesitylene sulphonyl was used for the protection of arginine.[33] The major problem in this case arises from the side reaction of tyrosine with products that are produced by cleavage of the arginine protecting group. In a synthesis of ostrich CLIP total deprotection including side-chain benzyloxycarbonyl and benzyl esters was achieved by this method in 60 min at 0 °C.

Use of the trifluoromethanesulphonic acid method has gained popularity following an impressive synthesis of ribonuclease A[103,104] in which this method was used for the final deprotection. In this case use of trifluoromethanesulphonic acid was superior to methanesulphonic acid, and the total removal of the 33 protecting groups was effectively achieved.

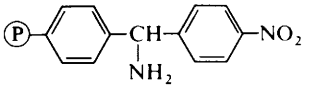
The use of trifluoroacetic acid for the final removal of acid-labile protecting groups is a generally used method. However, in many deprotections side reactions are observed and indeed in a synthesis of somatostatin[54] the presence of 1-t-butyltryptophan in the reaction product was observed when the deprotection was carried out in the presence of anisole and 2-methylindole. The nature of dimeric tryptophan derivatives produced by deprotection in trifluoroacetic acid has been investigated[105] using model tryptophan dipeptides. The rate of by-product formation was determined by t.l.c. and h.p.l.c. and the nature of the product confirmed by both n.m.r. and mass-spectral analysis.

A general, very comprehensive review of the side reactions involved in synthesis and deprotection has been mentioned previously.[8] The review covers side reactions produced by a variety of effects including oxidation and attack by electrophiles.

Repetitive Methods of Peptide Synthesis.—*Solid-phase Peptide Synthesis.* The development of the solid-phase method has continued with many new resins and techniques being described. The use of polyamide resins seems to have become a viable alternative to the conventional polystyrene-based synthesis, particularly

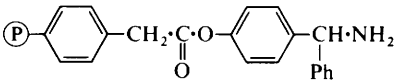
[102] T. Kimua, M. Takai, Y. Masui, T. Morikawa, and S. Sakakibara, *Biopolymers*, 1981, **20**, 1823.
[103] H. Yajima and N. Fujii, *Biopolymers*, 1981, **20**, 1859.
[104] H. Yajima and N. Fujii, *Chem. Pharm. Bull.*, 1981, **29**, 600.
[105] K. Hashizume and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 3806.

when Fmoc is used for α-amino protection. A short review[106] covering recent advances in the nature of the solid-phase supports used for polypeptide and polynucleotide synthesis has appeared.



(36)

Several modifications to existing resins have been made. A *p*-nitrobenzhydrylamine resin (36) has been developed and used in a synthesis of thymosin α_1 that utilized non-acidic conditions with Fmoc being used for intermediate amino-group protection.[107] Cleavage of the Fmoc group by piperidine in DMF was monitored by both ninhydrin and fluorometric methods. A most interesting feature of this synthesis is the step in which the peptide is removed from the resin; in this case cleavage is achieved by a catalytic transfer hydrogenolysis using palladium(II) acetate with cyclohexa-1,4-diene as the hydrogen donor. The author found that even in the presence of sulphur reasonable yields of cleaved peptides could be obtained. Model studies using acetylmethionine showed 64% cleavage from the resin under the conditions of catalytic transfer hydrogenolysis described.

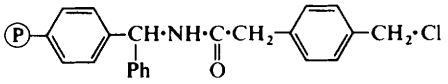


(37)

A multi-detachable benzhydrylamine resin (37) has also been designed and synthesized.[108] The resin linkage is stable to Tfa but may be cleaved by treatment with HF. Bases such as hydrazine and nucleophiles such as cyanide may also be used to liberate protected fragments from the resin. The resin is between 60 and 120 times more stable than the simple benzhydrylamine resin when treated with 50% Tfa, and a 0.014% loss in a 30 min deprotection cycle can be expected. The retro-phenyl ester embodied in the resin confers this Tfa stability and provides the alternative of liberating protecting fragments. Treatment with aqueous bisulphite or oxidative cleavage by $K_3[Fe(CN)_6]$ generates the peptide amide directly by 1—6 elimination. A *p*-methylbenzhydrylamine resin has also been developed,[109] the utility of this resin being demonstrated by the synthesis of the gastrin pentapeptide.

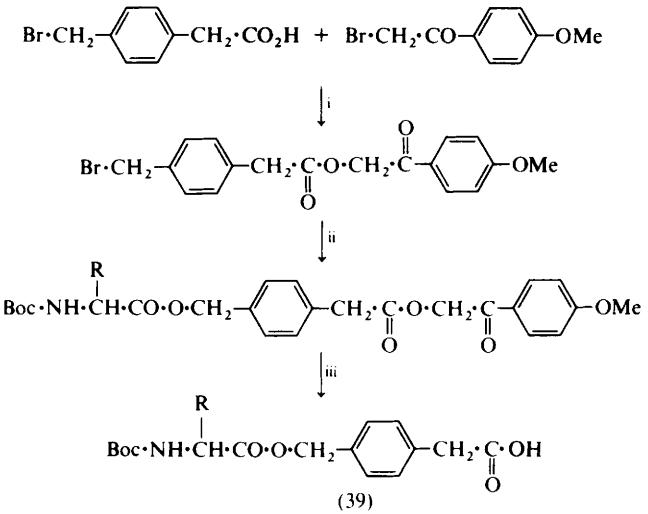
More details of the phenylacetamidobenzylpolystyrene (Pabresin) (38) have been reported.[110] The resin, which was used to synthesize histone H3 (39—43), is stabilized by an acetamido linkage that increases acid stability. Because of this increased acid stability the yield on HF cleavage is decreased as compared to a simple polystyrene resin; a considerable advantage, however, lies in the fact that fewer purification stages are required.

[106] W. Kawanobe, *Kagaku To Kogyo (Tokyo)*, 1982, **35**, 148.
[107] R. Colombo, *J. Chem. Soc., Chem. Commun.*, 1981, 1012.
[108] J. P. Tam, R. D. DiMarchi, and R. B. Merrifield, *Tetrahedron Lett.*, 1981, **22**, 2851.
[109] G. R. Matsueda and J. M. Stewart, *Peptides*, 1980, **2**, 45.
[110] E. Giralt, D. Andreu, M. Pons, and E. Pedroso, *Tetrahedron*, 1981, **37**, 2007.



(38) Pab resin

An improved method for the preparation of 4-(Boc-aminoacyloxymethyl)-phenylacetic acids (39) has been developed.[111] The synthetic route shown in Scheme 8 utilizes an intermediate protecting group that is removable either by

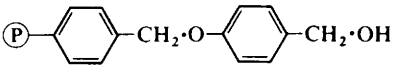


(39)

Reagents: i, KF; ii, Boc-AA-OH/KF; iii, *hv* or Zn/HOAc

Scheme 8

photolysis or by reduction. The acid derivative (39) is then coupled to the aminomethyl resin by the standard DCCI procedure. An improved preparation of the 4-alkoxybenzylalcohol resin (40) has appeared.[112] After a detailed study of the



(40)

kinetics of the reaction between chloromethyl resin and hydroxymethylphenol it was found that the best yield of the alkoxybenzyl resin was produced after an eight-hour reaction of 3 equivalents of hydroxymethylphenol with the chloromethyl resin in the presence of 3 equivalents of sodium methoxide, with dimethylacetamide as solvent. Under these conditions 1—6 elimination to produce the quinone methide and subsequent polymerization were reduced. Analysis showed that no chloromethyl resin remained and that loading with a protected amino-acid produced substitution at the level of 98% of the original chloromethyl sites.

[111] F. S. Tjoeng and G. A. Heavner, *Synthesis*, 1981, 897.
[112] G. Lu, S. Mojsov, J. P. Tam, and R. B. Merrifield, *J. Org. Chem.*, 1981, **46**, 3433.

Trifluoroacetic acid cleavage of a trial peptide after synthesis demonstrated that a very high recovery of peptide could be achieved.

The use of the alkoxybenzyl resin is elegantly demonstrated in a solid-phase synthesis of crystalline glucagon.[113] The synthesis utilized Bpoc for α-amino protection with the side chains being blocked by t-butyl-based protecting groups; histidine was protected as its Dnp derivative and arginine was introduced as nitroarginine. HF was ultimately used to deprotect the peptide, and a good recovery of arginine was obtained from nitroarginine. If hydrogenation was used, a less satisfactory recovery of arginine was observed. Purification using gel filtration and ion exchange gave the 29-residue peptide as a crystalline solid that was shown by gel electrophoresis and a variety of other techniques to be identical with the natural bovine material.

The ease of formation of diketopiperazines (DKP) when using acetamido- and nitrobenzamido-bridged polymer supports has been studied.[114] Boc-D-Val-Pro was synthesized (*a*) on a conventional Merrifield resin, (*b*) on the acetimido-bridged resin (Pab), and (*c*) on the nitrobenzamido-bridged resin. After Boc deprotection the free amino group was liberated by use of di-isopropylamine. All the resins showed DKP formation in parallel with hydroxy generation on the resin. (*a*) showed a very low rate of DKP formation, (*b*) was higher, and (*c*) the highest. The rate of DKP formation is therefore dependent on the electron-withdrawing character of the support, and in the case of (*c*) particular care must be exercised when introducing a third residue.

Polyamide supports have become increasingly important over the past few years, and a detailed account of the synthesis and application of the dimethylacrylamide-based resins used by Sheppard's group in Cambridge has appeared.[115] The original resins were prepared by copolymerization of the monomers (41), (42), and (43) in appropriate ratios, giving a functionalization of 0.3—0.4 milliequiv. g^{-1}.

$CH_2{=}CH{\cdot}CO{\cdot}NMe_2$

(41)

$CH_2{=}CH{\cdot}\overset{\overset{O}{\|}}{C}{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}NH{\cdot}\overset{\overset{O}{\|}}{C}{\cdot}CH{=}CH_2$

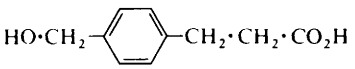
(42)

Boc-β-Ala-NH·$(CH_2)_6$·NH·CO·CH=CH_2

(43)

$CH_2{=}CH{\cdot}CO{\cdot}NMe{\cdot}CH_2{\cdot}CO_2Me$

(44)



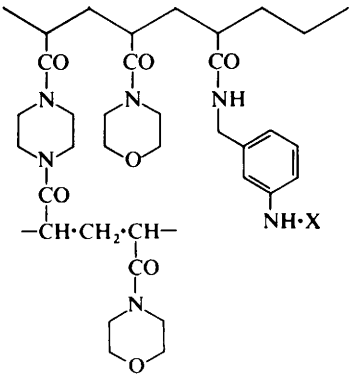
(45)

Some difficulties were encountered and ultimately the monomer (43) was replaced by the sarcosine derivative (44). This produced a fine-beaded polymer, with a resin loading of between 0.13 and 1 milliequiv. g^{-1}. The resin was converted into an amino form by overnight reaction at room temperature with ethylenediamine. A linkage agent (45) was then introduced prior to the addition of the first Boc amino-

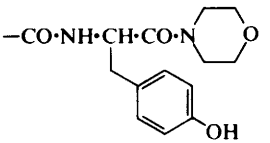
[113] S. Mojsov and R. B. Merrifield, *Biochemistry*, 1981, **20**, 2950.
[114] E. Giralt, R. Eritja, and E. Pedroso, *Tetrahedron Lett.*, 1981, **22**, 3779.
[115] R. Arshady, E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 529.

acid. The paper then illustrates the use of this resin for the preparation of acyl carrier protein (65—74), the synthesis being carried out with Boc for α-amino protection and the Boc amino-acids being introduced as their symmetrical anhydrides in a six-fold molar excess. The paper concludes that polyamides may frequently be used in areas where difficulties have been found with polystyrene resins.

Polyacryloyl morpholine-based matrices have also been synthesized and used for polypeptide synthesis.[116] [118] Polyacryloyl morpholine networks of the type shown in (46) generally have a relatively low loading level of 0.1—0.3 milliequiv. g^{-1}, and the matrix forms a highly solvated gel with properties closely similar to those of the peptide undergoing synthesis. The use of the resins was demonstrated by syntheses of leucine-enkephalin, $BF_3.Et_2O$ being used as the deprotecting reagent as this does not lead to collapse of the polyamide network. In this case a loading of 5 milliequiv. g^{-1} was used, and even at this loading ample space was available within the gel for growth of the peptide undergoing synthesis.[117] A



(46) X = $H_2^+Br^-$, CO·CH·CH$_2$–⟨⟩–OH, or CO–⟨⟩–CH$_2$·OH
NH·CO



(47)

related resin containing the unit (47) has also been prepared.[118] This resin, which shows the same gelation properties as those described for the other polyacryloyl resins, shows both polar and non-polar features within the same resin. Attachment of the growing peptide chain in this case is through a phenyl ester linkage utilizing the phenolic functions of tyrosine.

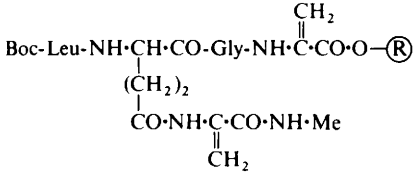
[116] R. Epton, S. J. Hocart, and G. Marr, *Polymer*, 1980, **21**, 481.
[117] R. Epton and A. Williams, *Int. J. Biol. Macromol.*, 1981, **3**, 334.
[118] M. Buckle, R. Epton, and G. Marr, *Polymer*, 1981, **21**, 582.

Other phenolic polymers based on styrene [119] and acrylonitrile [120] have been described. Both polymers are prepared by copolymerization with acetoxystyrene crosslinking with divinylbenzene. With the polystyrene-based phenolic polymer [119] functionalization of the resin is approximately 1 milliequiv. g^{-1}, and at this loading the resin shows good swelling properties in dichloromethane and DMF. Acylation of the resin is initially achieved through either hydroxysuccinamide or trichlorophenyl active esters; in the latter case the addition of HOBt may be used to increase the rate of coupling.

Physically supported acrylamide gels have also been used as supports in a continuous-flow solid-phase reactor.[121] Polymerization of the monomers (41), (42), and (44) within the pores of a macroporous inorganic support gave a new matrix that was resistant to compression. The fabricated kieselguhr pores are several thousand Å in diameter, and therefore the inorganic supported gel resembles the pure unsupported gel in chemical characteristics. The resultant loading of sarcosine is between 0.046 and 0.237 milliequiv. g^{-1}. When packed into a column little back-pressure resulted and a recirculation technique was used for synthesis incorporating continuous-flow u.v. monitoring. A synthesis of several gastrin fragments on this resin utilized Fmoc symmetrical anhydrides using 20% piperidine for deprotection.

A microporous copolystyrene resin crosslinked with 1% divinylbenzene has also been used under continuous-flow conditions.[122] In this case, the Pam-based resin was used for the synthesis of several test peptides with Boc or Fmoc for N^{α}-amino protection. Recycling through a detector was also employed and couplings were checked by reversed-phase h.p.l.c. Most of these continuous-flow techniques would be particularly adaptable to control by microprocessors or other similar devices.

The use of the unusual resin linkage/protecting group shown in (48) has been demonstrated.[123] On treatment with two equivalents of water and HCl in acetic acid the α,β-unsaturated amino-acid linkage is cleaved to give an amide. Thus, deprotection of (48) gave H-Leu-Asn-Gly-NH_2. Another unusual approach has been the application of the four-component condensation to solid-phase synthesis.[124] The procedure is illustrated by condensation of an isocyano polymer with 1-methyl-3-formylindole and appropriate protected carboxy and amino components.

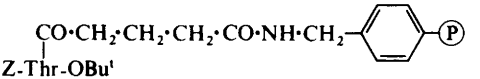


(48)

[119] R. Arshady, A. Ledwith, and G. W. Kenner, *Macromol. Chem. Phys.*, 1981, **182**, 41.
[120] A. Ledwith, M. Rahnema, and P. K. Sen Gupta, *J. Polym. Sci., Polym. Chem. Ed.*, 1980, **18**, 2239.
[121] E. Atherton, E. Brown, R. C. Sheppard, and A. Rosevear, *J. Chem. Soc., Chem. Commun.*, 1981, 1151.
[122] T. J. Lukas, M. B. Prystowsky, and B. W. Erickson, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 2791.
[123] K. Noda and E. Gross, *Z. Naturforsch., Teil B*, 1981, **36**, 1345.
[124] R. Arshady and I. Ugi, *Z. Naturforsch., Teil B*, 1981, **36**, 1202.

The use of 4-dimethylaminopyridine for enhancing the rate of DCCI or symmetrical anhydride couplings in solid-phase synthesis has been investigated.[125] The method gave near-quantitative couplings when hindered residues were examined and is clearly superior to the use of DCCI alone, DCCI/HOBt, or symmetrical anhydride couplings. It is becoming clear that caution should be exercised when using dimethylaminopyridine in conjunction with activated amino-acids, particularly when symmetrical anhydrides are used,[83, 125] as high levels of racemization may be encountered.

Anchoring of peptide chains through side-chain linkages to the resin provides an alternative means of attachment. Protected fragments linked through the side chains of glutamic acid or aspartic acid to a benzylester resin linkage have been examined.[126] Cleavage of the protected fragment from the resin may be achieved by aminolysis, giving the corresponding glutamine or asparagine peptides. Alternatively, serine and threonine may be linked by a succinimide linkage, as indicated in (49). In this case either hydrazinolysis or aminolysis can be used to liberate the protected threonine. Resins of the Pop type have recently been found to be the best supports for such work.



(49)

The use of the base-labile Fmoc protecting group has seen increasing usage in solid-phase synthesis. Fmoc amino-acid anhydrides have been prepared for use in synthesis using water-soluble carbodi-imides,[127] the isolated symmetrical anhydrides being claimed to be stable over a two-year period on storage at 0 °C. The Fmoc protecting group has found wide usage in conjunction with polyamide supports,[128] and in the syntheses of substance P and acyl carrier protein (65—74) the *p*-alkoxybenzyl resin linkage was used. In this work it is pointed out that the Fmoc amino-acids are not indefinitely stable in either DMF or DMA and that decomposition of, for example, Fmoc-glycine may reach the level of 14% after seven days. A comparison was made between the number of steps involved when using Fmoc in conjunction with t-butyl-based side-chain protection and the acid-labile (Boc) for α-amino protection with benzyl-based side-chain protection. In the preparation of substance P 23 separate operations were required in the case of the Fmoc system, whereas 41 stages were required with the Boc protection system; there is therefore considerable advantage in using the base-labile system. Other successful syntheses using the same strategy with alkoxybenzyl resins include the preparation of a 21-residue fragment of human proinsulin [129] and the synthesis of thymopoietin II,[130] the final deblocking from the resin in both cases being

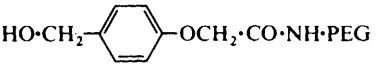
[125] S. S. Wang, J. P. Tam, B. S. H. Wang, and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 1981, **18**, 459.
[126] C.-I. Niu, S.-Z. Yang, A.-Q. Ma, Y.-N. Chen, Y.-Q. Jiang, and W.-T. Huang, *Biopolymers*, 1981, **20**, 1833.
[127] E. P. Heimer, C.-D. Chang, T. Lambros, and J. Meienhofer, *Int. J. Pept. Protein Res.*, 1981, **18**, 237.
[128] E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
[129] E. Atherton, W. Huebscher, R. C. Sheppard, and V. Woolley, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 833.
[130] R. Colombo, *Experientia*, 1981, **37**, 798.

achieved by treatment with aqueous trifluoroacetic acid. A variation on the Fmoc procedure using Fmoc amino-acid trichlorophenyl active esters (3 equiv.) and HOBt (1 equiv.) for coupling amino-acid residues has been published.[131] Generally, couplings of amino-acids required about one hour to reach completion as evidenced by the Kaiser test.

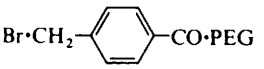
More conventional syntheses using Bpoc for α-amino protection and t-butyl-based protecting groups for side-chain protection in conjunction with alkoxybenzyl resins provided the elegant synthesis of crystalline glucagon described above[113] and the synthesis of a 42-residue peptide of nucleolar non-histone protein C23.[132] In the latter synthesis, however, benzyl was used for the protection of threonine and final removal of this benzyl group was by catalytic transfer hydrogenolysis in the presence of 1,4-cyclohexadiene as hydrogen donor. Even in the presence of methionine, at position 36 in this fragment, cleavage of the benzyl group may be achieved, provided that fresh catalyst is used.

It is interesting that the majority of solid-phase syntheses are carried out using the Boc, Bpoc, or Fmoc protecting groups. The use of other groups is comparatively rare, but the highly acid-labile Ddz group[133] has been used for a gel solid-phase synthesis of five fragments of the human-insulin B chain. The synthesis employs both Cys(Acm) and Cys(MBzl) for sulphur protection, as using these two groups provides differential protection for the cysteine residues. The adamantylmethylethoxycarbonyl (Adpoc) protecting group has also been used in a solid-phase synthesis of thymopoietin (32—36).[134] Amino-acids protected with the relatively hindered Adpoc protecting group were coupled in a double cycle with a 2.5-fold excess, employing DCCI as the coupling reagent. Deprotection was achieved by treatment with 10% trifluoroacetic acid in dichloromethane, the protecting group requiring 12 min for complete removal.

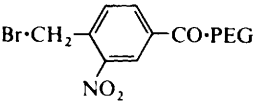
Other Repetitive Methods. The popularity of the liquid-phase method has increased, and several modified polyethylene glycols have been prepared. *p*-Alkoxybenzyl supports of the type shown (50) have been developed.[135] Peptides synthesized on this monofunctional polyethylene glycol are cleaved by treatment with Tfa in the presence of anisole, and they have been used for the synthesis of leucine- and methionine-enkephalin. Related resins of the type shown in (51) and (52) have also been prepared.[136] The first of these is acid labile and the second is



(50)

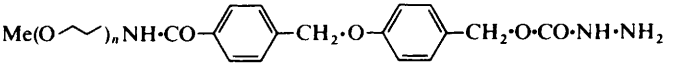


(51)

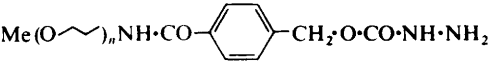


(52)

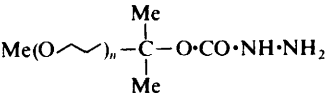
[131] K. M. Sivanandaiah and S. Gurusiddappa, *Synthesis*, 1981, 565.
[132] R. Colombo, *Bioorg. Chem.*, 1981, **10**, 219.
[133] C. Voss and C. Birr, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 717.
[134] G. Rampold, E. Lundanes, K. Folkers, W. Voelter, H. Kalbacher, and E. Bliznakov, *Z. Naturforsch., Teil B*, 1980, **35**, 1476.
[135] R. Colombo and A. Pinelli, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1385.
[136] V. N. R. Pillai, M. Mutter, E. Bayer, and I. Gatfield, *J. Org. Chem.*, 1981, **45**, 5364.



(53)



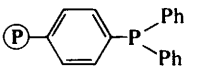
(54)



(55)

cleaved by photolysis. The three liquid-phase supports (53), (54), and (55) have been utilized for the preparation of protected peptide hydrazides.[137]

The repetitive excess mixed-anhydride method (the REMA method) has seen limited use in the preparation of hexapeptide extensions of proalbumin, using the Ddz protecting group for the α-amino functions.[138] The REMA method has also been used for the preparation of secretin [139] and a series of secretin analogues.[140] These preparations used Boc for N^{α}-amino protection, and monitoring of intermediates was carried out by reversed-phase h.p.l.c. A yield of 100% was only achieved on two occasions, but the majority of couplings showed between 90 and 100% yield. Isobutylchloroformate was used for activation in this REMA synthesis, and it appears that the possibility of wrong-way opening of the non-symmetrical anhydride is minimal when the peptides undergoing synthesis are of less than 20 residues. After HF deprotection for one hour at 0 °C in the presence of anisole the final product compared well with others prepared by different techniques.[139] [Aspartoyl^3]- and [β-aspartyl^3]-secretins were also prepared [140] by ring closure of the α-aspartic acid residue at position 3 to give the aspartoyl peptide, and subsequent rearrangement to the β-aspartic acid peptide was again monitored by h.p.l.c.



(56)

The resin-bound phosphine (56) [141] has been used in peptide synthesis in conjunction with carbon tetrachloride in the same way that triphenylphosphine is used in solution. The method appears to have several drawbacks and its use in synthesis has to be monitored carefully. Polymeric 2-mercaptopyridine and 2-mercaptonitrobenzene derivatives have been prepared by modification of poly-

[137] R. Colombo, *Tetrahedron Lett.*, 1981, **22**, 4129.
[138] C. Birr, K. Weigand, and A. Turan, *Biochim. Biophys. Acta*, 1981, **670**, 421.
[139] H. C. Beyerman, P. Kranenburg, W. M. M. Schaaper, and D. Voskamp, *Int. J. Pept. Protein Res.*, 1981, **18**, 276.
[140] D. Voskamp and H. C. Beyerman, *Int. J. Pept. Protein Res.*, 1981, **18**, 284.
[141] R. Appel and L. Willms, *Chem. Ber.*, 1981, **114**, 858.

styrene resin.[142] The resin-bound thiols may be used for the removal of Nps protection and as a means of preparing a polymeric active ester. Boc amino-acids react with the resin in the presence of DCCI to give the corresponding active ester, which is a potent acylating agent; the utility of these resin-bound active esters was demonstrated by a synthesis of leucine-enkephalin. Resin-bound *N*-hydroxysuccinimide active esters have also been used.[143, 144] In these cases methionine-enkephalin[143] and tuftsin and CCK-PZ octapeptide[144] have been prepared to demonstrate the application of the polymeric *N*-hydroxysuccinimide esters to peptide synthesis.

Monitoring Solid-phase Peptide Synthesis.—The use of ninhydrin[145] and of perchloric acid titration[146] for monitoring solid-phase reactions has been noted. However, in general, most researchers still prefer not to use any form of monitoring during solid-phase synthesis. The reaction of carbodi-imides with pyridine and barbituric acid or dimethylbarbituric acid has been found to yield a purple-red product with a very high extinction coefficient.[147] This colorimetric assay thus allows estimation of carbodi-imides in trace amounts, which may be useful in both solid-phase and solution synthesis.

^{13}C n.m.r. spectroscopy has been used as an analytical method when the poly(acryloylmorphine) gel resin has been used.[148] The swollen gel gave good n.m.r. spectra, and identification of the backbone elements was easily achieved. Boc deprotection using BF_3 was readily observed, and monitoring of the removal of other protecting groups is feasible. The synthesis and deprotection cycles in the preparation of a small peptide may thus easily be monitored by ^{13}C n.m.r., when a gel-type resin that swells in DMSO is being used.

Solid-phase Edman degradation of resin-bound peptides undergoing synthesis may also be used as a method of analysis.[149] When h.p.l.c. is used to identify the Pth derivatives, gross errors are easily seen, which is a warning that the synthesis should be recommenced. In the synthesis of a 117-residue peptide, spot checks were made at intervals of 10—15 residues, ensuring that the sequence carried by the resin agreed with the anticipated sequence.

Polymeric Peptides.—The mechanistic aspects of *N*-carboxyanhydride polymerization have been studied.[150-152] The first paper[150] deals with the coupling of dipeptides to an NCA and the second[151] with stereoselectivity in polymerization. The nature of the initiating amine seems to have a considerable effect on the stereoselectivity of NCA polymerization, and it appears that amino-acids react preferentially with an NCA of an amino-acid of opposite configuration. The nature of the product arising from the reaction of an NCA with the toluene

[142] M. Stern, A. Warshawsky, and M. Fridkin, *Int. J. Pept. Protein Res.*, 1981, **17**, 531.
[143] Y. A. Davidovich, A. V. Il'ina, and S. V. Rogozhin, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1981, 422.
[144] N. A. Samoilova, S. M. Andreev, O. M. Galkin, Y. A. Davidovich, and S. V. Rogozhin, *Bioorg. Khim.*, 1981, **7**, 1627.
[145] V. K. Sarin, S. B. H. Kent, J. P. Tam, and R. B. Merrifield, *Anal. Biochem.*, 1981, **117**, 147.
[146] M. Christensen, O. Schou, and V. S. Pedersen, *Acta Chem. Scand., Ser. B*, 1981, **35**, 573.
[147] M. Wilchek, T. Miron, and J. Kohn, *Anal. Biochem.*, 1981, **114**, 419.
[148] R. Epton, P. Goddard, and K. J. Ivin, *Polymer*, 1980, **21**, 1367.
[149] G. R. Matsueda, E. Haber, and M. N. Margolies, *Biochemistry*, 1981, **20**, 2571.
[150] H. Ohnishi, Y. Hashimoto, and Y. Imanishi, *Int. J. Biol. Macromol.*, 1981, **3**, 327.
[151] Y. Imanishi, H. Ohnishi, and Y. Hashimoto, *Int. J. Biol. Macromol.*, 1981, **3**, 97.

sulphonate salt or an amino-acid benzyl ester has been examined,[152] and the various tendencies to polymerization or step-wise addition have been examined. Polymerization appears to dominate when the side chain of the ester is bulkier than that of the NCA, and dipeptide formation occurs when the converse is true.

Although the NCA method is extensively used for homopolymers, other methods have to be used when small peptide fragments are to be polymerized. Recent examples of this are provided by the synthesis of polypeptides containing Lys(Z) and Glu(OBzl) in which Nps di- and tri-peptide nitrophenyl esters are polymerized after the removal of the Nps protection.[153] Polymerization by activation with diphenylphosphoryl azide has also been described;[154] in this case Ala-Gly was polymerized in DMSO over two days, using 1.3 equivalents of the azide and 2.6 equivalents of triethylamine.

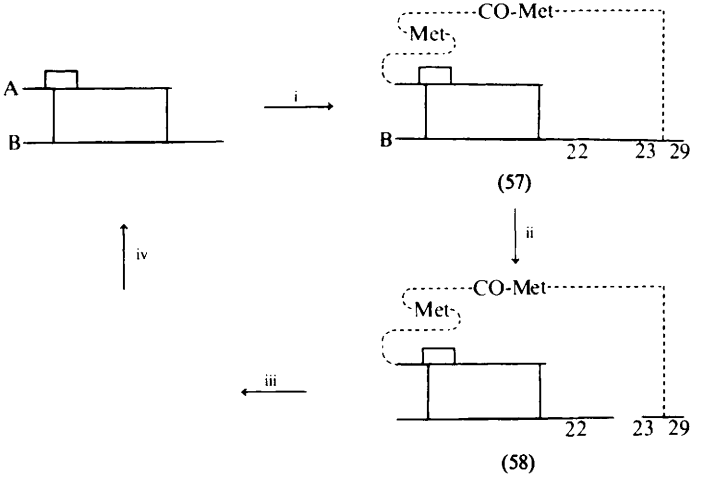
As the majority of polymeric peptides are prepared by established methods, their preparation has not been discussed in detail; however, the majority of syntheses reported in the literature are tabulated in Appendix I.

Semisynthesis.—The use of semisynthesis for the modification of natural hormones and enzymes continues to be of considerable interest as a route for the preparation of analogues of proteins. In the last year the variety of proteins studied or modified by semisynthesis has increased considerably. Two reviews have appeared: the first [155] is primarily concerned with work on insulin and pre-proinsulin and describes both the use of the mixed-anhydride method and enzymic synthesis in the formation of hybrid peptides. The second review [156] covers a wide variety of substrates including RNAse, cytochrome *c*, and insulin, in addition to many others. The logistics of semisynthetic programmes are considered, and monitoring of crucial stages by n.m.r. spectroscopy is reported.

Much work has been carried out on the synthesis of insulin or insulin analogues, the majority of the work being centred around the formation of the desoctapeptide B(23—30) insulin from insulin by means of tryptic cleavage at the arginine-22 residue.[157–160] After cleavage of the (23—30) fragment with trypsin new synthetic fragments can be coupled by reversing the action of tryptic cleavage. When either 50% DMF or 70% DMF–1,4-butanediol (1 : 1) was used as a solvent, a good yield of recombination was observed during coupling of synthetic protected octapeptides, trypsin being used to bring about coupling. Leucine [157] and alanine [158] analogues of bovine insulin were prepared in yields of just over 30%. In this case the increase in the rate of synthesis compared to that of cleavage is due to the rise in the apparent pK_a of the carboxy group of arginine without change in the pK_a of the amino group of the octapeptide. It is thought that this minimizes the energy required for proton transfer, thus facilitating coupling.

Intramolecular trypsin-catalysed reformation of the 22—23 amide bond of

[152] M. Oya and T. Takahashi, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 2705.
[153] T. Amiya, S. Itou, K. Van, and A. Teramoto, *Int. J. Biol. Macromol.*, 1981, **3**, 218.
[154] B. Nakajima and N. Nishi, *Polym. J.*, 1981, **13**, 183.
[155] H. Zahn, V. K. Naithani, H.-G. Gattner, E. E. Buellesbach, and P. M. Thamm, *Naturwissenschaften*, 1981, **68**, 56.
[156] I. M. Chaiken, *CRC Crit. Rev. Biochem.*, 1981, **11**, 255.
[157] K. Inouye, K. Watanabe, Y. Tochino, T. Kanaya, M. Kobayashi, and Y. Shigeta, *Experientia*, 1981, **37**, 811.
[158] K. Inouye, K. Watanabe, Y. Tochino, M. Kobayashi, and Y. Shigeta, *Biopolymers*, 1981, **20**, 1845.



Reagents: i, $CO(Met\text{-}ONp)_2$; ii, trypsin, pH 9.6; iii, trypsin, pH 6.5, Tris buffer: glycerol : DMSO (1 : 1 : 1); iv, CNBr

Scheme 9

insulin has been demonstrated in the manipulation of insulin shown in Scheme 9.[159] The hormone was initially crosslinked between the A1 amino and B29 lysine amino side-chain groups by reaction with the bisnitrophenyl ester of the methionine derivative shown in Scheme 9. The insulin derivative obtained (57) was subjected to tryptic cleavage at pH 9.6, resulting in the cleavage of the B(22—23) amide bond to give (58). (58) was then reconverted to (57), trypsin again being used; on this occasion the pH was adjusted to 6.5 and a mixture of Tris–glycerol and DMSO in equal proportions was used as solvent, the recombination giving yields in the region of 55—60%. The methionine bridge was then excised by the action of cyanogen bromide reliberating the intact insulin.

A specific activation of the arginine-carboxy group of the B chain of the desoctapeptide insulin has been described.[160] In this paper A1,B1,B29-Boc_3-insulin is degraded by trypsin, giving A1,B1-[desoctapeptide B(23—30)]insulin. The arginine-22 carboxy group is then coupled with phenylhydrazine, again by the action of trypsin at pH 6.5. On this occasion, using a mixture of DMSO, 2,4-butanediol, and water (35 : 35 : 30), these conditions give an 87% yield of the corresponding hydrazide; once again under these conditions the incoming hydrazine molecule is protonated. The phenylhydrazide is then oxidized to give a phenyldi-imide derivative that can then be coupled with a suitably protected octapeptide to give Boc_3-insulin. This may then ultimately be reconverted to insulin by treatment with Tfa. It appears that under these conditions some racemization is observed during the coupling using the di-imide-activated carboxy group.

[159] S.-C. Chu, C.-C. Wang, and D. Brandenburg, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 647.
[160] E. Canova-Davis and F. H. Carpenter, *Biochemistry*, 1981, **20**, 7053.

A modified method for the combination of the insulin A and B chains has appeared.[161] In the method the A and B chains are simultaneously subjected to reduction by mercaptoethanol in 8M urea at pH 8.6. Then, without isolation, oxidation is carried out at pH 10.6 prior to gel chromatography on Sephadex G25. The technique gives an improvement in yield over that generally observed, but the yields were found to be lower when modified analogues were studied.

Modified Kunitz proteinase inhibitors have also been prepared by use of the semisynthetic approach.[162] These inhibitors contain a disulphide bridge between cysteine at positions 14 and 38 and a lysine at position 15. Treatment with sodium borohydride followed by trypsin cleaved the bond between the lysine-15 and alanine-16. Subsequent reoxidation gave the inhibitor containing a cleaved (15—16) amide bond. Treatment with carboxypeptidase B then removed lysine at position 15 prior to coupling with valine methyl ester by the DCCI method. Then treatment with leucocyte elastase completed the peptide backbone, reforming the bond between valine-15 and alanine-16.

Semisynthetic modifications of human somatotropin have also appeared.[163, 164] In the first paper a non-covalent interaction of the natural amino-terminal fragments (1—134) with a synthetic analogue of the carboxy (140—182) fragment gave an analogue that was indistinguishable from natural growth hormone. In the second paper [164] thrombin was used to resynthesize the arginine-134–threonine-135 bond. This resynthesis, which was carried out on reduced carbamidomethylated human somatotropin, was carried out at pH 6 using 90% glycerol, the reconstituted somatotropin being obtained in 20% yield.

Enzyme analogues have also been prepared *via* a semisynthetic approach. [Isoleucine^{13}, homoserine^{20}]ribonuclease A was synthesized by combination of [Ile^{13}, Hse^{20}]-*S*-peptide lactone with natural *S*-protein.[165] The *S*-peptide analogue was obtained by cyanogen bromide cleavage of [Ile^{13}, Met^{20}]Gly-Gly-ribonuclease (1—22). The five-day recombination, which was monitored by h.p.l.c., showed a 7% yield of the active RNAse analogue.

Analogues of phospholipase A_2 have also been obtained using semisynthetic methods.[166] Cyanogen bromide was again used for cleavage of the natural peptide. In this case cleavage occurred at Met^8 to give the des-1,8-phospholipase A_2; the methionine was then reintroduced by coupling with Boc-Met-ONSu. After Boc cleavage the [Arg^6]-1,7 or [Asn^6]-1,7, both of which have been prepared by solid-phase synthesis, was coupled by an isobutylchloroformate mixed anhydride to give a yield of approximately 30%.

Enzyme-mediated Synthesis.—Enzymic synthesis of peptide bonds appears to be one of the most rapidly expanding areas, and indeed in the last section on semisynthesis it is clear that enzyme-mediated peptide-bond reformation is now one of the most important methods of semisynthetic coupling. The overall increase

[161] H.-G. Gattner, G. Krail, W. Danho, R. Knorr, H.-J. Wieneke, E. E. Buellesbach, B. Schartmann, D. Brandenburg, and H. Zahn, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1043.
[162] H. R. Wenzel and H. Tschesche, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 295.
[163] C. H. Li, J. Blake, C. H. K. Cheng, and M. D. Jibson, *Arch. Biochem. Biophys.*, 1981, **211**, 338.
[164] L. Graf and C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 6135.
[165] P. Hoogerhout and K. E. T. Kerling, *Recl. Trav. Chim. Pays-Bas*, 1981, **100**, 215.
[166] G. J. M. Van Scharrenburg, W. C. Puijk, M. R. Egmond, G. H. De Haas, and A. J. Slotboom, *Biochemistry*, 1981, **20**, 1584.

in activity in this area derives from the fact that for proteolytic enzymes in a mixed aqueous organic system the position of equilibrium shifts considerably from the position of hydrolysis to that of synthesis. One major advantage of enzymic synthesis is that the couplings should proceed without any possibility of racemization.

Several studies of the enzymes and conditions used in enzymic synthesis have been reported,[167–170] details of the kinetics have been investigated,[171,172] and the use of h.p.l.c. for monitoring protease-catalysed peptide synthesis has also been mentioned.[173] Two general reviews[174,175] covering choice of enzymes, kinetics, solvent compositions, and other variables have appeared.

A considerabe variety of enzymes have now been used, but trypsin remains one of the most widely used enzymes. Both trypsin[176] and papain[177] have been used for the preparation of Boc and Z hydrazides. In the first case, with aqueous DMF or DMSO as solvent, yields of up to 90% were recorded. A particularly useful application of enzymic synthesis has been the conversion of porcine to human insulin in one step.[178] This was achieved by transpeptidation of the terminal residue of the B chain of insulin with trypsin. In the presence of threonine t-butyl ester transpeptidation of the final residue of the B chain was observed when DMF–Tris at pH 5.8 was used as the solvent. After deblocking with Tfa-anisole, native insulin was obtained in 50—70% yield.

Catalysis by subtilisin, papain, and pepsin has also been studied.[179] In this investigation coupling of various benzyloxycarbonyl amino-acids or peptides with leucine-t-butyl ester or diphenylmethyl ester has been investigated using DMF as the cosolvent. Frequently it was found that the molecular size of the carboxy component was critical and that the amino component needed to be in a higher concentration than the carboxy. An investigation into couplings with papain in biphasic aqueous organic systems was also carried out.[180] Couplings of Boc-Phe-Phe-OEt or the corresponding acid with leucine-amide were studied and it was found that in immiscible solvent systems reaction was faster than when methanol was used. Yields varied between 71% for carbon tetrachloride and 80% for chloroform, ethyl acetate giving 74%, nitromethane 78%, and nitrobenzene 78%.

Chymotrypsin has also been used for the enzymic preparation of peptide bonds; generally it appears that tryptophan[181] or leucine[182] makes the best carboxy components. In the biphasic system ethyl acetate–water acetyltryptophan coupled

[167] A. N. Semenov, A. I. Gromov, and K. Martinik, *Bioorg. Khim.*, 1981, **7**, 867.
[168] K. Morihara, *Tanpakushitsu Kakusan Koso*, 1981, **26**, 1979.
[169] K. Martinek, A. N. Semenov, and I. V. Berezin, *Dokl. Akad. Nauk SSSR*, 1980, **254**, 121.
[170] P. Kuhl, S. Posselt, and H.-D. Jakubke, *Pharmazie*, 1981, **36**, 463.
[171] K. Martinek, A. N. Semenov, and I. V. Berezin, *Biochim. Biophys. Acta*, 1981, **658**, 76.
[172] K. Martinek and A. N. Semenov, *Biochim. Biophys. Acta*, 1981, **658**, 90.
[173] W. Kullmann and A. Fassberg, *J. Liq. Chromatogr.*, 1981, **4**, 1121.
[174] F. Brtnik and K. Jost, *Chem. Listy*, 1981, **74**, 951.
[175] K. Martinek and A. N. Semenov, *J. Appl. Biochem.*, 1981, **3**, 93.
[176] S. Yagisawa, *J. Biochem. (Tokyo)*, 1981, **89**, 491.
[177] J. L. Abernethy, C. M. Lovett, jun., G. F. Kuzmin, J. D. Kuhlberg, and W. A. Wilson, *Bioorg. Chem.*, 1981, **10**, 189.
[178] A. Jonczyk and H.-G. Gattner, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 1591.
[179] K. Morihara and T. Oka, *J. Biochem. (Tokyo)*, 1981, **89**, 385.
[180] G. Doering, P. Kuhl, and H.-D. Jakubke, *Monatsh. Chem.*, 1981, **112**, 1165.
[181] A. N. Semenov, I. V. Berezin, and K. Martinek, *Biotechnol. Bioeng.*, 1981, **23**, 355.
[182] A. Koennecke, R. Bullerjahn, and H.-D. Jakubeke, *Monatsh. Chem.*, 1981, **112**, 469.

readily with leucine-amide in the pH range 6—9. Both water-miscible and -immiscible cosolvents were studied in an investigation using immobilized chymotrypsin.[182] The characteristics of coupling when the enzyme was bound to either silica or enzacryl were similar to those observed when using the free enzyme. The best results were obtained with the silica-bound enzyme with leucine occupying the second position of the benzyloxycarbonyl dipeptide methyl ester, which was undergoing coupling with leucine-amide. Yields ranged from 60 to 70%, but when the dipeptide Z-Phe-Phe-OMe was used Z-Phe-Leu-NH_2 was produced as the main product owing to an intermediate substrate cleavage. Such degradative side reactions have been noted on other occasions in enzyme-mediated syntheses,[183] this type of side reaction being observed with papain, thermolysin, and chymotrypsin.

The sweet-tasting peptide aspartame has also been prepared by enzymic coupling using immobilized thermolysin in ethyl acetate–water at 40 °C.[184] On this occasion the enzyme was immobilized on Amberlite XAD-7 and, provided the immobilized enzyme was kept wet with water, coupling could easily be achieved when an ethyl acetate solution of Z-Asp-OH and H-Phe-OMe was mixed with the wet immobilized enzyme. The thermophilic proteolytic enzyme thermitase has also been used for peptide-bond formation.[185] On this occasion when a benzyloxycarbonyl dipeptide methyl ester was coupled with leucine-amide it was important that the second residue of the dipeptide was valine, alanine, or proline; however, no coupling was observed when the first amino-acid of the dipeptide was valine, isoleucine, or proline. The best couplings were observed when DMF or methanol was used with an appropriate buffer.

The enzyme clostripain has on one occasion been used to catalyse the reformation of cytochrome *c* from the haem (1—38) and apo (39—104) fragments.[186] In this recombination experiment the (1—38) and (39—104) portions obtained by a tryptic digestion of citraconylated cytochrome *c* were recombined using 90% glycerol as solvent. After chromatographic purification the pure native cytochrome *c* was re-isolated.

Purification Methods.—Many papers concerned with the purification of both protected and deprotected peptides have been published. The large number of references encountered in these papers are collected together in Appendix III.

3 Syntheses Achieved

A very large number of syntheses have been reported, but as many of these are syntheses of relatively small peptide hormones or solid-phase syntheses they are not all discussed individually; a full list is, however, given in Section 4, Appendix I.

The synthesis of insulin and various fragments and analogues has been the subject of much research. No new major synthesis of insulin has been reported, but a large number of preparations of a variety of fragments are reported in Appendix

[183] W. Kullmann, *J. Biol. Chem.*, 1981, **256**, 1301.
[184] K. Oyama, S. Nishimura, Y. Nonaka, K. Kihara, and T. Hashimoto, *J. Org. Chem.*, 1981, **46**, 5241.
[185] A. Koennecke and H.-D. Jakubke, *Monatsh. Chem.*, 1981, **112**, 1099.
[186] M. Juillerat and G. A. Homandberg, *Int. J. Pept. Protein Res.*, 1981, **18**, 335.

I. Two reviews of insulin work have appeared;[187, 188] the first [187] covers total syntheses of insulin and a study of structure–activity relationships and the second [188] is primarily a review of the work published by the Ciba-Geigy group. In this review the approach of selective disulphide-bond formation, which has provided a marked advance in the preparation of insulin and its analogues, is discussed in detail.

The syntheses of both porcine [189] and human [190, 191] big gastrin with the revised Pro^7-Pro^8-His^9 sequence have been reported. The porcine sequence was assembled by the same methodology that had been employed previously for the synthesis of the incorrect sequence.[192] Fragment coupling using DCCI/HOBt, or HONSu, gave the product, which was purified on Sephadex LH20 eluting with DMF prior to deprotection by treatment with Tfa in the presence of ethanedithiol. Chromatography on Sephadex G50 and AE-cellulose gave the pure hormone, which was shown by h.p.l.c. and by immunochemical methods to be identical to the natural porcine hormone. The synthesis of the revised human big gastrin I sequence [190, 191] was achieved by the Munich group who used a fragment condensation approach, coupling the (1—9), (10—14), and (15—34) fragments by a combination of DCCI/HONSu and azide methods. The synthesis, which used Nps for N^{α}-amino protection on acid deprotection with Tfa in the presence of ethanedithiol, gave the natural human big gastrin I after purification. Again, the product was shown by immunochemical methods and by h.p.l.c. to be identical to the natural human hormone.

The final stages of the work directed towards the synthesis of an analogue of hen egg-white lysozyme have now been reported.[193–197] Syntheses of the fragments of the second half of the lysozyme analogue utilized the same strategy as in earlier fragments, using acid-labile side-chain protection with benzyloxycarbonyl and Bpoc being used for N^{α}-amino protection. The acetamidomethyl group is again used for the protection of the thiol function of cysteine. The diphenylphosphinic mixed-anhydride procedure was used to particular advantage in the synthesis of the (118—129) fragment.[196] Combination of the residues spanning the sequence (76—129) was carried out by the DCCI/HONSu method. The product was highly insoluble and showed considerable tendency to aggregation in the fully protected state, making chromatography on Enzacryl K2 or Sephadex LH60 rather difficult. The two fragments (1—75) and (76—129) were then combined, again by the

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DCCI/HONSu method; the resulting fully protected (1—129) product was highly insoluble and was purified by a washing procedure prior to deprotection by treatment with trifluoroacetic acid in the presence of anisole and mercaptoethanol. After deprotection, purification by gel filtration and ion-exchange chromatography was carried out yielding a homogeneous (1—129) octa-Acm product. The subsequent attempts to remove the acetamidomethyl group using either mercury(II) or iodine deprotection gave poor results. In fact it appeared that only six out of the eight thiol groups could be liberated from the acetamidomethyl derivatives by treatment with mercuric acetate in 8M urea. This finding was the downfall of the synthesis as oxidation would be unlikely to give a significant amount of the correctly oxidized disulphide product even on a statistical basis.

Full details of the impressive two-man solution synthesis of ribonuclease A described in last year's Report have now been published.[103, 104, 198–205] The synthesis uses an unusual approach in that fragments are combined on a step-wise basis from the carboxy-terminal (110—124) fragment.[198] The assembled fragments carried the following side-chain protection: Lys(Z), Arg(Mbs), Cys(MBzl), Met(O), Glu(OBzl), Asp(OBzl), and in one position Glu(OBu^t). α-Amino protection was through the Z(OMe) group and carboxy-terminal protection was achieved by use of a benzyl ester function. Fragments that were assembled from the carboxy terminus were as follows: (110—124),[198] (9—124),[199] (69—124),[200] (41—124),[201] (21—124) and (1—20),[202] and the final total sequence.[203] On some occasions the Troc protecting group was used for intermediate hydrazide protection, being removed by treatment with zinc in acetic acid. Chromatographic purification of the fully blocked larger fragments was carried out on Sephacryl S200 with 5% water in DMSO as the solvent. As the peptide chains became longer it became more difficult to achieve acylation of the amino terminus. The initial routes coupled (1—20) with the (21—124) portion by the azide method, which had also been used at earlier stages. On this occasion acylation was extremely difficult, and it was apparent that a better yield could be obtained by addition of the (9—20) portion to the (21—124) fragment and subsequent addition of the (1—8) final amino-terminal fragment. However, a total of 30 equivalents of this fragment was required to bring about complete acylation. The use of Sephacryl S200 easily allowed removal of excess acylating species. Chromatographic purification yielded 0.98 g of ribonuclease in the fully protected form.

The most efficient removal of side-chain protecting groups was achieved by use of methanesulphonic acid in the presence of *m*-cresol. This deprotection regime was superior to the use of HF in the presence of *m*-cresol, but both systems gave satisfactory material on deprotection. Exhaustive purification was carried out initially by chromatography on Sephadex G75 followed by affinity chromatography on Sepharose 4B,5′-(4-aminophenylphosphoryl)uridine-2′(3′)-phos-

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phate. This gave a product that contained only a trace of impurity, which was finally removed by ion-exchange chromatography on CM-cellulose. The synthetic material was shown to be homogeneous and to have the same specific enzymic activity as natural ribonuclease A.

In later work the authors switched to the use of trifluoromethanesulphonic acid thioanisole Tfa as the deprotection medium.[103, 104, 204, 205] A few other slight modifications have been made to the work-up procedure, including the use of glutathione to facilitate the oxidation to give the correct disulphide linkages.[104]

A most impressive culmination of the year's publications on ribonuclease is the presentation of photographs of the actual crystals of the fully synthetic active enzyme.[103, 104, 204]

4 Appendix I: A List of Syntheses Reported During 1981

The syntheses are listed under the name of the peptide or protein to which they relate, as in previous years.

Peptide	*Ref.*
Natural Peptides, Proteins, Analogues, and Partial Sequences	
Actinomycin	
Actinomycin D analogues	206
Adrenocorticotropic hormone (ACTH)	
[Cys^{25}]ACTH (1—26) and derivatives	207
[Nle^{4}, Phe^{2}]human ACTH (1—38)	208
^{3}H-β-ACTH (1—24)	209
Ostrich CLIP [ACTH (18—39)]	33
Acyl carrier protein	
Acyl carrier protein (65—74)	128
Acyl carrier protein (65—74)	115
Acyl carrier protein (1—6)	210
Adipokinetic hormone	
Locust adipokinetic hormone	211
Alamethicin	
Alamethicin I	212
Alamethicin major component	213
Alamethicin	214
AM toxin	
AM toxin analogues	215
Amaninamide	
[Ile^{3}]amaninamide	216

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Peptide	*Ref.*
Angiotensin	
[Fal^5]angiotensin II	99
[Ile^5]angiotensin II	40
Ala-Pro-Gly-[Ile^3, Val^5]angiotensin II	217
[α-MeTyr^4]angiotensin II	218
Position-8 analogues of angiotensin II	219
Acyclic and cyclic angiotensin analogues	220
[Asn^1, azahomoVal^3]- and [Asn^1, azahomoVal^3, Ile^8]-angiotensin	221
Anorexogenic peptide	
Anorexogenic peptide	222
Anorexogenic peptide	223
Anorexogenic peptide	224
Apamin	
Apamin	48
Apamin	49
Aspartame	
Aspartame	184
Bradykinin	
Bradykinin analogues	225
Bradykinin	226
Photoaffinity-labelled bradykinin	227
Cyclic analogue of bradykinin	228
Tritiated bradykinin	229
[DehydroPhe^5]- and [dehydroPhe^6]-bradykinins	230
Bradykinin potentiating peptides	231
α-Bungarotoxin	
α-Bungarotoxin	232
Calcitonin	
Tritiated derivatives of human calcitonin	233
Carboxypeptidase A	
Carboxypeptidase A Zn-binding site, model	234
β-Casomorphin	
β-Casomorphin	235

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Peptide	*Ref.*
Chemotactic peptide	
Chemotactic peptide	236
Chemotactic peptide antagonist	237
Tripeptide chemotaxins and antagonists	238
Acylated chemotaxin antagonists	239
Cholecystokinin-pancreozymin (CCK-PZ)	
[Thr^{28}, Nle^{31}]- and [Thr^{28}, Leu^{31}]-CCK-PZ	45
CCK-PZ (24—33)	46
CCK-PZ octapeptide	144
Cytochrome *c*	
Cytochrome *c*	186
Dehydroaspartame	
Dehydroaspartame	240
Delta-sleep-inducing peptide	
Delta-sleep-inducing peptide analogues	241
[Phe^{1}]- and [Tyr^{1}]-delta-sleep-inducing peptide	242
Dermorphin	
Dermorphin analogues	243
Shortened dermorphin analogues	244
Dynorphin	
Dynorphin (1—13)	36
Dynorphin fragment analogue	245
Endorphin	
Equine β-endorphin	246
[Gly^{17}]-β-endorphin	73
Human β-endorphin analogues; substitution at positions 2, 9, 18, 27, and 31	247
Cystine-bridged analogues of human β-endorphin	248
Analogues of human β-endorphin (1—27)	249
[Gln^{8}]-β-endorphin	250
β-Endorphin model peptide	251
Enkephalin	
Retro-enkephalin	252

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Peptide	*Ref.*
[D-Ala^2, Leu(ol)^5]enkephalin	253
Di- and tri-peptide analogues of enkephalin	254
Analogues of leucine-enkephalin	255
Enkephalin analogues	117, 183, 256
Leucine-enkephalin	17, 18, 24, 65, 131, 135, 142, 148, 173, 257, 258
Methionine-enkephalin	22, 93, 127, 135, 143
Lys_2-linked cyclic enkephalin	43
Rigid enkephalin analogues	259
Enkephalin analogue, Tyr-D-Met(O)-Gly-MePhe(ol)	260
[β-Ferrocenylalanine^4]leucine-enkephalin	261
Tritiated enkephalin analogue FK33824	262
Enkephalin chloromethyl ketone derivatives	263
Cyclic leucine- and methionine-enkephalins	264
Cyclic enkephalin analogue	265
Disulphide-containing cyclic enkephalin analogues	266
TMV-enkephalin conjugates	267
[D-Ala^2, Car^4, Leu^5]- and [D-Ala^2, Car^4, Met^5]-enkephalin amides	268
Hydrophobic enkephalin analogues	269
Enkephalin-like pentapeptides containing *p*-(NO_2)Phe at position 4	270
[D-Ala^2]leucine-enkephalin amide	70
[D-Ala^2]enkephalin analogues with adamantylalanine at positions 4 and 5	271
D- and L-ferrocenyl-leucine-enkephalin	272
Chromophoric dehydro-enkephalins	273
Aminoxy analogues of leucine-enkephalin	274
Enkephalin analogues showing selectivity for μ- and δ-receptors	275
Ferredoxin	
Peptides related to ferredoxin	276

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Peptide	*Ref.*
Ferredoxin model peptide	277
Gastrin	
Chicken gastrin-releasing peptide	34
Gastrin-releasing peptide	35
Porcine-gut gastrin-releasing peptide	278
[Leu^{15}]gastrin (10—17)	121
Porcine big gastrin I	189
Human big gastrin I	190
Human big gastrin I	191
Glucagon	
Glucagon	113
Glucagon fragments	279
Des(1—4)-, des(5—9)-, des(10—15)-, des(16—21)-, des(22—26)-, and des(27—29)-glucagons	280
Glutathione	
Isoglutathione	281
S-(4-Azidophenacyl)glutathione as a photoaffinity label	282
Gramicidin S	
[^{14}C]Gramicidin G	283
Gramicidin S	66
Gramicidin S	98
Gratisin	
Gratisin	284
Hepatitis antigen	
Hepatitis β surface antigen (138—149)	285
Histones	
Histone H3 (39—43)	110
Immobilized model histone peptides	286
Lysine-containing histone models	287
Growth hormone	
(112—145) β-subunit of human chorionic gonadotropin (hCG)	288
hCG β-(112—145)	289
(6—13), (7—13), (8—13), and (9—13) fragments of human growth hormone (hGH)	290
hGH, non-covalent recombination (1—134)/(135—191)	291
Non-covalently linked hGH (1—134)/(135—191)	292

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Peptide	*Ref.*
(75—120)NH_2- and (73—128)GlyNH_2-hGH	293
hGH (27—44)	294
hCG β-(123—145)	295
Hydra-head activator peptide	
Hydra-head activator peptide	296
Immunoglobulins	
IgG V-H (2—118)	149
IgG (275—290) and IgG (281—290)	297
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Immunostimulating peptide FK156	298
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Insulin analogues	161
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Insulin analogues	187
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Insulin	188
Insulin B-chain fragments	133
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[Asn^{B12}]insulin	302
Des-B(25—30)-insulin	303
Bovine des-B(23—30)-insulin	160
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Insulin-related peptides	306
Insulin A(19—21)–B(15—27) disulphide	56
A1/B29 crosslinked insulin	159
A-Chain/B-chain crosslinked insulin derivative	307
[A1-N^{γ}-(4-Azidophenylacetyl)-D-α,γ-diaminobutyric acid]insulin	308
A1-αN-Trimethylinsulin	309
[Nle^{A2}]sheep insulin	310
[^{125}I-Tyr^{B1}]insulin	311
Des-Ala^{B30}-insulin	312

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Peptide	*Ref.*
Insulin A chain (1—5)	313
Insulin A chain (5—9)	314
Insulin A chain (13—16)	315
Insulin arginyl A-chain cyclic bisdisulphide	316
[Tyr^{24}, Ala^{25}]insulin B chain (1—25)	317
[Ala^{27}]insulin B chain (15—27)	318
Insulin B1,B29-Boc_2 B chain	319
Insulin C peptide	126
Insulin C-peptide fragment	320
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Insulin-like growth factor I, hybrid insulin	322
Interferon	
(1—20) lymphoblastoid interferon	323
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Partial retro–inverso analogues of LHRH	328
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Lysozyme (118—129)	196
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Peptide	*Ref.*
Melanocyte-stimulating hormone (MSH)	
[Phe(N_3)^{14}]-α-MSH	332
Tritiated α-MSH	333
MSH 2/7-position analogues	334
γ-MSH	335
Mellitin	
Mellitin-like cytotoxic peptide	336
Mosquito-attractant peptide	
Mosquito-attractant peptide	337
Myelin basic protein	
Myelin basic protein (human) (154—165)	338
Neurotensin	
Neurotensin analogues	339
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^{17}O-Labelled oxytocins	343
[Leu^7, Pro^8]oxytocin	344
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Human parathyroid hormone (1—84)	102
[Nle^{8,18}, D-Tyr^{34}]bovine parathyroid hormone (1—34)NH_2	346
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[Arg^6]phospholipase A_2	166
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Polymyxin B_1 analogue	347
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Proalbumin	
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Prothrombin	
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Bovine ribonuclease (41—124)	201
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Bovine ribonuclease A	204
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Substance P fragment	360
Substance P analogues	361
[D- and L-Ala^9]substance P (6—11)	362
[Glp^4]substance P (4—9)	363
Enzyme-resistant analogues of substance P	364
Thymopoietin	
Thymopoietin II	130
Thymopoietin (32—26)	134
Thymopoietin (24—36)/(30—36)	365
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Thymosin	
Thymosin α_1	107
Thymosin α_1 (14—28)	367
Fragments of thymosin α_1	368
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Peptide	*Ref.*
Tyrocidine A	
[Val^6]- and [Orn^7]-tyrocidine A	380
Ubiquitin	
Bovine ubiquitin (59—74)	381
Vasopressin	
Vasopressin	50
[Arg^7, Pro^8]vasopressin	344
^{13}C-Labelled vasopressin	345
Vasopressin analogues	382
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Poly[Lys(Z), Glu(OBzl)$_2$] and poly[Lys(Z), Glu(OBzl)]	153
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Nps-(Phe$_2$-Gly)$_n$-OEt (n = 1—6) and Nps-(Phe-Leu-Gly)$_n$ (n = 1—7)	398
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Poly-D-phenylglycine and poly-D-cyclohexylglycine	402
Poly[(hydroxypropyl)-glutamine-co-L-Trp]	403
Poly(δ-Orn)	404
L-Uracilylalanyl polypeptides	405
Enzyme Substrates and Inhibitors	
Angiotensin-converting enzyme inhibitors	406—410
Des-Pro^2-bradykinin, inhibitor of angiotensin-converting enzyme	411
Substrates for cathepsin G and chymotrypsin	412
Clostridiopeptidase inhibitor	413
Collagenase inhibitor	414
Dansylated porcine pepsin inhibitor	415
Fluorogenic elastase substrate	416
Fluorogenic peptide substrate for vitamin K-dependent blood-coagulation factor	417
Substrates for human-spleen fibrinolytic proteinase	418
Human-spleen fibrinolytic proteinase inhibitor	419
Inhibitor of pyroglutamylaminopeptidase	420
Kunitz proteinase inhibitor analogue	162
Leupeptin analogues	421
Pepstatin homologues (renin inhibitors)	422
Peptide diazomethyl ketones	423
Peptide inhibitors of thermolysin	424
Peptidyl dipeptidase inhibitor	425
Phosphonamidate dipeptides as inhibitors of carboxypeptidase A	426

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Renin inhibitor	427
Renin substrate	428
Spin-labelled leucine aminopeptidase inhibitor	429
Substrate for enkephalin-forming protease	430
Substrate for prolylcarboxypeptidase	431
Thrombin inhibitors, arylsulphonylarginine derivatives	432
Thrombin-like enzyme inhibitors	433
Thrombin substrate, Bz-Phe(*p*-NO_2)-Val-Arg-pNA	434
Trypsin-like protease inhibitors; *p*-guanidinobenzoic acid peptides	435
Glycopeptides	
Analogues of *N*-acetylmuramoyl-Ala-D-isoGln	436
N-Acylmuramoyl dipeptides	437
N-Acetylmuramoyl dipeptides	438
Adjuvant dipeptide (MDP)	439
Antigenic glycopeptides of asparagine	440
Antitumour glycopeptides	441
Asn-*N*-acetylglucosamine derivatives	442
[Asn^{18}(glucoside)]bovine pancreatic DNase (17—19) and (18—23)	443
D-Glucosaminic acid peptides	444
Peptides containing D-glucosaminic acid	445
Peptides containing α-D-glucopyranosylamine	446
Glycosylated peptides	447
Glycopeptides	448
Glycopeptides	449
Muramyl dipeptides	450
Muramyl dipeptides	451

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Peptide	*Ref.*
Muramyl dipeptide derivatives	452
Muramyl dipeptide derivatives with a multi-prenylacetyl group	453
Peptidylglycophospholipids	454
Serine glycopeptides	455
Miscellaneous Peptides	
Ac-Gly-Lys(For)-OMe	456
Aminobenzothiazole peptides	457
Arginyl dipeptides	458
Peptides of 2-aziridinecarboxylic acid	459
2-Aziridinecarboxylic acid peptides	460
Benzyloxycarbonyl endothiodipeptide esters	461
Bitter-tasting peptide, H-Asp-D-Phe-OMe	462
Boc-Pro-D-Pro-OMe	463
Peptides containing *N*-(carboxymethyl)amino-acids	464
^{11}C-Labelled peptides	465
cyclo(-D-Val-Pro-)	114
Peptidyl derivatives of cytidine	466
Dehydroamino-acid dipeptides	467
Derivatives of 2-*N*-acetyl-3-*N*-glycyl-L-2,3-diaminopropionic acid	468
3,5-Dinitrophthaloyl dipeptides	469
H-Leu-Ala-Gly-Val-OH	112
H-Leu-Ala-Gly-Val-OH	69
Mercaptoacylamino-acids	470
Mercaptoacylamino-acids	471
Mercaptoacylamino-acids	472
Nucleotidyl-(5′-*N*)-amino-acid esters	473
Nucleotripeptides	474
Porphyrins bearing peptide side chains	475

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Peptide	*Ref.*
Porphyrinyl peptides	476
cyclo(-Pro-Sar_2-)$_2$	477
^{17}O-Enriched amino-acid derivatives	478
Piv-Pro-(Me)Ala-NHPr^i	479
Zn^{II}-Binding cyclic heptapeptide (model for carbonic anhydrase)	480

5 Appendix II: Amino-acid Derivatives Useful in Peptide Synthesis

As before, this list includes both new derivatives and known ones for which new physical data or preparations have been reported. The recent practice of dividing the derivatives into two groups has been adhered to; those of the coded amino-acids are listed first, followed by those of other amino-acids.

Compound	*M.p.*/°C	$[\alpha]_D$*[1]	*Conc.*/g 100 cm^{-3}	*Solvent*	*Ref.*
Coded Amino-acids					
Alanine					
Boc-Ala-ONp-Pic-Tos-OH	123—125	−39.2*[2]	0.5	DMF	25
Boc-Ala-OPic	69—70	−32	1	DMF	39
Dnpy-Ala-OH	195	—	—	—	21
$(Fmoc\text{-}Ala)_2O$	125—126	−13.61	1	EtOAc	127
Tcroc-Ala-OH	127—128	—	—	—	22
Troc-Ala-OH	77	−18.4	3.5—4	EtOH	9
		−45.64	3.5	DMF	
TMSE-Ala-OH-Dcha	123—125	−11.7	1	80% HOAc	11
Z-Ala-ONp-Pic-Tos-OH	75—76	−24.8*[2]	0.5	DMF	25
Arginine					
H-Arg(Mds)-OH	120—122	+7.8	0.7	MeOH	38
H-Arg(Mtr)-OH	100—103	−4.8	1.3	MeOH	38
H-Arg(Pme)-OH	153—156	−5.5	0.9	MeOH	38
Z-Arg(Mds)-OH-Cha	140—141	+5.7	0.5	MeOH	36
Z-Arg(Mtr)-OH-Cha	195—197	+6.5	1.2	MeOH	38
Z-Arg(Pme)-OH-Cha	173—175	+5.8	1.3	MeOH	38
Asparagine					
Fmoc-Asn-ONp	188—189	−39.1*[3]	1.02	DMF	128
Aspartic Acid					
Dnpy-Asp-NH_2	134	—	—	—	21
DPA-Asp-OH	109—113	+9.3[a]	1	1M NaOH	48
Fmoc-Asp(OBu^t)-ONSu	129	−30.5	1	DMF	46
Troc-Asp-OH	146—147	−3.4	3.5—4	EtOH	9
		−31.97	3.5—4	DMF	
Cysteine					
Boc-Cys(Acm)-OBzl	92—93	—	—	—	47
Boc-Cys(Bu^t)-OH	89—90	−3	2	Acetone	51

*[1] $[\alpha]_D$ recorded at 589 nm unless indicated by superscript [a], 578 nm, or [b], 546 nm, being measured at 20—25 °C, except for *[2] measured at 19 °C and *[3] measured at 18 °C.

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Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc/g 100 cm^{-3}*	*Solvent*	*Ref.*
Boc-Cys(Dppm)-OEt	—	+15	1	EtOH	39
Boc-Cys(Dppm)-OH	Foam	+17	1	MeOH	39
Boc-Cys(Dppm)-OH-Dcha	202—204	+19	1.05	$CHCl_3$	39
Boc-Cys(Dppm)-OMe		+17	1.02	$CHCl_3$	39
Boc-Cys(Dppm)-OPcp		+2	1.2	$CHCl_3$	39
Boc-Cys(Npys)-OH-Dcha	153—155	−88.7	1	MeOH	57
Boc-Cys(TMB)-OH	101—103	−11.5	0.7	HOAc	50
Boc-Cys(TMB)-ONp	136—137	−50.7	1	DMF + 1% HOAc	50
HCO-Cys(Bu^t)-OH	149—150	+39	2	MeOH	51
Fmoc-Cys(Bzl)-OH	127—128	−30	2	DMF	342
Fmoc-Cys(Bzl)-ONp	118—119	−48	1.2	DMF + 1% HOAc	342
H-Cys(Bu^t)-OBu^t-HCl	202—203	—	—	—	51
H-Cys(Bu^t)-OBzl-Tos-OH	166—168	—	—	—	51
H-Cys(Bu^t)-OEt-HCl	141—142	+20.5	2	EtOH	51
H-Cys(Bu^t)-OH-HCl	204	+6.35	2	5M HCl	51
H-Cys(Bu^t)-OMe-HCl	120	+18.5	2	MeOH	51
H-Cys(Dppm)-OH	154—157	+51	2.1	1M HCl	39
H-Cys(SBu^t)-OBu^t-Tos-OH	126—128	−79	0.5	MeOH	54
		−93.6[b]	0.5	MeOH	
H-Cys(TMB)-OH	235—238	+9.4	0.5	MeOH	50
H-Cys[MeO-CO-$(CH_2)_3$]-OBu^t-HCl	113—115	−7.8	1	MeOH	384
		−3.4	0.5	DMF	
Nps-Cys(Bu^t)-OH-Dcha	159	—	—	—	51
Pht-Cys(Bu^t)-OH	154	—	—	—	51
Tos-Cys(Bu^t)-$NHNH_2$	149—151	+62	2	Acetone	51
Tos-Cys(Bu^t)-OH	168—170	+28	2	EtOH	51
Tos-Cys(Bu^t)-OMe	87—88	—	—	—	51
Z-Cys(Bzl)-ONp-Pic-Tos-OH	68—71	−42.2	0.5	DMF	25
Z-Cys(Bu^t)-OH-Dcha	162—163	—	—	—	51
Z-Cys(Bu^t)-ONp	64—65	−14	2	Acetone	51
Z-Cys(Dppm)-OH-Dcha	170—172	+26.5	1	$CHCl_3$	39
Z-Cys(Dppm)-OTcp		+16	1	MeOH	39
Z-Cys(TMB)-OCp	143—144	+20.1	0.4	EtOAc	50
Z-Cys(TMB)-OH	122—123	−12.3	1	EtOH	50
		−36.9	1	DMF	
Z-Cys(TMB)-ONp	147—149	−56.6	1	DMF	50
Glutamine					
Fmoc-Gln-ONp	192—193	−46.5*[3]	1.01	DMF	128
Glutamic acid					
Dnpy-Glu-OH	144	—	—	—	21
DPA-Glu-OH	152—153	−22.7[a]	1	MeOH	481
Troc-Glu-OH	74	−12.4	3.5—4	EtOH	9
		−22.81	3.5—4	DMF	
Glycine					
Boc-Gly-ONp-Pic-Tos-OH	125—126	—	—	—	25
Dnpy-Gly-OH	220	—	—	—	21
DPA-Gly-OH	152—154	—	—	—	481
$(Fmoc-Gly)_2O$	165—166	—	—	—	127
Fmoc-Gly-OTcp	146	—	—	—	131
Troc-Gly-OH	123—125	—	—	—	9

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Compound	*M.p.*/°C	$[\alpha]_D$*[1]	*Conc.*/g 100 cm^{-3}	*Solvent*	*Ref.*
Z-Gly-ONp-Pic	114—115	—	—	—	25
Histidine					
Boc-His(Boc)-ONp	141—142	−23.2	1	Dioxan	16
Boc-His(Dppm)-$NHNH_2$	92—93	+5	1	MeOH	39
Boc-His(Dppm)-OH	—	+10	1	MeOH	39
Boc-His(Dppm)-OMe	—	+2	1.2	EtOH	39
Boc-His(π-Bom)-OH	155	+6.9	0.5	MeOH	40
Boc-His(π-Br-Bom)-OH	74	−9.4	1	MeOH	40
Dnpy-His-OH	255	—	—	—	21
Z-His(Dppm)-OH	109—111	+10.5	0.9	MeOH	39
Z-His(Dppm)-OMe	—	+4	1	MeOH	39
Isoleucine					
Troc-Ile-OH-tBA	178—179	—	—	—	9
Z(OMe)-Ile-ONb	115—118	−12.5	0.4	THF	483
Leucine					
DPA-Leu-OH	111—113	−49.3[a]	1	MeOH	481
Troc-Leu-OH-tBA	168—169	—	—	—	9
Z-Leu-ONp-Pic-Tos-OH	81—83	−21.6	0.5	DMF	25
Z(OMe)-Leu-OPcp	108—110	−18.3	0.9	THF	483
Z-Leu-OTcrom	94—97	−9.5	3.1	$CHCl_3$	22
Lysine					
Nps-Lys(Adoc)-OH-Dcha	154	−24.1	1	$CHCl_3$	54
		−26.5[b]	1	$CHCl_3$	
Nps-Lys(Adoc)-ONSu	130—131	−36.86	1	$CHCl_3$	54
		−44.0[b]	1	$CHCl_3$	
H-Lys(Pac)-OH	232—234	+17.5	0.9	1M HCl	31
Troc-Lys(Troc)-OH-tBA	137	—	—	—	9
Troc-Lys(Z)-OH-tBA	131	—	—	—	9
Z-Lys(Boc)-OBu^t	Oil	—	—	—	36
Z-Lys(Pac)-OH	134—135	+2.4	1	MeOH	31
		−3.3	1	Dioxan	
Z-Lys(Troc)-OH-tBA	120	—	—	—	9
Methionine					
Boc-Met-OTcrom	98—100	—	—	—	22
DPA-Met-OH	—	−43[a]	1	MeOH	481
Troc-Met-OH	80—81	−12.3	3.5—4	EtOH	9
		−23.8	3.5—4	DMF	
Z(OMe)-Met-OMe	40—43	−30.8	0.7	MeOH	483
Z(OMe)-Met-ONSu	85—87	−13.6	0.9	THF	483
Z(OMe)-Met-OPcp	113—115	−13.4	0.4	THF	483
Phenylalanine					
Boc-Phe-OBzl	82	−13.5[a]	1	EtOH	412
Boc-Phe-ONp-Pic	117—118	−46.6	0.5	DMF	25
DPA-Phe-OH	154—156	+8.8[a]	1	EtOH	481
$(Fmoc\text{-}Phe)_2O$	152—153	—	—	—	127
Fmoc-Phe-OTcp	186.5	−55	1	DMF	131
Fmoc-Phe-OTcrom	102—107	—	—	—	22
Pht-Phe-OMTM	52	−169.4	1	EtOH	484
Tcroc-Phe-OEt	102—107	+31.3	1.3	$CHCl_3$	22
Tfa-Phe-OMTM	63	−39.0	1	EtOH	484

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Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc./g 100 cm^{-3}*	*Solvent*	*Ref.*
Troc-Phe-OH	129—130	+4.5	3.5—4	EtOH	9
		−28.95	3.5—4	DMF	
Z-Phe-ONp-Pic-Tos-OH	115—116	32.4	0.5	DMF	25
Proline					
DPA-Pro-OH	127—130	+8.8[a]	1	EtOH	481
TMSE-Pro-OH-Dcha	145—148	−28.3	1	MeOH	11
Troc-Pro-OH	64—65	−44.27	3.5—4	EtOH	9
		−41.90	3.5—4	DMF	
Z-Pro-ONp-Pic-Tos-OH	95—98	−39.0	0.5	DMF	25
Serine					
Boc-Ser(Bzl)-NH_2	97	+19.19	1	MeOH	243
Dnpy-Ser-OH	196	—	—	—	21
DPA-Ser-OH	128—132	−8.3[a]	1	1M NaOH	481
H-Ser(Bzl)-NH_2-Tfa	98	—	—	—	243
Z-Ser-NH_2	110—112	+8.5	1	MeOH	243
Z-Ser-OMTM	64	−20.4	1	EtOH	484
Tryptophan					
DPA-Trp-OH	125—128	+4.7[a]	1	MeOH	481
TMSE-Trp-OH	100—105	−24.7	1	DMF	11
TMSE-Trp-ONSu	119—121	−56.7	1	MeOH	11
Z-Trp-OMTM	127—128	−23.0	1	EtOH	484
Tyrosine					
Boc-Tyr(Cac)-OH	238—240	−19.6[a]	1	DMF	43
Boc-Tyr(Cac)-OH-Dcha	182	+39.7[a]	2.4	DMF	43
Dnpy-Tyr-OH	195	—	—	—	21
[Fmoc-Tyr(Bu^t)]$_2$O	94—96	−3.88	1	EtOAc	127
Fmoc-Tyr(Me)-OH	162	−28.5	1	DMF	364
Fmoc-Tyr-ONp	124—126	−20	1.5	$CHCl_3$ + 1% HOAc	242
H-Tyr(Cac)-OH	246	−0.9[a]	1	HCO_2H	43
Tyr(Cac)-NCA	179	−109.4[a]	1	Dioxan	43
Xan-Tyr(Cac)-NCA	144	+86.0[a]	1	Benzene	43
Z-Tyr(Bzl)-ONp-Pic-Tos-OH	135—136	−30.2	0.5	DMF	25
Valine					
(Fmoc-Val)$_2$O	173—174	+2.8	1	$CHCl_3$	127
Troc-Val-OH	80.5—81	−3.2	3.5—4	EtOH	9
		−8.9	3.5—4	DMF	
Other Amino-acids					
Adamantylalanine (Ada)					
H-Ada-OMe-HCl	155	+24.8	1	MeOH	271
D-H-Ada-OMe-HCl	155	−24.7	1	MeOH	271
Thiolalanine					
Boc-Ala-SH-Dcha	140—142	+6.4	4.3	EtOH	72
D-Alanine					
Boc-D-Ala-OH	81	+23.1	1	MeOH	268
(Fmoc-D-Ala)$_2$O	125—126	+12.07	1	EtOAc	127
β-Alanine					
DPA-*β*-Ala-OH	110—112	—	—	—	481
N-Methylalanine					
H-(Me)Ala-OTce-HBr	189—190	−12.58	2	DMF	374
Aminoisobutyric acid (Aib)					
Ac-Aib-OH	194—195	—	—	—	213

Compound	*M.p.*/°C	$[\alpha]_D$*[1]	*Conc.*/g 100 cm^{-3}	*Solvent*	*Ref.*
Boc-Aib-OH	117—118	—	—	—	213
Aziridine-2-carboxylic acid (Azy)					
Trt-(3-Me)Azy-OMe	103—107	−79.7	1.1	THF	459
Cyclohexyl-leucine (Cle)					
Boc-Cle-OH	125—128	—	—	—	125
Diaminopimelic acid (Dpm)					
L$^{\alpha}$,D$^{\alpha'}$-Boc$^{\alpha'}$-Dpm-(NHNHBoc)$^{\alpha'}$	200 (dec.)	+29.5	0.5	MeOH	298
L$^{\alpha}$,D$^{\alpha'}$-Z$^{\alpha}$-Boc$^{\alpha'}$-Dpm-(NHNHBoc)$^{\alpha}$	146—148	+18.8	1	MeOH	298
L$^{\alpha}$,D$^{\alpha'}$-Z$^{\alpha}$-Dpm-(NHNHBoc)$^{\alpha'}$	194—196	−20.8	0.5	MeOH	298
Diaminopropionic acid (Dap)					
Boc-Dap(H)-OH	198—200	−2.7	1	HOAc	485
Boc-Dap(Z)-OH-Dcha	177—178	+9.8	1	MeOH	485
Z-Dap(Boc)-OH	144—146	−9.3	1	MeOH	485
Z-Dap(Boc)-OH-Dcha	191—192	+7.1	1	MeOH	485
Z-Dap(H)-OH	228—230	−7.8	0.4	1M NaOH	485
Dehydroalanine (Dha)					
Boc-Dha-OH	94—95	—	—	—	215
Hexafluorovaline (Fal)					
H-Fal-OBzl-HCl	147—148	—	—	—	99
Tfa-DL-Fal-OH-$\frac{1}{2}H_2O$	123.5—125.5	—	—	—	99
D-Glutamine					
Boc-D-Gln-OH	115—117	+3.1	1.06	EtOH	308
Pyroglutamic acid (Glp)					
Z-Glp-OH	135—136	−29.5	1	MeOH	482
3′-Iodotyrosine (Ity)					
Boc-Ity-$NHNH_2$	188—188.5	+26*[2b]	1	MeOH	44
Boc-Ity-OH-Dcha	124—125	+34*[2b]	1	MeOH	44
Boc-Ity-OMe	101—103	+19*[2b]	1	MeOH	44
Boc-Ity-ONSu	169—170	+20*[2b]	1	HOAc	44
H-Ity-OMe-HCl	194.5—197	+8*[2b]	1	MeOH	44
Z-Ity-$NHNH_2$	211.5—213	−7*[2b]	1	DMF	44
Z-Ity-OH	123—124	+12*[2b]	1	MeOH	44
Z-Ity-OH-Dcha	164—165	+37*[2b]	1	MeOH	44
Z-Ity-OMe	99.5—101	+2*[2b]	1	MeOH	44
Neopentylglycine (Neo)					
Boc-Neo-OH	98	−16.8	1	MeOH	486
H-Neo-NH_2-HCl	217 (dec.)	+9.5	1	MeOH	486
H-Neo-OMe-HCl	160	+17.9	1	MeOH	486
Ornithine (Orn)					
Boc-Orn(Z)-$NHNH_2$	110	−10.8	1	MeOH	404
Boc-Orn(Z)-OH-Dcha	110	−11.5	1	MeOH	404
Boc-Orn(Z)-OMe	66—68	−8	1	MeOH	404
D-Phenylalanine					
DPA-D-Phe-OH	144—147	−9.5[a]	1	EtOH	481
β-Phenylalanine					
Boc-β-Phe-OH	122—123.5	+49.1	1	MeOH	231
		+47.0[a]	1	MeOH	
N-Methylphenylalanine					
Fmoc-(Me)Phe-OH	Oil	−39.6	1	DMF	364

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Compound	*M.p./°C*	$[\alpha]_D$*[1]	*Conc./g 100 cm⁻³*	*Solvent*	*Ref.*
Sarcosine (Sar)					
Fmoc-Sar-OH	43—45	—	—	—	364
D-Tyrosine					
Boc-D-Tyr-OMe	102—109	−10.5	2	MeOH	487

6 Appendix III: Purification Methods

Methods of purification for protected amino-acids and peptides are given: the list also includes reference to the purification of free peptides.

Technique	*Ref.*
High-performance Liquid Chromatography	
H.p.l.c. of angiotensins	488
Separation of Co^{III}-bis(ethylenediamine)amino-acid complexes by reversed-phase h.p.l.c.	489
H.p.l.c. of dansyl amino-acids	490
Dansylation of amino-acids for h.p.l.c.	491
H.p.l.c. of diphenylindenoylthiohydantoin derivatives	492
Enantiomer resolution by h.p.l.c.	493
H.p.l.c. of enkephalins	494
Separation of enkephalins and endorphins on Sep-Pak reversed-phase h.p.l.c. cartridges	495
H.p.l.c. of β-lipotropin and β-EP analogues	496
Prep. h.p.l.c. of ferricrocin	497
H.p.l.c. of glucagon and secretin	498
Resolution of DL-amino-acid glycosyl derivatives by reversed-phase h.p.l.c.	499
H.p.l.c. of linear gramicidins	500
H.p.l.c. of iodinated peptide hormones	501
H.p.l.c. of immunosuppressive peptide	502
H.p.l.c. separation of bovine and porcine insulins	503
H.p.l.c. of lysozyme tryptic fragments	504
Reversed-phase h.p.l.c. of peptide Pd^{II} complexes	505
H.p.l.c. of phosphoamino-acids	506

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Technique	*Ref.*
Purification of bovine PTH by reversed-phase h.p.l.c.	507
PTH amino-acid separation by h.p.l.c.	508
H.p.l.c. of peptides and proteins on porous silica columns	509
H.p.l.c. for separation of large polypeptides	510
Peptide purification by mixed-mode h.p.l.c.	511
Peptide separation by hydrophobic h.p.l.c.	512
Analytical and prep. h.p.l.c. on a macroreticular anion-exchange resin	513
Reversed-phase h.p.l.c. of peptides on radially compressed flexible-walled columns	514
Reversed-phase h.p.l.c. of peptides on radially compressed columns	515
Peptide purification by reversed-phase h.p.l.c.	516
Ligand-exchange h.p.l.c.	517
H.p.l.c. of peptides on chemically bonded hydrocarbon-based stationary phases	518
H.p.l.c. of protected peptides on Sephadex LH20	519
High-pressure gel-permeation chromatography of native proteins on TSK-SW	520
Solvents for peptide h.p.l.c.	521
Triethylammonium formate buffers for h.p.l.c. of oligopeptides	522
Buffer systems for h.p.l.c. of peptides	523
Retention times of peptides in h.p.l.c.	524
Gas–Liquid Chromatography	
α-Hydroxy-acid esters as chiral stationary phases for g.l.c.	525
Glass-capillary g.c. on optically active supports	526
Amino-acid derivatives as chiral stationary phases for g.l.c.	527
Enantiomer separation by g.l.c. on chiral stationary phases	528
Enantiomer separation on g.l.c.	529
G.c. separation of enantiomers on chiral phases	530
Enantiomer separation by circulation g.c.	531
Enantiomer separation on chiral phases, using stainless-steel capillary g.c.	532
G.c. of amino-acid derivatives, using glass capillary columns	533

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Technique	*Ref.*
Retention of Tfa-Aa-OBu^t derivatives on Chromosorb GHP(GLC)	534
G.c. of heptafluorobutyryl isobutyryl esters of tryptophan	535
Preparation of amino-acids for g.l.c.	536
Ion-exchange, Liquid, and Thin-layer Chromatography	
Amino-acid separation on β-cyclodextrin polyurethane resins	537
Ion-exchange chromatography of amino-acid and peptide copper(II) salts	538
Peptide–ampholyte interaction during purification	539
Liquid chromatography on porous polystyrene–divinylbenzene	540
Liquid chromatography of amino-acid enantiomers	541
Partition chromatography of β-lipotropin and β-EP analogues	542
Ligand-exchange chromatography on Spheron gels	543
Ligand-exchange chromatography on chemically bonded chiral phases	544
Ligand-exchange chromatography for separation of underivatized amino-acid enantiomers	545
Reversed-phase chromatography of dipeptides (t.l.c.)	546
T.l.c. on RP-2, -8, and -18 plates impregnated with dodecylbenzene sulphonic acid	547
T.l.c. of polypeptides on silanized silica gel	548

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4

Peptides with Structural Features not Typical of Proteins

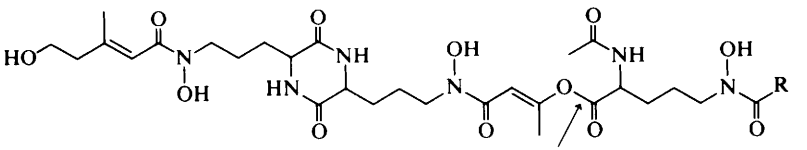
BY P. M. HARDY

1 Introduction

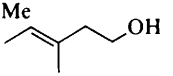
The area of coverage of this Report remains the same as that of last year except for the omission of the section on *S*-alkyl cysteinyl peptides as such. The bulk of this section concerned the leukotrienes, but since this year's work on these substances contains little new of interest with respect to peptide chemistry the topic has not been covered. Conformational studies of cyclic peptides larger than 2,5-dioxopiperazines have not this year been separated from other aspects of their chemistry; it has proved more convenient to consider cyclopeptides purely in order of their ring size. A few naturally occurring compounds have been included that, strictly speaking, do not have the requisite amino-acid content; however, their inclusion is considered justified by the fact that they arise by modification of protein amino-acids and do contain amide bonds.

2 Cyclic Peptides

2,5-Dioxopiperazines (Cyclic Dipeptides).—A new peptide containing a dioxopiperazine ring, triornicin (1a), has been isolated from *Epicoccum purpurascens*.



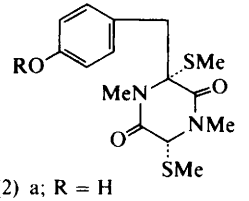
(1) a; R = Me

b; R = 

An acetyl function replaces the (*E*)-5-hydroxy-3-methyl-2-pentenoyl group of desferricoprogen (1b), earlier isolated from the same fungus. Cleavage of triornicin with methanolic base gives two fragments, N^{α},N^{δ}-diacetyl-N^{δ}-hydroxyornithine and the known siderophore dimerumic acid [cleavage position shown by arrow in (1)]. When grown in an iron-deficient medium, the fungus excretes triornicin into its environment as the iron-free ligand. Desferricoprogen does not possess the slight but significant anti-tumour effect shown by triornicin.[1]

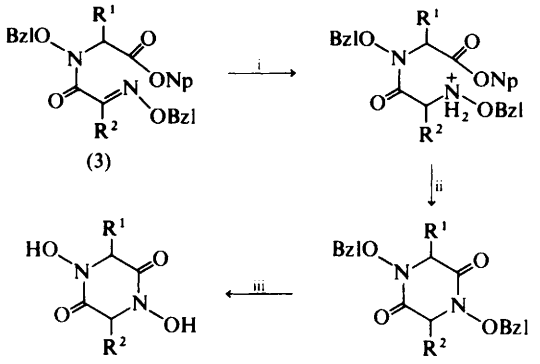
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Two new metabolites of *Gliocladium deliquescens*, which has already yielded gliotoxin and bisdethiobis(methylthio)gliotoxin, have been characterized as a phenol (2a) and its $\lambda\lambda$-dimethylallyl ether (2b).[2]



(2) a; R = H
b; R = $Me_2C{=}CHCH_2{-}$

Optically pure dioxopiperazines have been obtained in good yield on refluxing for 3 h dipeptide methyl, ethyl, or benzyl esters in 2-butanol containing 0.1M acetic acid, and this route is claimed to be superior to other methods. Where proline was the *N*-terminal residue, 1—2M acetic acid was required.[3] Cyclic dipeptide extrusion from dipeptides attached to α-(3-nitrobenzamido)benzylpolystyrene is much faster than from the original Merrifiled resin or a phenylacetamido-bridged polystyrene resin.[4] An investigation of the effects of heating an aqueous solution of Ile-Gly and Gly-Ile at 132 °C over the pH range 2—10 has shown that transient dioxopiperazine formation is important in determining the extent of racemization in the neutral pH region.[5] Although the ethyl esters of *NN'*-bis(benzyloxy)dipeptides resist ring closure to cyclic dipeptide derivatives, the corresponding *p*-nitrophenyl esters cyclize well, opening up an efficient route for the synthesis of 1,4-dihydroxy-2,5-dioxopiperazines (Scheme 1). When R^1 and R^2 are alkyl groups, the reduction of (3) proceeds with some chiral induction.[6] All



(3)

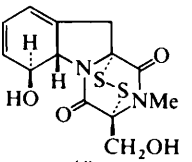
R^1, R^2 = H, Me, or Pr^i; ONp = $OC_6H_4NO_2$-*p*

Reagents: i, $Me_3N{\cdot}BH_3$, HCl, dioxan; ii, pyridine, CH_2Cl_2; iii, Pd/C, H_2

Scheme 1

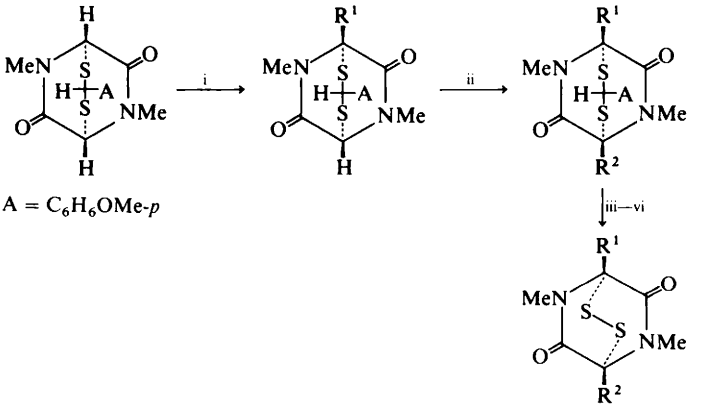
[2] J. R. Hanson and M. A. O'Leary, *J. Chem. Soc., Perkin Trans. 1*, 1981, 218.
[3] K. Suzuki, Y. Sasaki, N. Eudo, and Y. Mihara, *Chem. Pharm. Bull.*, 1981, **29**, 233.
[4] E. Giralt, R. Eritja, and E. Pedroso, *Tetrahedron Lett.*, 1981, 3779.
[5] S. Steinberg and J. L. Bada, *Science*, 1981, **213**, 544.
[6] J. D. M. Herscheid, J. H. Colstec, and H. C. J. Ottenheijm, *J. Org. Chem.*, 1981, **46**, 3346.

four stereoisomers of *cyclo*(-Tyr-Arg-) have been prepared by acetic acid-catalysed cyclization of the linear dipeptide methyl esters. The LL isomer proved the most potent in inducing analgesia when administered intracerebrally into mice, being five times as active as Tyr-Arg, which occurs naturally in bovine brain.[7]



(4)

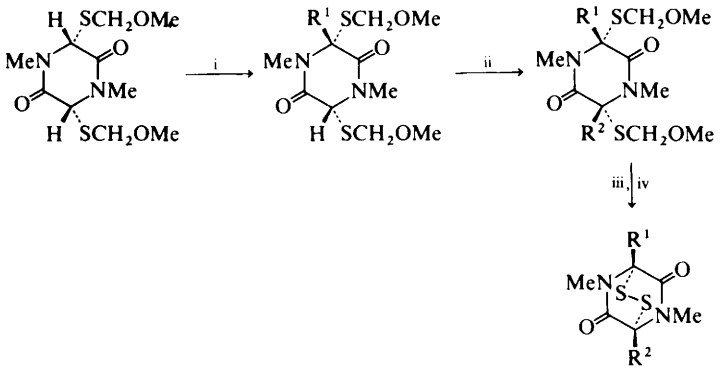
Full details of the total synthesis of gliotoxin (4) have been published. In this paper two general methods for the synthesis of epidithiapiperazinediones are described (Schemes 2 and 3). The route described in Scheme 2 is more suitable for



A = C_6H_6OMe-*p*

Reagents: i, $LiNiPr_2$, THF, −78 °C, R^1Br; ii, as for i but R^2Br; iii, *m*-chloroperbenzoic acid, 0 °C; iv, Me_2S; v, 70% perchloric acid in MeOH; vi, $NaHCO_3$

Scheme 2

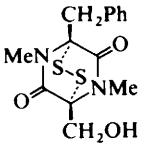


Reagents: i, $LiNiPr_2$, THF, −76 °C, R^1Br; ii, as for i but R^2Br; iii, BCl_3; iv, I_2

Scheme 3

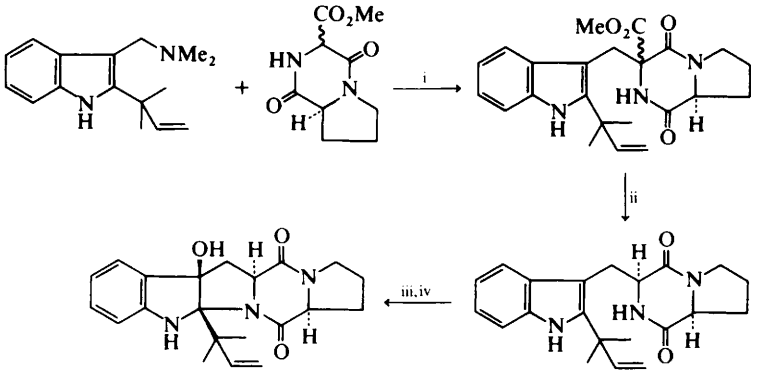
[7] Y. Sosaki, Y. Akutsu, K. Suzuki, S. Sakurada, and K. Kisara, *Chem. Pharm. Bull.*, 1981, **29**, 3403.

multi-stage synthesis of complex molecules because of the stability of the thioacetals under various conditions, and it was used in preparing gliotoxin. The other route is more suited to the large-scale synthesis of simple epidithiapiperazinediones, *e.g.* hyalodendrin (5), because of the simplicity of procedures and



(5)

high yields.[8] The chiral total synthesis of brevianamide E (6) *via* deoxybrevianamide E has also been accomplished, showing that natural material has the

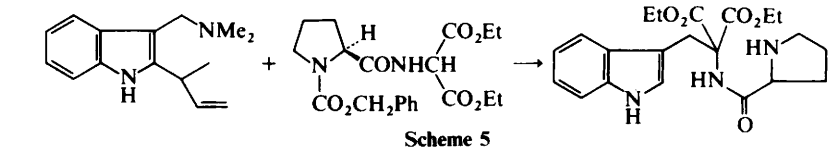


(6)

Reagents: i, NaH, DMF, 55—60 °C; ii, DMSO, $MgCl_2$, 130 °C; iii, *hν*, MeOH, Rose Bengal, 3 h, O_2; iv, Me_2S

Scheme 4

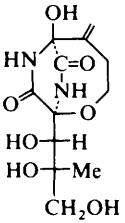
(4a*S*,5a*R*,10a*S*,11a*S*) configuration (Scheme 4). The final oxidative photocyclization went in a yield of 42%.[9] An alternative synthesis of deoxybrevianamide E has been reported; like the previous synthesis, in the key step a gramine derivative is condensed with a dipeptide, but in this case it is an open-chain derivative not a dioxopiperazine (Scheme 5).[10]



Scheme 5

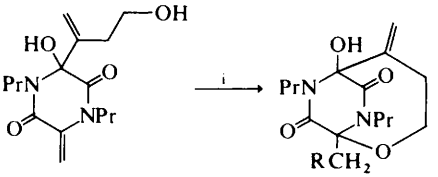
A number of groups have now demonstrated a variety of approaches to the synthesis of the bicyclic system of bicyclomycin (7), an antibiotic from *Streptomyces sapparonsesis* active against Gram-negative bacteria whose structure was

[8] F. Fukuyama, S.-I. Nakatsuka, and Y. Kishi, *Tetrahedron*, 1981, **37**, 2045.
[9] T. Kametani, N. Kanaya, and M. Ihara, *J. Chem. Soc., Perkin Trans. 1*, 1981, 959.
[10] R. Ritchie and J. Saxton, *Tetrahedron*, 1981, **37**, 4295.



(7)

determined by *X*-ray in 1974.[11] The conditions for cyclization and the structural elements involved differ in each case. Cyclization with pyridinium hydrobromide perbromide (Scheme 6) gave a 65% yield of material with the desired skeleton, but

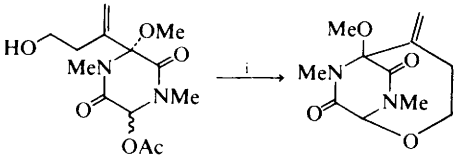


(8) a; R = Br
b; R = SePh

Reagent: i, $PHBr_3$, Py, CH_2Cl_2, 20 °C or PhSeCl, Py, CH_2Cl_2, 70 °C

Scheme 6

attempts to elongate the side chain of the product (8a) by S_N2 substitution of the bromine failed. Cyclization with phenylselenyl chloride gave a slightly better yield (of 8b), and the selenium-containing side chain in this case could be converted to an aldehyde group.[12] A product lacking the side chain but incorporating the exocyclic alkene group (9) has been prepared *via* cyclization with pyridinium



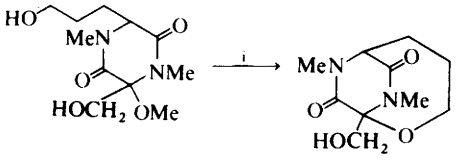
(9) 50%

Reagent: i, pyridinium tosylate, CH_2ClCH_2Cl, 80 °C, 2 h

Scheme 7

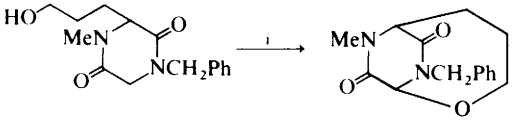
tosylate (Scheme 7).[13] Two other syntheses also follow the same pattern in as much as the ether link is constructed last in the completion of the bicyclic system; in one case cyclization is mediated by camphorsulphonic acid (Scheme 8),[14] and in the other *N*-bromosuccinimide is initially used to effect bromination, cyclization occurring as a subsequent step under the reaction conditions used (Scheme 9).[15]

[11] T. Takuma, S. Koda, T. Miyoshi, and Y. Marimoto, *Bull. Chem. Soc. Jpn.*, 1974, **47**, 18.
[12] T. Fukuyama, B. D. Robins, and R. A. Sachleben, *Tetrahedron Lett.*, 1981, 4155.
[13] S. Nakatsuka, K. Yoshida, and T. Goto, *Tetrahedron Lett.*, 1981, 2009.
[14] R. M. Williams, *Tetrahedron Lett.*, 1981, 2341.
[15] C. Shin, Y. Sato, and J. Yoshimura, *Tetrahedron Lett.*, 1981, 2401.



Reagent: i, camphorsulphonic acid, MeCN, reflux

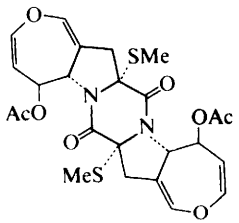
Scheme 8



Reagent: i, *N*-bromosuccinimide, $CHCl_3$, reflux 1 h

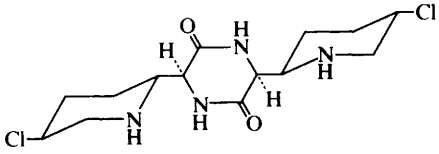
Scheme 9

cyclo(-L-[^{15}N]Phe-L-[1-^{13}C]Phe-) is incorporated intact into bisdethiobis-(methylthio)acetylaranotin (10) by *Aspergillus terreus*, but neither *cyclo*(-L-Phe-D-Phe-) nor *cyclo*(-D-Phe-D-Phe-) is an efficient precursor.[16] The oxepin systems are thought to arise *via* ring opening of arene oxides followed by epoxidation and cyclization.[17]



(10)

A number of *X*-ray studies on dioxopiperazines have been reported. In *cyclo*(L-Ser)$_2$ the side chains are in quasi-axial positions directed towards each other across the ring. There is no direct interaction between the side chains, both oxygen atoms of the hydroxyl groups being engaged in intermolecular hydrogen bonds within the crystal.[18] Full details of the crystal structure of the antineoplastic compound *cyclo*(streptolutyl)$_2$ (11), as its sulphate pentahydrate, have appeared. Both piperidine rings are chair forms, with the chlorines in equatorial positions. The dioxopiperazine ring itself is in a twist-boat conformation, with the amide bonds



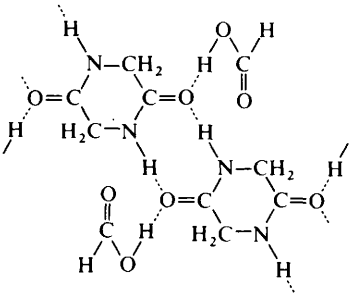
(11)

[16] M. I. P. Boente, G. W. Kirby, and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 619.

[17] R. Nagarajan, L. L. Huckstep, D. H. Lively, D. C. Delong, M. M. Marsh, and N. Neuss, *J. Am. Chem. Soc.*, 1981, **103**, 2980; J. W. Moncrief, *ibid.*, p. 6517.

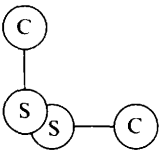
[18] G. G. Fava, M. F. Belicchi, R. Marchelli, and A. Dossewa, *Acta Crystallogr., Sect. B*, 1981, **37**, 625.

appreciably twisted from planarity as a result of repulsion between the bulky and charged side chains.[19] In *cyclo*-L-cystine the dioxopiperazine ring is also in a twisted boat form, again with significantly non-planar peptide bonds. The intramolecular disulphide bridge has a negative chirality with χ_3 angle of $-94°$.[20] The heterocyclic ring of the formic acid complex of 2,5-dioxopiperazine itself is almost exactly planar except for the hydrogen atoms attached to the α-C. There are eight-membered rings formed by hydrogen bonds (12); in the O—H···O bonds there is a hydrogen-to-acceptor-oxygen distance of 1.73 Å.[21]



(12)

^{1}H and ^{13}C n.m.r. studies of *cyclo*(L-Thr)$_2$ and *cyclo*(L-*allo*-Thr)$_2$ in water and DMSO show conformational differences. In the former the peptide ring is puckered to hold the side chains more nearly axial than is the case with the latter, although the degree of ring folding is not large. Hydrogen bonding between the two hydroxyl groups is not an important factor in stabilizing the conformers.[22] C.d. and ^{1}H n.m.r. studies of *SS'*-bis-[*cyclo*(-L-Cys-L-Sar-) indicate that the proline-containing peptide equilibrates between two rotamers around the CSSC bond in MeOH, but a P-helix type of conformation (13) predominates in

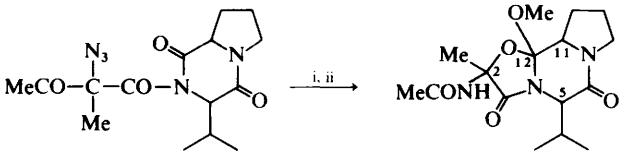


(13)

acetonitrile. The sarcosine-containing peptide rotates freely in DMSO, but a relatively stable P-helix exists in MeOH or $CHCl_3$–Tfa (9 : 1).[23] The infrared spectra of CCl_4 solutions of the sporidesmins have been examined. Marked differences in the 3500—3600 cm^{-1} region indicate differences in intramolecular hydrogen bonding.[24]

An *X*-ray study of a cyclol produced in low yield by photolysis of a dioxopiperazine (14) (Scheme 10) shows it to have (2*S*, 5*S*, 11*R*, and 12*S*)

[19] R. B. Van Dreche, *Acta Crystallogr., Sect. B*, 1981, **37**, 93.
[20] K. I. Varughese, C. T. Lu, and G. Kartha, *Int. J. Pept. Protein Res.*, 1981, **18**, 88.
[21] G. Kartha, K. I. Varughese, and C. T. Lue, *Acta Crystallogr., Sect. B*, 1981, **37**, 1798.
[22] K. D. Kopple and V. Narutis, *Int. J. Pept. Protein Res.*, 1981, **18**, 33.
[23] N. Ueyama and T. Araki, *Biopolymers*, 1981, **20**, 2485.
[24] J. W. Ronaldson, *Aust. J. Chem.*, 1981, **34**, 1215.

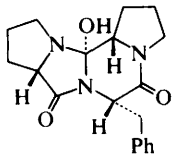


(14)

Reagents: i, *hν*, MeOH; ii, H^+, MeOH

Scheme 10

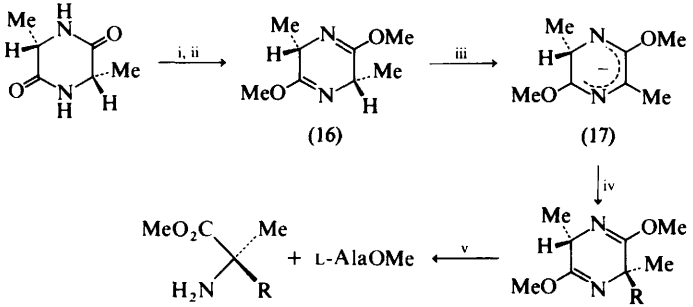
stereochemistry; it therefore differs at two centres from ergotamine. This cyclol is thought to be produced through an acylimine intermediate.[25] The first azacyclol to be obtained free of protecting groups has been prepared by cyclization of Pro-Phe-Pro-ONp in an aqueous medium. An 8.5% yield of the product (15) was



(15)

obtained, and its structure was verified by *X*-ray analysis. The cyclol slowly decomposes on standing in solution at 20 °C, and unlike *N*-protected analogues it does not show tautomerism with the aminoacyldioxopiperazine form. Attempts to prepare a cyclol from Phe-Pro-Pro-ONp failed.[26]

Dioxopiperazines have been further exploited as intermediates for the synthesis of various types of amino-acid. Treatment of *cyclo*(L-Ala)$_2$ with trimethyloxonium fluoroborate gives the bis-lactim ether (16), which can be alkylated in good chemical yield with more than 90% diastereoselectivity, allowing preparation of α-methylamino-acids (Scheme 11).[27] The lithiated bis-lactim ether (17) also reacts



Reagents: i, $[Me_3O]^+BF_4^-$; ii, H_2O, pH 7; iii, Bu^nLi; iv, RX; v, 2 equiv. 0.25M HCl, 25 °C

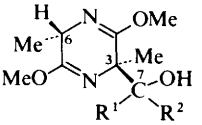
Scheme 11

[25] M. Przybylska and F. R. Ahmad, *Acta Crystallogr., Sect. B*, 1981, **37**, 168.

[26] G. Lucente, F. Pinnen, G. Zanotti, S. Cerrini, W. Fedali, and E. Gavuzzo, *Tetrahedron Lett.*, 1981, **22**, 3671.

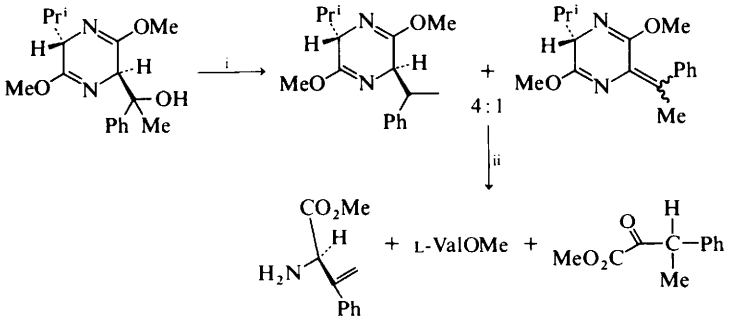
[27] U. Schollkopf, W. Hartwig, U. Groth, and K. O. Westphalen, *Liebigs Ann. Chem.*, 1981, 696.

with aldehydes or ketones with 81—95% asymmetric induction at C-3, the (*R*) configuration again predominating (18). With aldehydes and unsymmetrical



(18)

ketones, C-7 of the adduct becomes a chiral centre of (*R*) configuration with an asymmetric induction of 47—73%. Hydrolysis yields a β-hydroxyamino-acid,[28] and dehydration to give (*R*)-α-vinylamino-acids can be effected (Scheme 12). The bis-lactim ether of *cyclo*[-(*S*)-(*OO*-dimethyl-α-methyldopa)-Gly-] is also suitable for the synthesis of α-vinylamino-acids. Using acetone in a reaction similar to Scheme 12, (*R*)-3-amino-3-methyl-3-butenoic acid methyl ester with an enantiomeric excess of 88% was obtained.[29]



Reagents: i, $SOCl_2$, 2,6-lutidine; ii, 0.25M HCl

Scheme 12

cyclo(-L-Phe-L-His-) has been found to catalyse the asymmetric addition of hydrogen cyanide to benzaldehyde with the highest optical yield yet obtained for a non-enzymic catalyst. After a reaction time of half an hour, the enantiomeric excess of the product, rich in the (*R*) antipode, was 90%. Longer reaction times showed some racemization to be occurring, indicating the actual enantioselectivity to exceed 90%.[30]

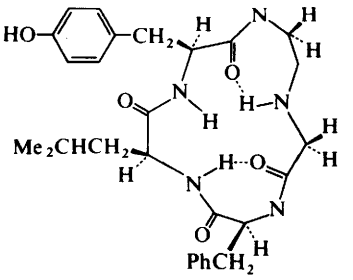
Larger Cyclic Peptides.—The cyclotripeptide *cyclo*(-L-Ala-Gly-ε-aminocaproyl-) has been prepared and its conformation examined, theoretical calculations suggesting that the molecule should exist predominantly as a type II β-bend.[31] A 48% yield was obtained after a *p*-nitrophenyl ester cyclization, and examination by c.d. and ^1H n.m.r. indeed showed a type II β-bend, with a rather bent NH···O=C bond in the aminocaproyl residue. There are small amounts of

[28] U. Schollkopf, U. Groth, and W. Hartwig, *Liebigs Ann. Chem.*, 1981, 2407.
[29] U. Schollkopf and U. Groth, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 977.
[30] J.-I. Oku and S. Inoue, *J. Chem. Soc., Chem. Commun.*, 1981, 229.
[31] G. Némethy, J. R. McQuie, M. S. Pottle, and H. A. Scheraga, *Macromolecules*, 1981, **14**, 1975.

molecules with type I and type II β-bends.[32] I.r. and Raman spectral studies confirm the predominant conformation.[33] A linear peptide with the same amino-acid sequence, Boc-L-Ala-Gly-Aca-OMe, contains significant amounts of type II β-bend structures.[32] A 270 and 500 MHz ^{1}H n.m.r. investigation of *cyclo*(L-Pro)$_3$ revises a number of previous spectral assignments. The ^{1}H-coupling constants are best interpreted in terms of an α^+ (envelope) conformation. The high mobility of the γ-CH_2 group easily transforms this structure into twist conformations.[34]

An analogue of the cytostatic cyclic tetrapeptide chlamydocin has been synthesized based on the observation that *cyclo*(-Gly-L-Phe-D-Pro-L-Ala-) adopts a conformation in $CHCl_3$ closely related to that of chlamydocin. A 2,4,5-trichlorophenyl ester cyclization gave a 14% yield of *cyclo*[-Gly-L-Phe-D-Pro-L-Dap-(β-Boc)-]. However, both this product and a derivative in which the side-chain protecting group is *N*-maleoylglycyl have little of the biological activity of chlamydocin.[35] An *X*-ray determination of the crystal structure of dihydrotentoxin, *cyclo*(-L-Leu-D-MePhe-Gly-L-MeAla-), shows its conformation to differ little from that of *cyclo*(Sar)$_4$. The energetic cost of placing two peptide bonds in the *cis* conformation is apparently more than balanced by the more favourable disposition of extra-annular alkyl groups.[36]

Three groups have synthesized cyclic analogues of enkephalin. The heterodetic compound [D-Dys^2, L-Cys^5]enkephalinamide and its D,D diastereoisomer proved 37.9 and 73.3 times, respectively, as potent as [Met^5]enkephalin, being highly resistant to enzymic degradation.[37] Two homodetic analogues, *cyclo*(-Tyr-Gly-Gly-Phe-Leu- or -Met-), have been prepared by azide cyclizations in 12% yield, the Gly-Gly bond being the last to be formed. Poor water solubility prevented bioassay, but ^{1}H n.m.r. spectroscopy shows a predominant conformation containing two γ-turns (19), as previously found in *cyclo*(-Phe$_3$Gly$_2$-). Comparison of



(19)

[32] R. Deslauriers, D. J. Evans, S. J. Leach, Y. C. Meinwald, E. Minasian, G. Némethy, I. D. Rae, H. A. Scheraga, R. L. Somarjai, E. R. Stimson, J. W. Van Nispen, and R. W. Woody, *Macromolecules*, 1981, **14**, 985.

[33] F. R. Mayfield, J. Banderar, S. Krimm, D. J. Evans, S. J. Leach, G. Némethy, and H. A. Scheraga, *Macromolecules*, 1981, **14**, 997.

[34] H. Kessler, A. Friedrich, and W. E. Hull, *J. Org. Chem.*, 1981, **46**, 3892.

[35] D. H. Rick, R. D. Jasensky, G. C. Mueller, and K. E. Anderson, *J. Med. Chem.*, 1981, **24**, 567.

[36] P. N. Swepston, A. W. Cordes, L. F. Kuyper, and W. L. Meyer, *Acta Crystallogr., Sect. B*, 1981, **37**, 1139.

[37] P. W. Schiller, B. Eggimann, J. Dimaio, C. Lemieux, and T. M. D. Nguyen, *Biochem. Biophys. Res. Commun.*, 1981, **101**, 337.

these results with those of the zwitterionic linear enkephalins in DMSO suggests a possible $\lambda\lambda$-conformation for the latter as well, instead of the previously proposed conformation containing only one β-turn.[38,39] [Met^5]enkephalin and $ACTH_{4-10}$ both decrease acquisition of the step-up active avoidance response when injected i.p. into rats. Both contain a Phe and a Met residue, so *cyclo*(-Phe-Met-ε-Ahx-), *cyclo*(-D-Phe-Met-ε-Ahx-), *cyclo*(-Phe-D-Met-ε-Ahx-), and *cyclo*(-Phe-Met(O_2)-ε-Ahx-) have been prepared, using diphenylphosphorylazide for the cyclizations, for comparison purposes. All except the methionine sulphone-containing analogue showed some biological activity, and *cyclo*(-Phe-Met-ε-Ahx-) was also effective after oral administration.[40] ^{1}H and ^{13}C n.m.r. spectral studies on *cyclo*(-Val-Pro-Gly-Val-Gly-), prepared by a *p*-nitrophenyl ester cyclization, indicate the presence of a *cis*-Val^1-Pro^2 peptide bond in water. A strong intramolecular H-bond between the Val^{1}NH and Gly^{3}C=O group forms a type II β-turn placing the Val^4 and Gly^5 residues within the turn. Two minimum-energy conformations were theoretically derived, one of which agrees very well with the solution conformation proposed.[41]

A solid-state ^{13}C n.m.r. examination of *cyclo*(-D-Phe-Pro-Gly-D-Ala-Pro-) and *cyclo*(Gly-Pro-Gly)$_2$ shows that the two conformationally distinct Pro residues in each compound exhibit a clear distinction of the C_β signals. This is not shown by *cyclo*(Gly-Pro-Gly)$_2$ in aqueous solution.[42] The crystal structure of *cyclo*(-Gly-His-Gly-Ala-Tyr-Gly-) shows a type I β-turn at Ala-Tyr and a type II′ β-turn at Gly-His. The overall conformation is very similar to that reported earlier for *cyclo*(-Gly-Ala-Gly-Ala-Ala-Gly-), although the hydration and H-bonding patterns are entirely different. In solution, ^{1}H n.m.r. indicates that a single backbone is not dominant in either DMSO or H_2O.[43] *X*-Ray analysis of *cyclo*(Gly-D-Leu-Leu)$_2$ shows two molecules in the asymmetric unit. Both molecules have backbones in the form of two type II′ β-turns linked by extended glycyl residues. Five molecules of water and one of ethanol are present in the cell, and the hydrophobic leucyl side chains are segregated from the solvent molecules and the solvated peptide bonds.[44]

The effect of amino-acid sequence upon the formation of β-turns has been examined in a series of cyclic hexapeptides of the form *cyclo*(X-Pro-Y)$_2$, where X and Y were chosen from Gly, L- or D-Ala, L- or D-Phe, and L-Val. The presence of β-turns was detected by n.m.r., and the results led to the formulation of the following set of 'rules'.

(1) The sequence L-Pro-Gly or L-Pro-D-Y leads to type II β-turns.
(2) The sequence Gly-L-Pro-L-Y (where Y is not L-Val) leads to type I β-turns.
(3) The sequence Gly-L-Pro-L-Val or D-X-L-Pro-L-Y leads to type II′ β-turns.

A survey of β-turns in proteins revealed frequencies and positions of amino-acid occurrences in β-turns consistent with these rules, confirming cyclohexapeptides to

[38] H. Kessler and G. Holzemann, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 124.
[39] H. Kessler and G. Holzemann, *Liebigs Ann. Chem.*, 1981, 2028.
[40] H. Rigter, H. N. Grevan, J. W. Van Nispen, and J. L. Martinez, *Biochem. Biophys. Res. Commun.*, 1981, **98**, 424.
[41] M. A. Khaled, C. M. Venkatachalam, H. Sugano, and D. W. Urry, *Int. J. Pept. Protein Res.*, 1981, **17**, 23.
[42] L. G. Pease, M. H. Frey, and S. J. Opella, *J. Am. Chem. Soc.*, 1981, **103**, 467.
[43] C.-H. Yang, J. N. Brown, and K. D. Kopple, *J. Am. Chem. Soc.*, 1981, **103**, 1715.
[44] K. I. Varughese, G. Kartha, and K. D. Kopple, *J. Am. Chem. Soc.*, 1981, **103**, 3310.

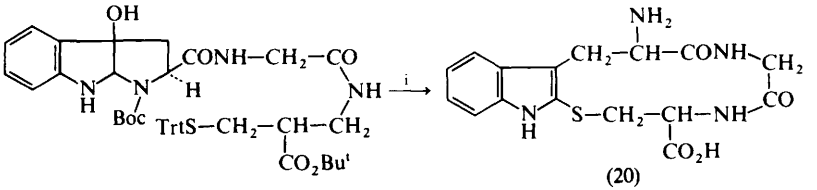
be good models for regions of a protein where the peptide chain reverses direction.[45] In cyclic hexapeptides of the type *cyclo*(L-X-L-Pro-D-Y)$_2$, which exist in solution predominantly in two forms of C_2 average symmetry, stability of the all-*trans* form has been found to decrease as X is changed through the series Gly, L-Ala, L-Leu, and L-Val (Y = Gly, D-Ala, or D-Phe). For a given set of side chains, the stability of the all-*trans* form increases as the polarity of the solvent decreases, consistent with models in which all the C=O and NH groups are accessible for solvation in the two-*cis* form but where two C=O and two NH groups are somewhat sequestered in the all-*trans* form.[46]

A redetermination of the crystal structure of ferrichrome A at low temperature has shown the same disorder in parts of the structure found in the earlier investigation. *X*-Ray also shows that the peptide conformation in alumichrome A (aluminium desferri-ferrichrome A) is identical to that in ferrichrome A. N.m.r. work indicates that substitution of Al^{III} for Fe^{III} also does not change the solution conformation.[47] A number of cyclic oligomers of the repeating pentapeptide sequence of elastin, *cyclo*(Val-Pro-Gly-Val-Gly)$_n$ (n = 2—6), have been synthesized by *p*-nitrophenyl ester monomeric cyclizations. Yields were 32—35% except for n = 3, when an 80% yield was obtained. The conformations of the cyclopentapeptide (whose preparation was reported earlier) and of the cyclodecapeptide clearly differ from the linear pentapeptide, but those for n = 3—6 are quite similar, in particular for n = 3 or 6.[48] Six minimum-energy structures have been theoretically derived for *cyclo*(Val-Pro-Gly-Val-Gly)$_3$, three of which are consistent with ^{1}H n.m.r. results and one of which is similar to the *X*-ray structure of the crystal reported earlier.[49] The one-bond C_α,H-coupling constants in cyclic di-, tetra-, hexa-, and octa-peptides strongly support the idea that the conformational term that modulates the coupling constant is composed of two independent terms. There is a positive contribution from the neighbouring nitrogen p_z-orbital and a negative contribution for the carbonyl π-system. Application to the conformational analysis of *cyclo*(L-Pro-Gly)$_3$, *cyclo*(D-Ala$_2$-Ala-D-Ala-Ala$_2$)$_2$, and *cyclo*(Sar)$_8$ has been discussed.[50]

Di-Z^δ-Orn-gramicidin S has been prepared by a one-pot monomeric cyclization using (5-nitropyridyl)diphenylphosphinate in 82% yield.[51]

Cyclic Peptides from *Amanita* Species.—Etherification of α-amanitin with tritiated methyl iodide has given radiolabelled material of specific activity ~4 Ci mmol^{-1} whose inhibition capacity for RNA polymerase II is very similar to that of α-amanitin itself. This derivative has been used successfully in binding assays and in radioimmunology. Reaction of α-amanitin with long-chain alkyl bromides gives lipophilic ethers with an increased capacity for cell penetration. *O*-Hexyl- and *O*-decyl-α-amanitin proved more toxic *in vivo* than α-amanitin, although their

[45] L. M. Gierasch, C. M. Deber, V. Madison, C.-H. Niu, and E. R. Blout, *Biochemistry*, 1981, **20**, 4730.
[46] K. D. Kopple, S. K. Sarkar, and G. Giacometti, *Biopolymers*, 1981, **20**, 1291.
[47] D. Van der Helm, J. R. Baker, R. A. Loglury, and J. D. Ekstrand, *Acta Crystallogr., Sect. B*, 1981, **37**, 323.
[48] D. W. Urry, T. L. Trapane, H. Sugano, and K. U. Prasad, *J. Am. Chem. Soc.*, 1981, **103**, 3080.
[49] C. M. Venkatachalam, M. A. Khaled, H. Sugano, and D. W. Urry, *J. Am. Chem. Soc.*, 1981, **103**, 2372.
[50] H. Egli and W. Van Philipsborn, *Helv. Chim. Acta*, 1981, **64**, 976.
[51] T. Mukaiyama, K. Kamekawa, and Y. Watanabe, *Chem. Lett.*, 1981, 1367.

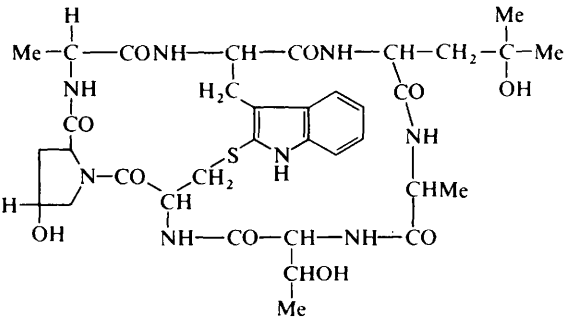


(20)

Reagent: i, Tfa, 2 h, 20 °C

Scheme 13

affinity to RNA polymerase II is much reduced. Alkylation in these ethers occurs at the indole phenolic hydroxyl rather than the dihydroxyleucine hydroxyls.[52] The cyclic thioether peptide 2-mercapto-L-tryptophanyl-glycylcysteine cyclic (1 → 3) sulphide (20) shows a Cotton effect ~290 nm in its c.d. spectrum that is nearly a mirror image of that of phallotoxin (21) itself. An *X*-ray structure analysis of

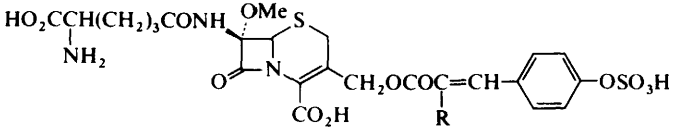


(21)

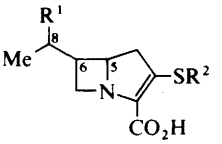
the *N*-*p*-bromobenzenesulphonyl derivative of (20) shows M-helicity, indicating phallotoxin to possess P-helicity. Although the ^{1}H n.m.r. spectrum of (20) confirms M-helicity, helicity could not be unambiguously assigned to phallotoxin on ^{1}H n.m.r. evidence. Cyclization to give the model compound (20) was effected by the Savige–Fontana reaction in 24% yield (Scheme 13).[53] F-Actin, $[A(SH)_5]_n$, prepared from rabbit skeletal muscle, reacts with 2,4-dinitrophenylglutathionyl-disulphide (DNP-SSG) to give $[A(SH)_3(SSG)_2]_n$. One thiol group reacts rapidly (~20 min), and the other more slowly (~200 min); the former has been identified as Cys-373. Phalloidin, which binds to polymeric actin, shields one of these two reactive thiol groups from reaction with DNP-SSG; in this it resembles ATP. It is thought that in both cases a conformational change in F-actin that exposes a second thiol group is suppressed.[54]

Highly Modified Cyclic Peptides.—A number of new β-lactam antibiotics have been characterized. *Streptomyces organonensis* has yielded organomycin A (22a), a close relative of the earlier-known cephomycin A (22b). The latter is significantly

[52] H. Faulstich, H. Trischumann, Th. Wieland, and E. Wuff, *Biochemistry*, 1981, **20**, 6498.
[53] Th. Wieland, B. Beijer, A. Seeliger, J. Dabrowski, G. Zanotti, A. E. Tonelli, A. Gieren, B. Dederer, V. Lamm, and E. Hadicke, *Liebigs Ann. Chem.*, 1981, 2381.
[54] H. Blackholm and H. Faulstich, *Biochem. Biophys. Res. Commun.*, 1981, **103**, 125.



(22) a; R = H
b; R = OMe

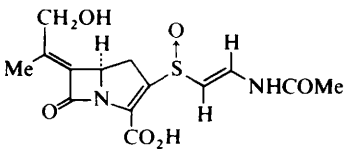


	R^1	R^2	Configurations			β-Lactam configuration
			C-5	C-6	C-7	
A	OH	$CH_2CH_2NHCOMe$	*R*	*R*	*S*	*cis*
B	OH	CH=CHNHCOMe	*R*	*R*	(*S*)	*cis*
C	OH	$CH_2CH_2NHCOMe$	*R*	*S*	*S*	*trans*
D	OH	CH=CHNHCOMe	*R*	*S*	(*S*)	*trans*
E	$-OSO_3H$	CH=CHNHCOMe	(*R*)	*R*	(*S*)	*cis*
F	$-OSO_3H$	$CH_2CH_2NHCOMe$	(*R*)	*R*	(*S*)	*cis*
Thienomycin	OH	$CH_2CH_2NHCOMe$	*R*	*S*	*R*	*trans*

( ) signifies an inferred configuration

(23)

less stable than organomycin A in aqueous solution at pH 7.5.[55] A new family of β-lactam antibiotics, the epithienamycins (23), has been isolated from *Streptomyces flavogriseus*. Enzymically deacetylated epithienamycin A has the properties of an isomer of the already known compound thienamycin.[56] A third Streptomyces, *S. takunonensis*, is the source of a new carbapenem antibiotic asparenomycin A (24), which is unique in having a substituted ethylidene side



(24)

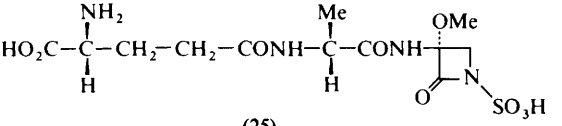
chain. A strong inhibitor of various types of β-lactamase, it is active against a broad range of Gram-negative and -positive organisms. The structure was determined by *X*-ray crystallography.[57] Conformational-energy calculations on the family of β-lactam antibiotics, which includes thienamycin, PS-5, and 1-oxa- and 1-thiapenems, indicate the bicyclic system to be highly rigid in all cases and to favour only

[55] H. Gushima, S. Watanabe, T. Saito, T. Sasaki, H. Eiki, Y. Oka, and T. Osono, *J. Antibiot.*, 1981, **34**, 1512.

[56] P. J. Cassidy, G. Albers-Schonberg, R. T. Goegelman, T. Miller, B. Arison, E. O. Stapley, and J. Birnbaum, *J. Antibiot.*, 1981, **34**, 637.

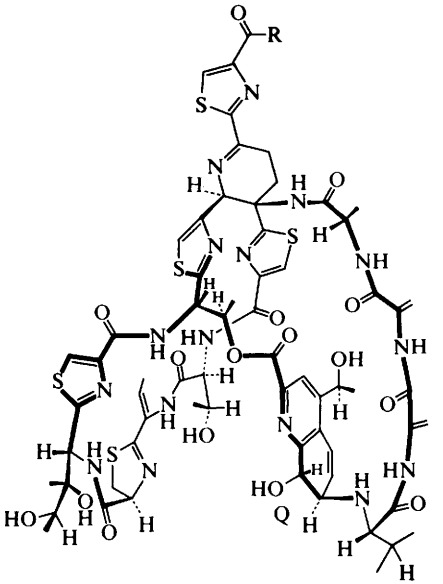
[57] K. Tanaka, J. Shoji, Y. Terui, N. Tsuji, E. Kondo, M. Mayama, Y. Kawamura, T. Mattari, K. Matsumobo, and Y. Yoshida, *J. Antibiot.*, 1981, **34**, 909.

one conformation. This contrasts with the penicillins, where the five-membered rings assume two puckered conformations. In agreement with *X*-ray results, this study predicts that the lactam peptide bond is more planar in 1-carbapenems than in 1-thiapenems.[58] A structurally novel water-soluble acidic β-lactam antibiotic, sulfazecin (25), has been found to occur in *Pseudomonas acidophila.* Hydrolysis yields D-alanine and D-glutamic acid.[59]



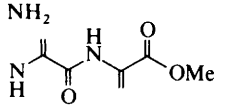
(25)

The structures of four new thiostrepton-type antibiotics have been established. The major constituent of the antibiotic complex from *Micromonospora arborensis* has proved very close in structure to thiostrepton itself, differing only in possessing a thioamide bond in place of an amide bond and a piperidine ring in place of a tetrahydropyridine ring. The molar mass was determined by ^{252}CF plasma-desorption m.s.[60] The structures of siomycins B and C from *Streptomyces sioyaensis* (26a and 26b) and thiostrepton B from *S. azureus* (26c) have been



(26) a; R = NH_2

b; R =



c; R = NH_2; contains Ile-Ala instead of Val-DeAla

[58] T. K. Vasudevan and V. S. R. Rao, *Biopolymers*, 1981, **20**, 865.

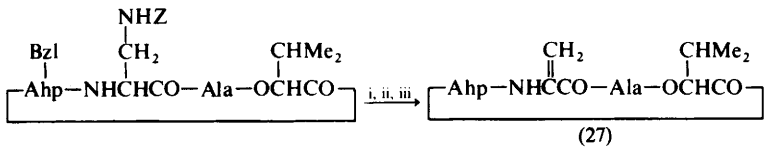
[59] M. Asai, K. Haibora, M. Muroi, K. Kintaka, and T. Kishi, *J. Antibiot.*, 1981, **34**, 621.

[60] M. S. Puar, A. K. Ganguly, A. Alfonso, R. Brambilla, P. Mangiaracina, O. Sarre, and R. D. Macfarlane, *J. Am. Chem. Soc.*, 1981, **103**, 5231.

determined by 270 MHz ^1H and 25 MHz ^{13}C n.m.r. studies.[61] Siomycin A has been converted into half esters with dicarboxylic acids [$HO_2C(CH_2)_{2-10}CO_2H$] to try to improve its water solubility. The sodium salts in some cases showed better therapeutic effects in treating infected mice than did the parent compound.[62]

3 Cyclic Depsipeptides

All four stereoisomers of the cyclic depsidipeptide *cyclo*(-Trp-Hmp-) (Hmp = 2-hydroxy-4-methylpentanoic acid) have been prepared as analogues of the bitter principle *cyclo*(-Trp-Leu-) obtained from casein hydrolysates. Cyclization yields of 32, 11, 11, and 46% were obtained for the LL, LD, DL, and DD stereoisomers, respectively, using 1-succinimidyl esters. Chymotrypsin was found to cleave the LL and LD isomers at moderate rates, but the DL and DD forms were attacked only very slowly. A folded conformation for two of the analogues similar to that occurring in *cyclo*(-Trp-Leu-) was indicated in their ^1H n.m.r. spectra by the unusually highfield shifts of the $C_\beta H$ of L-Hmp in the LL isomer and the $C_\alpha H$ proton of D-Hmp in the LD case.[63] The synthesis of AM-toxin III (27), one of the host-specific phytotoxic metabolites produced by *Alternaria mali*, has been reported. Methods earlier used in the preparation of AM-toxins I and II were not applicable because of the phenolic group. The dehydroalanine residue was formed by use of a Hofmann degradation of 2,3-diaminopropionic acid (Scheme 14) instead of a β-elimination reaction on serine. The method was tested out successfully first with a sequence containing a lower homologue of Ahp.[64]



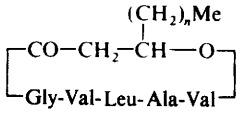
(27)

Ahp = L-2-amino-5-(*p*-hydroxyphenyl)pentanoic acid
Z = benzyloxycarbonyl

Reagents: i, H_2/Pd, HCl, DMF; ii, MeI/$KHCO_3$; iii, heat, $KHCO_3$

Scheme 14

Three new cyclohexadepsipeptides, isariins B, C, and D (28), have been isolated from the fungus *Isaria felina*. In isariins C and D L-alanine replaces the L-valine



(28) Isariin $n = 8$
Isariin B and C $n = 5$
Isariin D $n = 3$

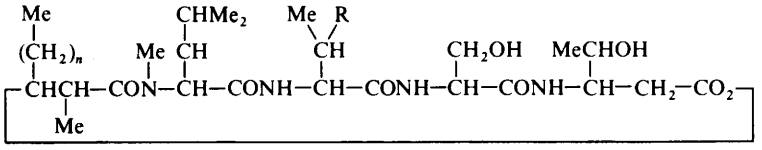
[61] K. Tori, K. Tokura, Y. Yoshimura, Y. Terui, K. Okabe, H. Otsuka, K. Matsushita, F. Inagaki, and T. Miyazawa, *J. Antibiot.*, 1981, **34**, 124.

[62] K. Tokura, K. Hayoshi, K. Okabe, K. Tori, Y. Yoshimura, M. Mayama, S. Matura, and H. Otsuka, *J. Antibiot.*, 1981, **34**, 800.

[63] K. Miyazaki, A. Yasutake, N. Nishino, H. Aoyagi, T. Kato, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1981, **17**, 118.

[64] T. Kanmera, H. Aoyagi, M. Waki, T. Kato, N. Izumiya, K. Noda, and T. Ueno, *Tetrahedron Lett.*, 1981, 3625.

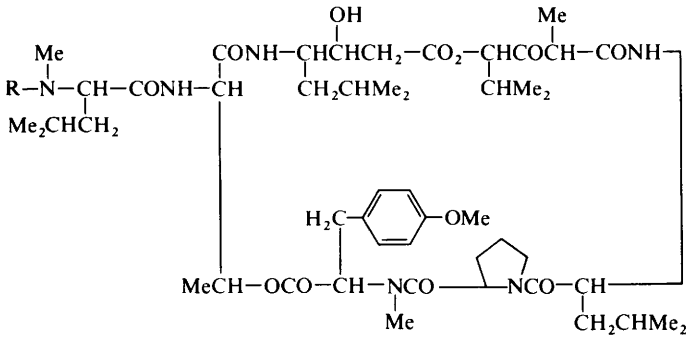
residues of isariin B. Each was sequenced by m.s. after opening the ring with methanolic KOH and esterifying with diazomethane. Unlike isariin itself and isariin B, isariins C and D possess insecticidal activity against *Galleria melonella* larvae.[65] The structures of four minor components of the globomycin complex of antibiotics from *Streptomyces hygroscopicus* have been elucidated (29). They have



(29)

Globomycin	R	*n*
A_2	Et	3
A_3	Me	5
A_{4a}	Et	6
A_{4b}	Me	7

four amino-acids in common, the fifth being either valine or *allo*-isoleucine.[66] A new class of antiviral and cytotoxic depsipeptides has been isolated from a Caribbean tunicate of the family *Didemnia* (genus *Tridemnum*). The didemnius (30) were sequenced by m.s. after partial hydrolysis on mixtures of components.[67,68]



(30) Didemnin

a; R = H

b; R = MeCHOH—CON—CO

c; R = MeCHOH—CO—

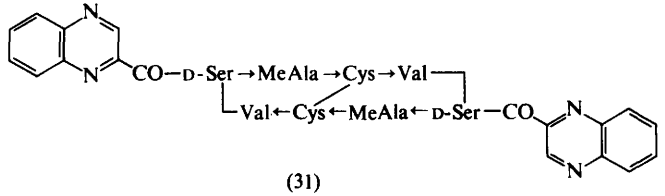
[MeAla^2, MeAla^6]des-*N*-tetramethyltriostin A (31), an analogue of the bicyclic octadepsipeptide triostin A, has been synthesized to explore the mode of binding of this antibiotic to nucleic acids. The 2-quinoxalinecarbonyl groups were added at

[65] G. Deffieux, D. Merlet, M.-A. Baute, and A. Neven, *J. Antibiot.*, 1981, **34**, 1266.

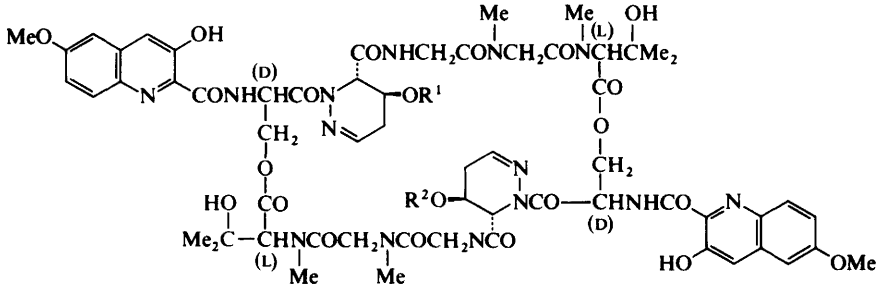
[66] S. Onoto, H. Ogino, and S. Inouye, *J. Antibiot.*, 1981, **34**, 1416.

[67] K. L. Rinehart, jun., J. B. Loer, J. C. Cook, jun., S. A. Mizsak, and T. A. Scahill, *J. Am. Chem. Soc.*, 1981, **103**, 1857.

[68] K. L. Rinehart, jun., J. B. Gloer, R. G. Hughes, H. E. Renis, J. P. McGovren, E. B. Swynenberg, D. A. Stringfellow, S. A. Kuentzel, and L. H. Li, *Science*, 1981, **212**, 933.



(31)

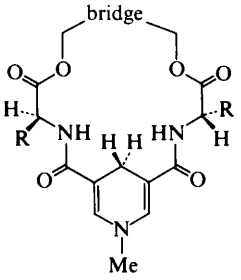


(32) BBM-928
a; R^1 = COMe, R^2 = COMe
b; R^1 = COMe, R^2 = H
c; R^1 = H, R^2 = H

the last stage after formation of both the peptide and disulphide ring systems.[69] A series of cyclic decadepsipeptides (32), which in gross structure resemble the echinomycins, have been isolated from *Actinamadura luzonensis*. They lack the S-containing crosslink of the cyclo-octadepsipeptide echinomycins and contain the unusual amino-acids L-β-hydroxy-*N*-methylvaline and *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid.[70] A ^{1}H n.m.r. study has shown the existence of a ternary complex (stoicheiometry 1 : 1 : 1) of valinomycin, K^+, and an anion such as a protonopheric uncoupler of oxidative phosphorylation, *e.g.* 2,4-dinitrophenol, when these components are partitioned between an organic solvent and water. It is suggested that there is a synergistic action of valinomycin and the uncoupler in the transport of K^+ and H^+ across membranes.[71] Valinomycin has also been used as a test compound for the use of selective irradiation of ^{1}H for the simultaneous and complete assignment of both the ^{1}H and ^{13}C resonances in peptide n.m.r. spectra. It is pointed out that stepwise selective irradiation of ^{1}H resonances can be employed for peptide sequencing.[72]

It was shown in 1979 that the compound (33a) reduces activated carbonyl compounds to the corresponding alcohols at 20 °C in the presence of Mg^{2+}, giving enantiomeric excesses of *S*-isomer of 64—86%.[73] A series of structural analogues (33b) have now been prepared in which the component amino-acids and the length of the bridge have been varied in order to try to approach the selectivity of

[69] M. K. Dhaon and R. K. Olsen, *J. Org. Chem.*, 1981, **46**, 3493.
[70] M. Konishi, H. Ohkuma, F. Sakai, T. Tsuno, H. Koshiyama, T. Naito, and H. Kawaguchi, *J. Antibiot.*, 1981, **34**, 148.
[71] K. Yoshikawa and H. Terada, *J. Am. Chem. Soc.*, 1981, **103**, 7788.
[72] M. A. Khaled and D. W. Urry, *J. Chem. Soc., Chem. Commun.*, 1981, 230.
[73] J. D. DeVries and R. M. Kellogg, *J. Am. Chem. Soc.*, 1979, **101**, 2759.

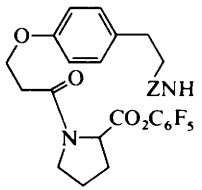


(33) a; bridge = $-(CH_2)_2O(CH_2)_2-$, R = Me_2CH
b; bridge = $-(CH_2)_{4-12}-$ or $-(CH_2)_2[O(CH_2)_2]_{1-3}-$,
R = combinations of the side chains of Val, Phe, and Pro

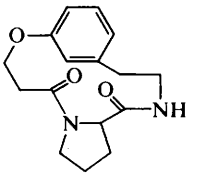
NAD(P)H. It was found that the length and shape of the bridge can be varied extensively without greatly decreasing the enantiomeric excess as long as 10 linking atoms are not exceeded. When both amino-acid residues are proline, however, no chirality is induced.[74]

4 Peptide Alkaloids

Construction of the ansapeptide ring of the peptide alkaloids by ring closure at the proline carboxyl group is claimed to give better results than ring closure at the proline imino group. The compound (34) could be cyclized in 50% yield by adding it slowly to a stirred suspension of Pd/C in dioxan at 95 °C, containing



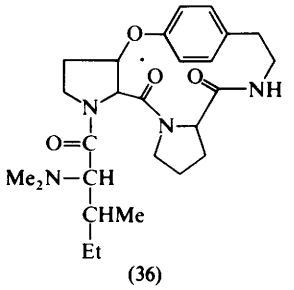
(34) Z = benzyloxycarbonyl



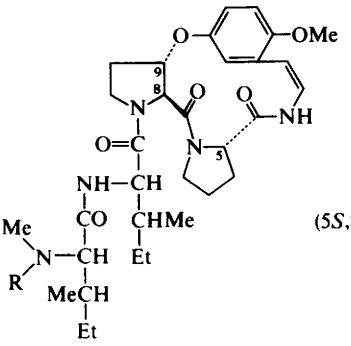
(35)

4-pyrrolidinopyridine as catalyst, into which hydrogen was passed. Cyclization apparently occurs while the peptide is still adsorbed on the catalyst. The presence of a small amount of ethanol (2%) in the solvent is crucial to efficiency. The yield drops dramatically if alcohol is omitted, if its concentration exceeds 5%, if the temperature drops below 75 °C, or if other bases are used as catalyst. Under the optimum conditions a small amount of cyclodimerization occurs; in ethyl acetate at 75 °C cyclodimer is the major product (45%). A ring containing a *meta* aromatic bridge (35) is more easily obtained (80% yield) by a similar pentafluorophenyl ester cyclization.[75] Using this method of ring closure, the total syntheses of dihydrozizyphine G (36), zizyphine A (37a), and *N*-acetylzizyphine B (37b) have been accomplished. Key steps in the synthesis of the latter two compounds are outlined in Scheme 15. As ring closure with the aromatic ketone itself proceeds poorly, it

[74] P. Jonin, C. B. Troostwijk, and R. M. Kellogg, *J. Am. Chem. Soc.*, 1981, **103**, 2091.
[75] U. Schmidt, H. Griesser, A. Lieberknecht, and J. Talbiersky, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 280.



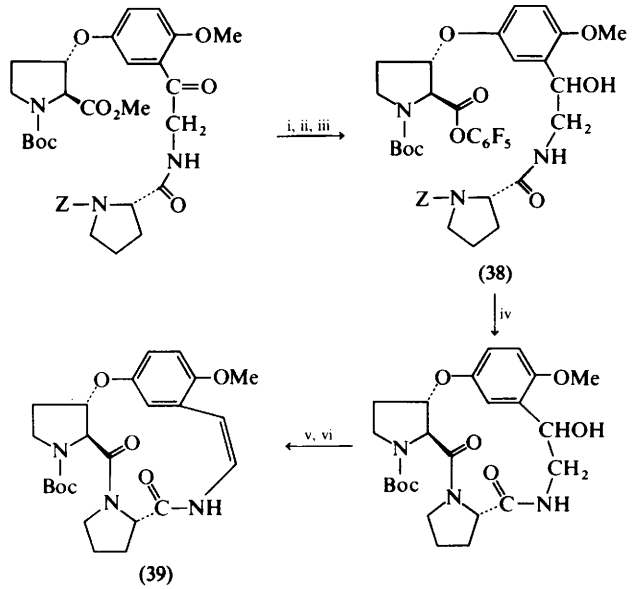
(36)



(5*S*,8*S*,9*S*)

(37) a; R = Me
b; R = MeCO

was reduced (although non-stereoselectively) before cyclization. At the cyclization stage the pentafluorophenyl ester (38) consisted of a mixture of four diastereoisomers. Only two of the resulting cyclopeptides underwent elimination to



(38)

(39)

Reagents: i, $NaB(CN)H_3$; ii, OH^-, H_2O; iii, C_6F_5OH, *NN'*-dicylohexylcarbodi-imide; iv, Pd/C, dioxan; v, Bu_3P, *o*-nitrophenylselenocyanate; vi, H_2O_2

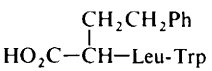
Scheme 15

the olefin (39) under the conditions used, and the two products were separated by silica-gel chromatography. The required (5*S*,8*S*,9*S*) isomer was identified by its large negative optical rotation.[76]

[76] U. Schmidt, A. Lieberknecht, H. Griesser, and J. Hausler, *Angew. Chem., Int. Ed. Engl.*, 1980, **20**, 281.

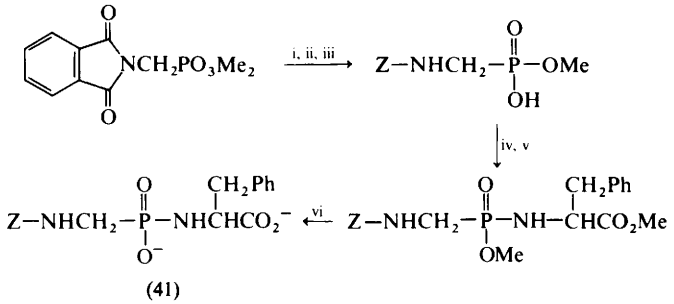
5 Linear Peptides

The design principle earlier effective for generating inhibitors of angiotensin-converting enzyme, *i.e.* the use of *N*-carboxymethyldipeptides, has now been successfully extended to thermolysin inhibitors. The most potent compound discovered was the dipeptide derivative (40), thought to be the (*S,S,S*) isomer,



(40)

whose inhibition was fully developed within 30 seconds of addition.[77] An analogue of the carboxypeptidase A substrate Z-Gly-Phe has been prepared in which the scissile peptide bond has been replaced by a tetrahedral phosphonamidate group. This dipeptide (41), synthesized according to Scheme 16, is a competitive and reversible inhibitor of the enzyme that is not hydrolysed by it.[78]



(41)

Reagents: i, N_2H_4; ii, ZCl; iii, NaOH; iv, $SOCl_2$; v, PheOMe; vi, LiOH

Scheme 16

N^2-Acetyl-N^3-glycyl-L-2,3-diaminopropionic acid amide and ethyl ester have been prepared as analogues of N^{α}-acetyl lysine amide and ethyl ester. They were cleaved at 1.3% and 5.2%, respectively, of the rates of hydrolysis of the comparable lysine compounds.[79]

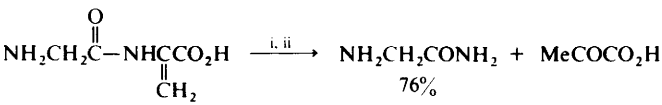
Five papers concern $\alpha\beta$-dihydroamino-acid residues in peptides. The first use of the *N*-chlorination–dehydrochlorination method in the synthesis of a dehydrodipeptide has been reported during the preparation of a dehydrothyroliberin, pGlu-Δ^2-Phe-Pro-NH_2. Other methods for preparing the required intermediate Z-pGlu-Δ^2-Phe proved less efficient.[80] A procedure for the cleavage of dehydroalanine-containing peptides using mercuric acetate at neutral or slightly acidic pH has been devised (Scheme 17). Trimethylamine-borane is added in order to precipitate elemental mercury. Application to Boc-Ala-ΔAla-Asp$(OMe)_2$, the

[77] A. L. Maycock, D. M. Desousa, L. O. Payne, J. ten Brocke, M. T. Wu, and A. A. Patchett, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 963.

[78] N. E. Jacobsen and P. A. Bartlett, *J. Am. Chem. Soc.*, 1981, **103**, 654.

[79] Y. Minematsu, S. Kang, M. Waki, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 297.

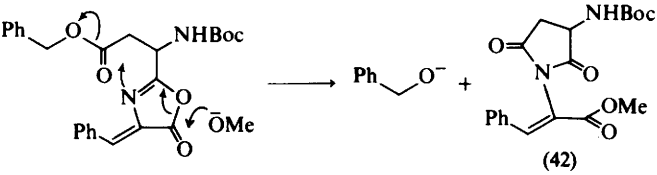
[80] M. D. Grim, V. Chauhan, Y. Shimohigashi, A. J. Kolar, and C. H. Stammer, *J. Org. Chem.*, 1981, **46**, 2671.



Reagents: i, $Hg(OAc)_2$ in DMF or 50% aq. MeOH; ii, trimethylamine-borane

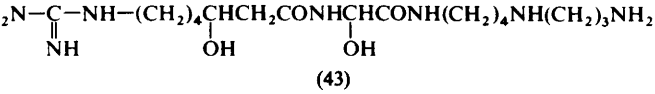
Scheme 17

only peptide yet examined, gave a 40% yield.[81] Dehydroaspartame, Asp-Δ^2-Phe-OMe, has been synthesized by DDQ oxidation of the dipeptide azlactone and subsequent methanolysis. In the absence of 4-(dimethylamino)pyridine as catalyst, methanolysis produced the succinimide derivative (42) (Scheme 18), leading to the



Scheme 18

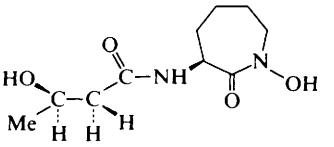
formation of β-aspartyldehydrophenylalaninamide as well as the desired product. Dehydroaspartame proved tasteless, in agreement with the current picture of the sweetness receptor.[82] Full details of approaches to the synthesis of didehydrodipeptides by direct condensation of dehydrocomponents or by base-catalysed elimination of β-mesylate groups have appeared.[83] The use of saturated analogues as references for the measurement of the u.v. spectra of dehydropeptides has been advocated. This double-beam approach eliminates the interference by the strong absorption of peptide bonds below 240 nm. The effects of *Z* or *E* configuration on these u.v. spectra have also been discussed.[84]



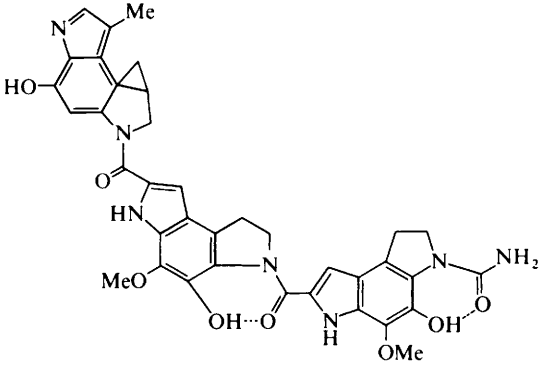
(43)

Both the structure and synthesis of spergualin (43), an anti-tumour antibiotic from a strain closely related to *Bacillus laterosporus*, have been reported.[85] The hydroxy component involved in the ester linkage of mycobactin T, cobactin T (44), has also been synthesized as a first step in the synthesis of analogues aimed at producing inhibitors of pathogenic mycobacteria.[86] The first synthesis of a naturally occurring tripeptide from *Penicillium chrysogenum*, L-α-aminoadipyl-L-seryl-D-valine, has also been accomplished.[87] A structurally more unusual tri-

[81] A. S. B. Edge and P. Weber, *Int. J. Pept. Protein Res.*, 1981, **18**, 1.
[82] S. W. King and C. H. Stammer, *J. Org. Chem.*, 1981, **46**, 4780.
[83] C. Shin, Y. Yonezawa, M. Takahashi, and Y. Yoshimura, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 1132.
[84] Y. Shimohigashi, J. W. Dunning, jun., M. A. Grim, and C. H. Stammer, *J. Chem. Soc., Perkin Trans. 2*, 1981, 1171.
[85] H. Umezawa, S. Kondo, H. Inuma, S. Kunimoto, Y. Ikeda, H. Iwasawa, D. Ikeda, and T. Takeuchi, *J. Antibiot.*, 1981, **34**, 1622, 1625.
[86] P. J. Maurer and M. J. Miller, *J. Org. Chem.*, 1981, **46**, 2835.
[87] J. E. Shields, C. S. Campbell, K. S. Doyle, R. D. Miller, and H. Neuss, *Helv. Chim. Acta*, 1981, **64**, 2587.

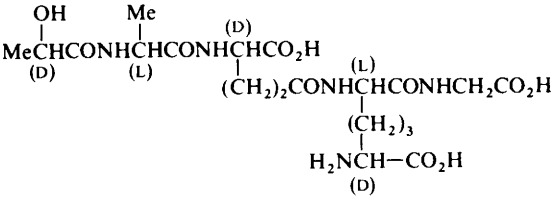


(44)



(45)

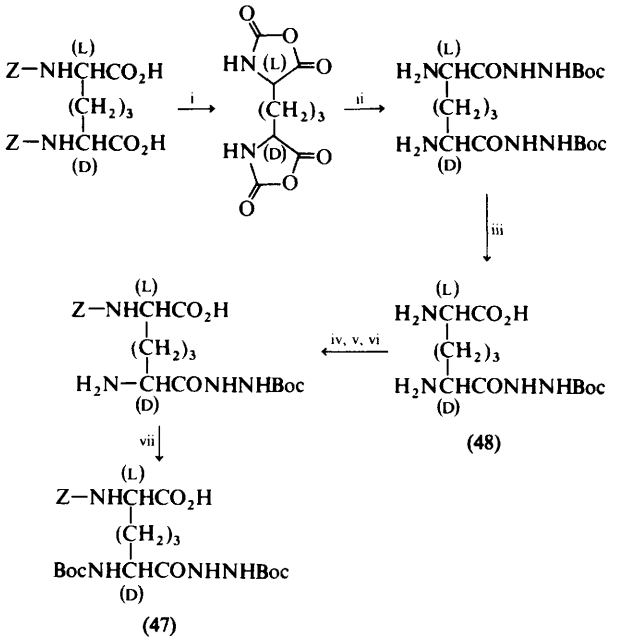
peptide CC-1065 (45) is produced by *Streptomyces zelensis*. This potent anti-tumour agent binds to DNA without intercalation, and the conformation shown by *X*-ray crystallography fits very well in either the major or the minor groove of DNA, the curve of the molecule mimicking the turn of the DNA helix. The cyclopropyl ring can be placed in close proximity to the amino group of an adenine residue for alkylation. In one of the two forms found in the crystal there is an amide group which is extremely distorted from planarity. Previously the most distorted amide bonds have been reported in highly strained ring compounds.[88]



(46)

The preparation of the immunostimulating tetrapeptide FK-156 (46), isolated from *Streptomyces olivaceogriseus*, has been described. Construction of the key intermediate (47) in the synthesis required differentiation of the two pairs of amino-acid functions in *meso*-2,2′-diaminopimelic acid (Scheme 19). The selective carbobenzyoxylation of (48) is thought to be the first example of the copper complex of an α-amino-acid hydrazide being more stable than the copper complex

[88] C. G. Chidester, W. C. Krueger, S. A. Mizsak, D. J. Duchamp, and D. G. Martin, *J. Am. Chem. Soc.*, 1981, **103**, 7629.



Reagents: i, PCl_5, CH_2Cl_2; ii, $BocN_3$, MeCN; iii, aminopeptidase from *Streptomyces sapparonensis*; iv, $CuCl_2$; v, ZCl; vi, H_2S; vii, $(Boc)_2O$, NEt_3, dioxan, H_2O

Scheme 19

of the free α-amino acid.[89] The replacement of phenylalanine by carboranylalanine (Car) in the enkephalin analogues [D-Ala^2, Car^4, Leu^5]- and [D-Ala^2, Car^4, Met^5]-enkephalin enhances *in vitro* activity twofold, owing to, it is thought, the electrophilic aromatic properties of Car rather than its hydrophobic or space-filling character.[90] Replacement of phenylalanine or leucine by adamantylalanine (Ada) was also explored; [Ala^2, Ada^5]enkephalin proved three times as active as leucine-enkephalin *in vitro*, but analogues containing Ada^4 were nearly inactive.[91] An ester of a diastereoisomeric mixture of enkephalins, HCl-D/L-Tyr-Gly-Gly-D/L-Phe-Leu-OBu^t (49), has been prepared by an unusual route involving intermediates containing the aromatic amino-acids bound up in β-lactam systems (Scheme 20). The β-lactam rings are cleaved by catalytic hydrogenation at 50 °C but are stable under the same conditions at 20 °C.[92]

A number of ester and amide derivatives of edeine A have been prepared. The antimicrobial activities of these compounds are comparable to that of the native antibiotic, indicating that the presence of the free carboxyl group is not essential for biological activity.[93] A series of oligosarcosines containing two to seven

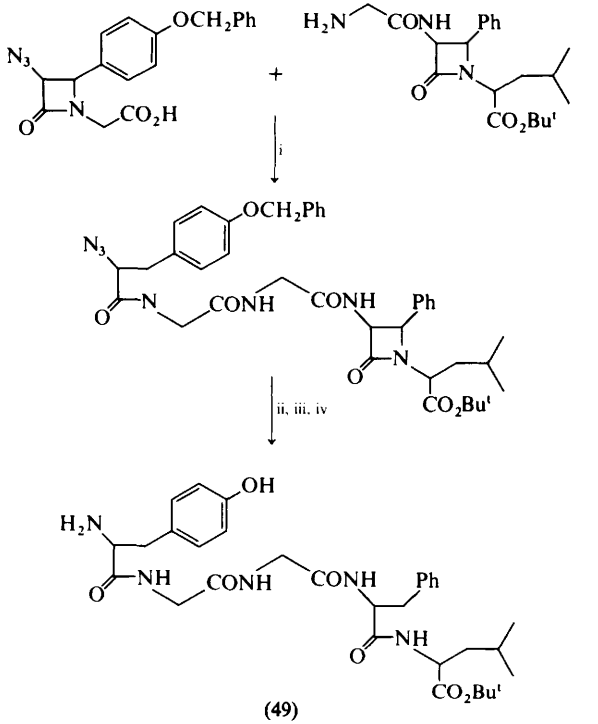
[89] K. Hemmi, H. Takeno, S. Okada, O. Nakaguchi, Y. Kitawa, and M. Hashimoto, *J. Am. Chem. Soc.*, 1981, **103**, 7026.

[90] R. Schwyzer, K. Q. Do, A. N. Eberle, and J. L. Fauchere, *Helv. Chim. Acta*, 1981, **64**, 2079.

[91] K. Q. Do and R. Schwyzer, *Helv. Chim. Acta*, 1981, **64**, 2084.

[92] K. Hatanaka, R. Abe, and I. Ojima, *Chem. Lett.*, 1981, 1297.

[93] J. Mazerski, H. Wojciechowska, W. Zgoda, B. Woynarowska, and E. Barowski, *J. Antibiot.*, 1981, **34**, 28.

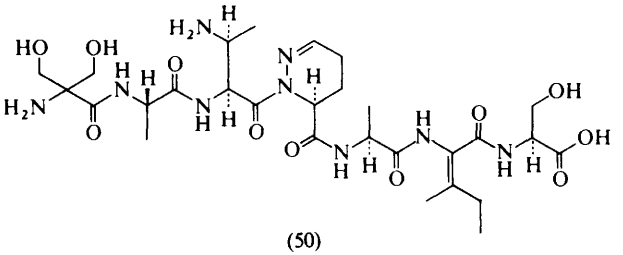


(49)

Reagents: i, *NN'*-dicyclohexylcarbodi-imide, 1-hydroxybenzotriazole; ii, H_2, Pd, 20 °C; iii, HCl, 1 equiv.; iv, H_2, Pd/C, 50 °C

Scheme 20

amino-acid units flanked by an electron donor (*p*-dimethylaminoanilide) and an electron acceptor (3,5-dinitrobenzoyl) have been synthesized. They showed distinct charge-transfer absorption in $CHCl_3$ and EtOH solution at concentrations low enough to eliminate the contribution of intermolecular charge-transfer interaction. Maximum intramolecular effect occurred in the tetrapeptide.[94] A new

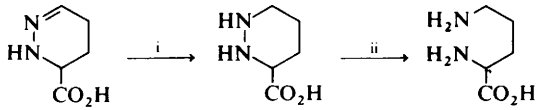


(50)

peptide antibiotic antrimycin (50) has been isolated from *Streptomyces xanthocidicus*. Acidic hydrolysis gives the uncommon amino-acids 2,2-bis(hydroxymethyl)glycine and (2*S*,3*S*)-L-2,3-diaminobutanoic acid, but the heterocyclic

[94] M. Sisido, Y. Kanazawa, and Y. Imanishi, *Biopolymers*, 1981, **20**, 653.

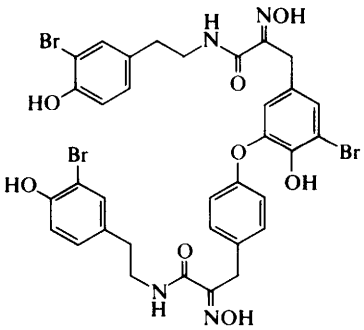
amino-acid was destroyed. Alkaline hydrolysis, however, furnished 2,3,4,5-tetrahydropyridazine-3-carboxylic acid, whose structure was confirmed by a reduction to L-ornithine (Scheme 21).[95]



Reagents: i, $NaBH_3CN$, MeOH; ii, H_2/Pt, AcOH

Scheme 21

Dehydrophenylalanine has been incorporated into three analogues of bradykinin in place of phenylalanine; [Δ^2Phe^5]bradykinin was 23 times as potent as bradykinin when given intravenously, [Δ^2Phe^8] material was less potent, and the [Δ^2Phe^{5,8}] analogue was comparable in activity. All three seem to be more resistant than bradykinin to enzymic degradation during passage through the pulmonary vascular bed. During the solid-phase synthesis of these compounds, addition of Boc-Gly-Δ^2Phe and Boc-Pro-Δ^2Phe using *NN'*-dicyclohexylcarbodiimide required extended coupling times.[96] The structure and synthesis of tremerogen A-13, a peptidal sex hormone of the fungus *Tremella mesenterica*, have been reported. Micro-scale Edman degradation established the sequence (51), which is very different from tremerogen A-10 isolated in 1978 by the same group.



(51)

Thermolysin preferentially cleaves the Gly-Val bond. In the synthesis the farnesyl chain was added to cysteine as the final step. Formation of a *C*-terminal amide gives a product biologically more potent than the free acid.[97] The thallium acetate complex of gramicidin A in dioxan has been studied by ^{205}Tl n.m.r. The resonant frequency of the Tl^+ ion in the complex is the lowest ever observed for the ^{205}Tl nucleus. A rather weak interaction between the cation and the functional groups

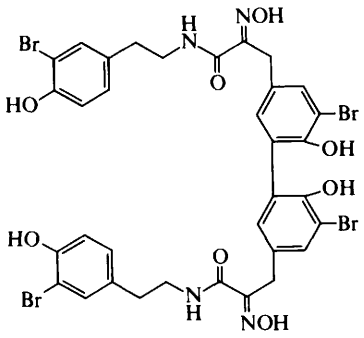
[95] N. Shimada, K. Morimoto, M. Naganawa, T. Takita, M. Hamada, K. Maeda, T. Takeuchi, and H. Umezawa, *J. Antibiot.*, 1981, **34**, 1613.

[96] G. H. Fisher, P. Berryer, J. W. Ryan, V. Chauhan, and C. H. Stammer, *Arch. Biochem. Biophys.*, 1981, **211**, 269.

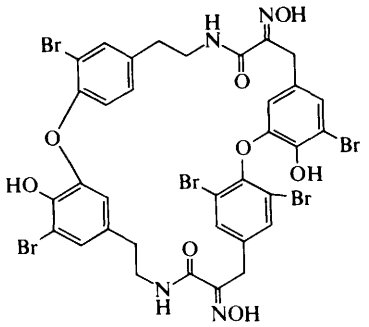
[97] C. Kitada, M. Fujino, Y. Sakagami, M. Yoshida, A. Isogai, and A. Suzuki, *Agric. Biol. Chem.*, 1981, **45**, 1049.

responsible for ion binding is suggested. The cation-binding site also appears to be at the entrance to the channel of the helical molecule.[98]

Seven novel metabolites, each originating from 4 bromotyrosine units, have been isolated from the sponge *Ianthella basta*. Three are derived from a single phenol oxidative coupling, *e.g.* (51) and (52), and four from double phenol oxidative couplings, *e.g.* (53). These compounds, the bastadins, show potent *in vitro* antimicrobial activity against Gram-positive organisms.[99]



(52)



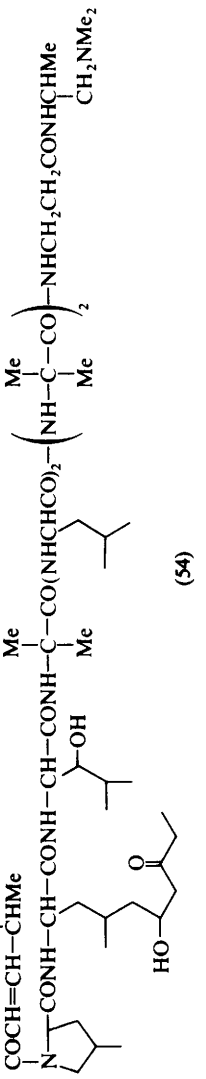
(53)

Peptides Containing α-Aminoisobutyric Acid.—The number of known antibiotics rich in α-aminoisobutyric acid residues continues to grow. *Paecilomyces lilacinus* has yielded P168 (54), a strong inhibitor of the growth of phytopathogenic fungi. Amongst its component amino-acids are *cis*-4-methyl-L-proline, *threo*-β-hydroxyleucine, and (2*S*,4*S*)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid. On acidic hydrolysis the latter undergoes cyclization, and a pipecolic acid derivative (55) is formed.[100] Trichopolyns I and II (56a and b) from *Trichoderma polysporum* differ from each other only in one residue, valine replacing isoleucine

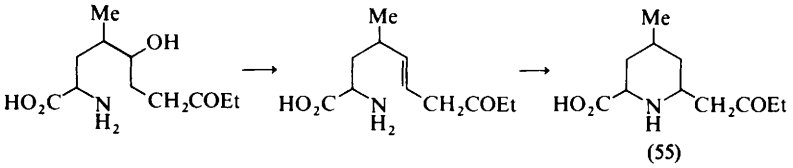
[98] J. F. Hinton, G. L. Turner, and F. S. Millet, *J. Magn. Reson.*, 1981, **45**, 42.

[99] R. Kazlauskas, R. O. Lidgard, P. T. Murphy, R. J. Wells, and J. F. Bount, *Aust. J. Chem.*, 1981, **34**, 765.

[100] A. Isogai, A. Suzuki, S. Higashikawa, S. Koyama, and S. Tamura, *Agric. Biol. Chem.*, 1980, **44**, 3029, 3033; *ibid.*, 1981, **45**, 1023.

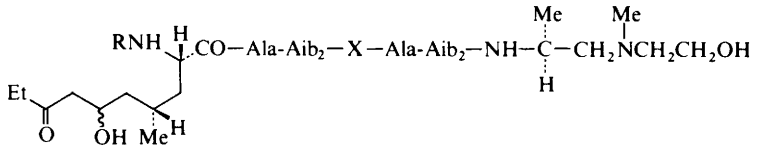


(54)



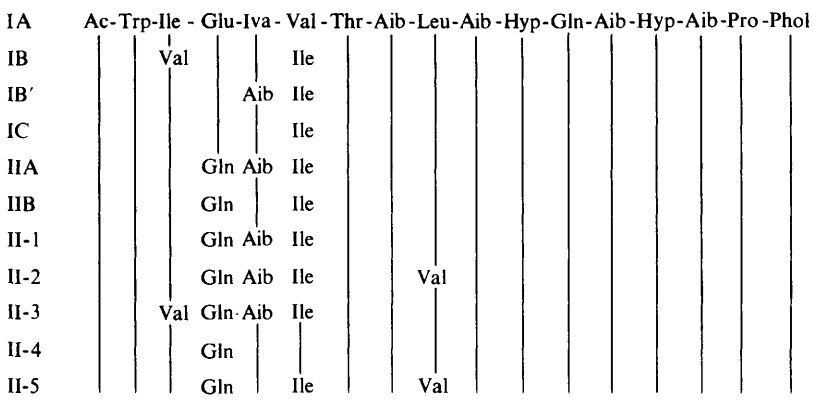
(55)

Scheme 22



(56) a; X = Ile, R = (*R*)-2-methyldecanoyl-Pro-
b; X = Val, R = (*R*)-2-methyldecanoyl-Pro-

in the latter. On acid hydrolysis the same pipecolic acid derivative obtained from P168 was isolated, Michael addition apparently following dehydration (Scheme 22).[101] Fast atom bombardment mass spectrometry has been applied to the zervamycins, a hitherto intractable group of peptaibophols. Structures of 11 components have now been reported (57). This group contains tryptophan and



(57) Replacement amino-acids only shown

threonine, not known in other peptaibophols. The presence of tryptophan has earlier resulted in poor results from electron-impact and field-desorption mass spectrometry of the intact peptides.[102]

No fewer than three total syntheses of the major component of alamethicin have been completed this year. Two of these are solution-phase syntheses,[103, 104] each

[101] T. Fujita, Y. Takaishi, A. Okamura, E. Fujita, K. Fuji, N. Hiratsuka, M. Kamatsu, and I. Arita, *J. Chem. Soc., Chem. Commun.*, 1981, 585.

[102] K. L. Rinehart, jun., L. A. Gaudioso, M. L. Moore, R. C. Pandey, M. Barber, R. D. Sedgwick, R. S. Bardoli, A. N. Tyler, and B. N. Green, *J. Am. Chem. Soc.*, 1981, **103**, 6517.

[103] T. M. Balasubramanian, N. C. E. Kendrick, M. Taylor, G. R. Marshall, J. E. Hall, I. Vodyanov, and F. Reusser, *J. Am. Chem. Soc.*, 1981, **103**, 6127.

[104] R. Nagaraj and P. Balaram, *Tetrahedron*, 1981, **37**, 1263.

employing the *NN'*-dicyclohexylcarbodi-imide–1-hydroxybenzotriazole (DCC/HOBt) method to couple the fragments (see Figure), with benzyloxycarbonyl [104]

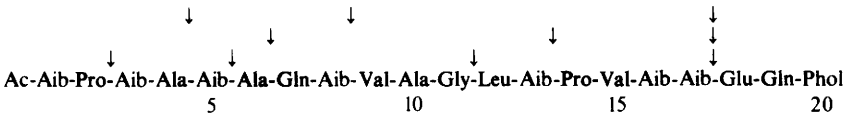
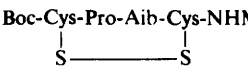


Figure *Fragments used in the synthesis of alamethicin*[103, 104, 106]

or t-butyloxycarbonyl [103] protection of amino groups. In one of these syntheses 48 h reaction times were found necessary for obtaining reasonable yields where Aib-containing peptides were concerned, and in the prevention of Boc-Gln-Aib-OBzl diphenylphosphoryl azide was used to prevent the dehydration of the Gln caused by the use of DCC/HOBt. Acetyl-Aib was added last as the acetyl group had been found to be quite labile during catalytic hydrogenation operation.[103] In the other synthesis dipeptide fragments containing Aib were avoided as their deprotection lead to dioxopiperazine formation. It was noted that the reduction in conformational freedom imposed by the presence of Aib residues facilitated the ready crystallization of even large fragments of alamethicin.[104] In preparing the *N*-terminal pentapeptide, racemization of the proline residue on coupling Z-Aib-Pro to Aib-OMe using DCC alone was indicated, although this was only detected by n.m.r. at the pentapeptide level.[105] In the solid-phase synthesis three fragments (see Figure) were prepared on Merrifield resin using Boc-amino-acids and DCC couplings with no additive; longer than usual reaction times were needed for the addition of Aib residues. The fragments were then joined using DCC/HOBt couplings to the *C*-terminal resin-bound tripeptide, which was assembled in solution and then bound to the support through the glutamyl γ-carboxyl.[106]

The *X*-ray conformational evaluation of a number of peptides containing Aib residues in general accords with expectations. The bridged tetrapeptide (58) adopts



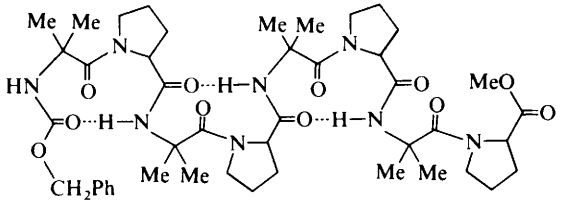
(58)

a highly compact conformation with two intramolecular 4 → 1 H-bonds, one involving the methylamide NH, stabilizing a 3_{10} backbone helix. The S—S bridge adopts a right-handed twist with a dihedral angle of 82°.[107] The crystal structures of four peptide fragments of alamethicin, the largest being Boc-Pro-Aib-Ala-Aib-Obzl, show the Aib conformation to agree with theoretical predictions excellently considering the differing environments. It is concluded that crystal-packing forces do not play an important role in determining conformation for this residue.[108]

[105] R. Nagaraj and P. Balaram, *Tetrahedron*, 1981, **37**, 2001.
[106] B. F. Gisin, D. G. Davis, Z. K. Barowska, J. E. Hall, and S. Kobayashi, *J. Am. Chem. Soc.*, 1981, **103**, 6373.
[107] B. V. V. Prasod, A. Rawi, and P. Balaram, *Biochem. Biophys. Res. Commun.*, 1981, **103**, 1138.
[108] G. D. Smith, V. Z. Pletnev, W. L. Duax, T. M. Balasubramanian, H. E. Bosshard, E. W. Czerwinski, N. E. Kenrick, F. Scott Matthews, and G. R. Marshall, *J. Am. Chem. Soc.*, 1981, **103**, 1493.

Tosyl-(Aib)$_5$-OMe folds into a 3_{10} helix containing 3 (4 → 1) hydrogen bonds. When crystallized from aqueous methanol containing $LiClO_4$ the packing of the molecules in the crystal gives a hydrophobic channel, minimum diameter 5.2 Å. When crystallized from MeOH alone there are no channels, although only the molecular packing is different.[109] By contrast, the undecapeptide model of alamethicin Boc-Ala-Aib-Ala-Aib-Ala-Glu(OBzl)-Ala-Aib-Ala-Aib-Ala-OMe is reported to have nine of its amino-acid residues in an α-helix, only the two *C*-terminal residues being bent away in a β-turn. The α-helices are coupled head to tail in the crystal to form antiparallel chains, the CO groups of Aib[8] and Ala[9] being on the periphery of the molecule and acting as acceptors for intermolecular H-bonding with the NH groups of Ala[1] and Aib[2] of a neighbouring molecule.[110]

N.m.r. studies of Aib peptides, however, are all interpreted in terms of 3_{10} helical structures. This is the case with Z-(Aib-Pro)$_4$-OMe, for which the conformation (59) is suggested.[111] A 270 MHz ^{1}H n.m.r. study of 1—6 and 7—13



(59)

fragments of alamethicin also supports 3_{10} helical conformations, and a structure has been proposed for the alamethicin backbone that involves a 3_{10} helical segment from residues 1—10, followed by a slight expansion at 11—14 to one turn of an α-helix, which then tightens to a 3_{10} helix again from 14—17. The polar charged tail Glu-Gln-Phol is likely to be flexible. The 'flexibility' of the Gly-Leu (11—12) segment may provide a structural hinge allowing the molecule to interconvert between two conformations that have different spatial orientations of the rigid helical segments.[112] A similar study on the *N*-terminal decapeptide of suzukacillin indicates a 3_{10} helix with some flexibility in the Val[5]-Ala[6] region.[113]

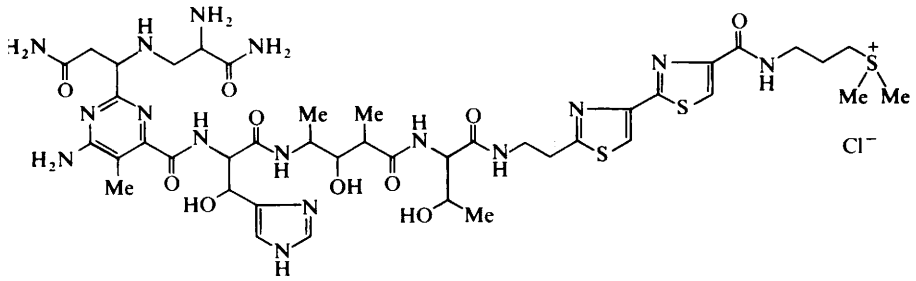
Theoretical calculations also suggest that for Aib residues the 3_{10} helix is more stable than the α-helix, a situation which should prevail in solution as well as the crystal.[114] A review summarizing conformational aspects of alamethicin and related compounds has been published.[115] Alamethicin and fragments containing at least 13 residues have been shown to be uncouplers of oxidative phosphorylation in rat-liver mitochondria. Structural requirements are similar to those observed for ionophoretic activity.[116]

[109] Ch. P. Rao, N. Shamala, R. Nagaraj, C. N. R. Rao, and P. Balaram, *Biochem. Biophys. Res. Commun.*, 1981, **103**, 898.
[110] T. Butters, P. Hutter, G. Jung, N. Pauls, H. Schmitt, G. M. Sheldrick, and W. Winter, *Angew. Chem., Int. Ed. Engl.*, 1981, **103**, 889.
[111] Y. V. Venkatachalapathi and P. Balaram, *Biopolymers*, 1981, **20**, 1137.
[112] R. Nagaraj and P. Balaram, *Biochemistry*, 1981, **20**, 2828.
[113] M. Iqbal and P. Balaram, *J. Am. Chem. Soc.*, 1981, **103**, 5548.
[114] Y. Paterson, S. M. Rumsey, E. Benedetti, G. Némethy, and H. A. Scheraga, *J. Am. Chem. Soc.*, 1981, **103**, 2947.
[115] R. Nagaraj and P. Balaram, *Acc. Chem. Res.*, 1981, **14**, 356.
[116] M. K. Matthew, R. Nagaraj, and P. Balaram, *Biochem. Biophys. Res. Commun.*, 1981, **98**, 548.

Conformational Studies.—The crystal structure of Boc-α-MePhe-Val-OBzl shows the conformation of the α-MePhe backbone to differ from that of Phe peptides, falling in the region of left-handed helices and coinciding with the observed conformation for Aib.[117] The latest *X*-ray examination in the series looking at proline conformations in dipeptides where proline is flanked by two amide bonds concerns pivaloyl-Gly-Pro-NHMe·H_2O,[118] and Boc-D-Ala-D-Glu-OH·H_2O, a compound that inhibits D-Ala-D-Ala carboxypeptidases, has also been examined.[119] In the structure of Z-Ala-D-Phe-Pro-OH·H_2O the lone water molecule is shown by *X*-ray analysis to be hydrogen-bonded to both the carbonyl of the Z group and to one of the oxygen atoms of the carboxyl group. This is the first observation of an internal water bridge in a linear peptide, and it provides a model for water-mediated chain reversal.[120] A c.d. and i.r. study of Boc-Pro-D-Ala-Leu and Boc-Pro-D-Ala-Val shows a type IV β-turn, the first confirmation of a structure predicted theoretically for peptides containing L–D sequences. If Boc is replaced by acetyl or the *C*-terminal residue is proline, the conformation reverts to a type II β-turn.[121] The crystal structure of Boc-Val-Pro-Gly-Gly-OBzl, which contains a repeating peptide sequence of elastin, shows also a type II β-turn, the intramolecular H-bond being (Val^1)C=O···H—N(Gly^4). The valine side chain adopts a gauche conformation.[122]

6 Glycopeptides

Glycopeptide Antibiotics.—The total synthesis of deglycobleomycin A_2 (60) has been accomplished. Bleomycin A_2 is an anti-tumour antibiotic used clinically in



(60)

the treatment of squamous cell carcinoma and malignant lymphoma, and the synthetic deglyco material proved identical with a component found in trace amounts in the culture broth of bleomycin fermentation. A fragment condensation assembly of amino-acids was employed (Scheme 23).[123] The disaccharide unit

[117] P. Van Roey, G. D. Smith, T. M. Balasubramanian, and G. R. Marshall, *Acta Crystallogr., Sect. B*, 1981, **37**, 1785.

[118] P. A. Aubry, G. Boussard, and M. Marraud, *Acta. Crystallogr., Sect. B*, 1981, **37**, 1474.

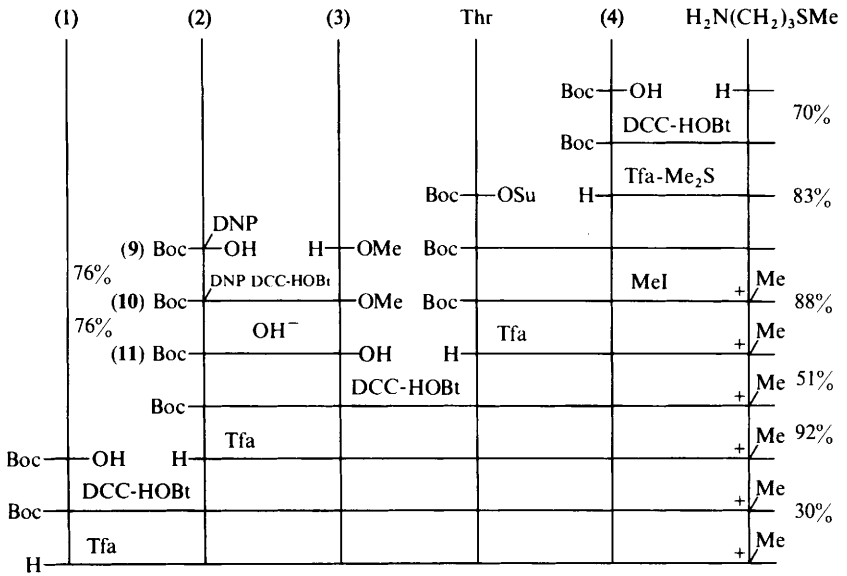
[119] P. O. Dideberg, J. Lamotte, L. Dupont, and L. Christaens, *Acta Crystallogr., Sect. B*, 1981, **37**, 1150.

[120] C. M. K. Nair, R. Nagaraj, S. Ramaprasad, P. Balaram, and M. Vijayan, *Acta Crystallogr., Sect. B*, 1981, **37**, 597.

[121] V. S. Ananthanrayanan and N. Shyamasundar, *Biochem. Biophys, Res. Commun.*, 1981, **102**, 295.

[122] H. Ayato, I. Tanaka, and T. Ashida, *J. Am. Chem. Soc.*, 1981, **103**, 6869.

[123] T. Takita, Y. Umezawa, S.-I. Saito, H. Morishima, H. Umezawa, Y. Muraoka, M. Suzuki, M. Otsuka, S. Kobayoshi, and M. Ohno, *Tetrahedron Lett.*, 1981, **22**, 671.



(1) = pyridoblamic acid; (2) = *erythro*-β-hydroxy-L-histidine; (3) = (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid; (4) = 2′-(2-aminomethyl)-2,4′-bithiazole-4-carboxylic acid

Scheme 23

found in bleomycin A_2, 2-*O*-(α-D-mannopyranosyl)-L-gulopyranose, has also been synthesized.[124] It has proved possible to prepare deglycobleomycin by mild acidic hydrolysis of the copper complex of bleomycin B_2; a N → O acyl migration occurs during the hydrolysis, but this reaction is subsequently reversed. Nine products in all were formed and separated by ion-exchange chromatography.[125] The oxidation of bleomycin–Fe–DNA complex by oxygen is known to lead to the damage of DNA. A n.m.r. and e.s.r. study of the deglycobleomycin A_2–Fe^{II} suggests that the carbonyl portion of the sugar does not directly co-ordinate with ferrous iron, and the iron binding consists of a rigid 5-5-5-6 ring-member chelate (61).[126] An analogue (62) of the aminepyrimidineimidazole region of bleomycin is capable of binding Fe^{II} to yield an oxygen-sensitive complex at physiological pH.[127]

The complex obtained from the hydrolysis of either of the isomeric green or orange Co^{III} bleomycins has been shown to contain the *N*-terminal tetrapeptide of bleomycin A bound to the cation in a manner identical with that of the peptide in the bleomycin metabolite complex Cu^{II}–(P-3A), indicating that the as yet unidentified functional group that differentiates the isomers is not part of the *N*-terminal tetrapeptide.[128] A ^1H n.m.r. study of the Zn^{II}–bleomycin A_2–poly(dA-

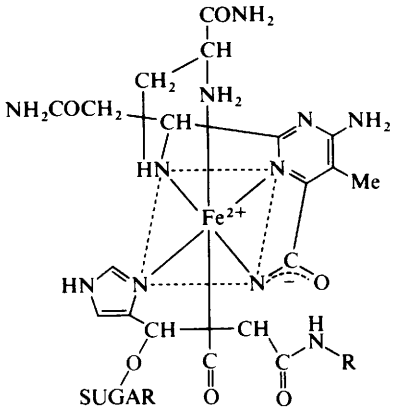
[124] T. Tsuchiya, T. Miyake, S. Kageyama, S. Umezawa, M. Umezawa, and T. Takita, *Tetrahedron Lett.*, 1981, **22**, 1413.

[125] Y. Muraoka, M. Suzuki, A. Fujii, Y. Umezawa, H. Naganawa, T. Takita, and H. Umezawa, *J. Antibiot.*, 1981, **34**, 353.

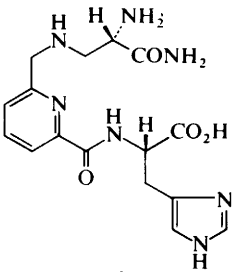
[126] Y. Sugiura, T. Suzuki, Y. Muraoka, Y. Umezawa, T. Takita, and H. Umezawa, *J. Antibiot.*, 1981, **34**, 1232.

[127] M. Otsuka, M. Yoshida, S. Kobayashi, and M. Ohno, *J. Am. Chem. Soc.*, 1981, **103**, 6986.

[128] M. Tsukayama, C. R. Ramall, F. S. Santillo, and J. C. Dabrowiak, *J. Am. Chem. Soc.*, 1981, **103**, 458; J. C. Dabrowiak and M. Tzukayama, *ibid.*, p. 7543.



(61)



(62)

dT) ternary complex shows retention of the general characteristics of binary Zn^{II}–bleomycin A_2 and bleomycin A_2–nucleic acid complexes. There appear to be three distinct regions in the antibiotic. First, there is the amino terminus, up to and including the disaccharide and hydroxyhistidine residues, whose resonances are easily perturbed by metal interactions. Secondly, there is the *C*-terminal dipeptide, whose resonances are displaced only by nucleic acid interactions. Thirdly, there is the methylvaleric-threonine dipeptide, sensitive to both types of interaction.[129]

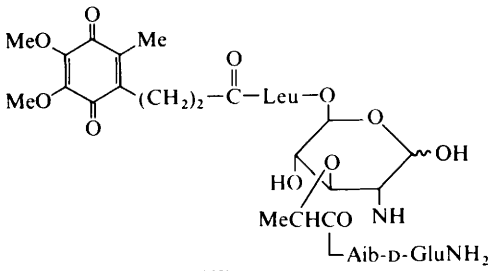
A ^1H n.m.r. study of nucleic acid complexes of a fragment of bleomycin suggests a structure in which the thiazole rings are intercalated in the duplex and stack preferentially on the purines. Both thiazole protons may extend into the minor groove with the ammonium group binding to the phosphate anion of DNA.[130] Field-desorption and fast-atom-bombardment mass spectrometry have both been applied to bleomycin and the results compared.[131] Theoretical calculations on Zn^{II}–bleomycin have been made, and one conclusion is that the groups co-ordinated to the zinc are considerably constrained in their conformational freedom.[132]

Full details of the detailed assignment of the ^{13}C n.m.r. spectra resonances of ristocetins A and B have appeared, defining hitherto doubtful configurations at anomeric carbons of the carbohydrate portion.[133] All but four of the sixty-six carbon-atom resonances of vancomycin have been assigned in a ^{13}C n.m.r. investigation.[135]

Other Glycopeptides.—Twenty-two analogues of *N*-acetylmuramyl-Ala-D-isoGlN (MDP) have been synthesized. Replacement of the alanine residue by basic or

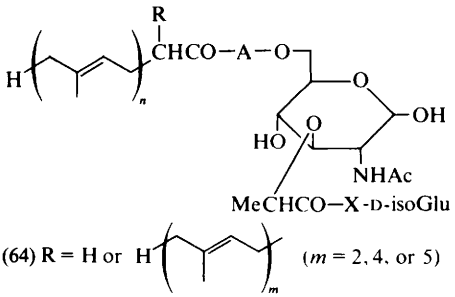
[129] J. D. Glickson, R. P. Pillai, and T. T. Sakai, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 2927.
[130] S. H. Lin and A. P. Grollman, *Biochemistry*, 1981, **20**, 7589.
[131] A. Dell, H. R. Morris, M. D. Levin, and S. M. Hecht, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 730.
[132] G. M. Crippen, N. J. Oppenheimer, and M. L. Connolly, *Int. J. Pept. Protein Res.*, 1981, **17**, 156.
[133] M. P. Williamson and D. H. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1483.
[134] A. Bongini, J. Feeney, M. P. Williamson, and D. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1981, 201.
[135] K.-I. Kamisango, I. Saiki, Y. Tanio, S. Kobayashi, T. Fukuda, I. Sekikawa, I. Azuma, and Y. Yamamura, *Chem. Pharm. Bull.*, 1981, **20**, 1644.

aromatic amino-acids lowers biological activity, but substitution by Glu, Met, Gln, Ile, or Leu gives compounds with similar, or slightly enhanced, activity with respect to MDP.[135] Of twelve MDP derivatives containing quinonyl groupings (63) proved the most potent.[136] Phosphatidylethanolamine has been linked to the



(63)

C-6 of the 6-deoxymuramyl moiety of MDP in an attempt to prepare an MDP derivative whose adjuvant activity may not require on oily vehicle. Tested in guinea pigs, using egg albumin as antigen, in saline it induced more delayed-type hypersensitivity than MDP in water-in-oil emulsion.[137] A similar bioassay of a series of multi-prenylacetylamino acylated MDP derivatives (64) showed five to be



(64) R = H or (*m* = 2, 4, or 5)

n = 2—8

A = *β*-Ala or -Leu, X = Ala or Aib

more active than MDP itself.[138] Five carbohydrate analogues of MDP have been prepared, and tests showed that replacement of OH at C-4 or C-6 by H or Cl leads to almost complete loss of immunological acitivity.[139]

Two glycopeptides, *β*-D-Glc*p*-NAc-(1 → 4)-Asn-Phe-Phe-Trp-Lys and *β*-D-Glc$_{\Delta}$-NAc-(1 → 4)-Asn-Phe-Phe-Trp-Lys, which contain the 5—9 amino-acid sequence of somatostatin, have been prepared by solid-phase synthesis. It is proposed to extend the peptide chain to that of somatostatin; the sugar has been incorporated to try to increase the biological half-life of the peptide, other substitutions on the

[136] I. Fukuda, S. Kobayashi, H. Yukimasa, I. Imada, M. Fujino, I. Azuma, and Y. Yamamura, *Chem. Pharm. Bull.*, 1981, **29**, 2215.

[137] R. K. Jain, C. M. Gupta, and W. Anand, *Tetrahedron Lett.*, 1981, **22**, 2317.

[138] T. Fukuda, S. Kobayashi, H. Yukimasa, S. Terao, M. Fujino, T. Shiba, I. Saiki, I. Azuma, and Y. Yamamura, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 3530.

[139] A. Hasegawa, E. Tanahashi, Y. Goh, M. Kiso, and I. Azuma, *Carbohydr. Res.*, 1981, **92**, 75.

asparaginyl side chain being known to affect biological activity.[140] The solid-phase synthesis of [β-D-Glc-NAc-*p*-(1 → 3)-Ser[13]]somatostatin has also been reported by the same group, but no biological activity is given.[141] *N*-Acetyl-lactosaminyl-L-asparagine has been prepared as a starting material for the synthesis of larger glycopeptides.[142]

[140] S. Lavielle, N. C. Ling, and R. C. Guillemin, *Carbohydr. Res.*, 1981, **29**, 221.
[141] S. Lavielle, N. C. Ling, R. Saltman, and R. C. Guillemin, *Carbohydr. Res.*, 1981, **89**, 229.
[142] M. A. E. Shaban and R. W. Jeanloz, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 3570.

5

Chemical Structure and Biological Activity of Hormones and Related Compounds

With contributions by D. BRANDENBURG, D. H. COY, G. W. HARDY, M. MANNING, A. OLMA, J. RAMACHANDRAN, P. D. ROY, D. SAUNDERS, W. H. SAWYER, AND A. SCHÜTTLER

1 Hypothalamic Releasing Hormones

By D. H. Coy

Thyrotropin-releasing Hormone (TRH).—Some progress continues to be made in the dissociation of the CNS activities of TRH (<Glu-His-Pro-NH_2) from its classical effects on TSH release. Nutt *et al.*[1] described the synthesis of several TRH analogues in which modifications to the *N*- and *C*-termini gave large increases in CNS activity without concomitant increases in hormonal effects. The best analogue contained a larger six-membered aminoadipic acid (Aad) ring in place of <Glu together with thiazolidine carboxamide in place of Pro-NH_2. This peptide exhibited 35 times the noradrenergic CNS activity of TRH whilst having the same hormonal activity. Problems encountered during its synthesis included instability of Thz residues towards normal Tfa treatment during the α-Boc removal, which was overcome by using HCl–EtOAc treatment at −25 °C. DCC–HOBt-mediated coupling of <Aad to His(DNP)-Thz-NH_2 resulted in diketopiperazine formation by the dipeptide when the reaction was carried out in DMF; however, good yields of the tripeptide were obtained in CH_2Cl_2.

Over the past few years the peripheral gastrointestinal biological properties of TRH have also been receiving considerable attention. Perhaps related to these studies have been recent observations that a tripeptide <Glu-His-Gly-OH, isolated from anorexic women,[2] has potent inhibitory effects on gastrin, gastric acid, and insulin release produced by sham feeding in dogs.[3] There appear to be important therapeutic implications for this in the area of ulcer treatment and appetite control with more active and long-acting analogues of this peptide, particularly since it is devoid of the TSH and prolactin-releasing activities of TRH itself. Indeed, viewed as an analogue of TRH, this peptide is yet another example of the specificity that can rather readily be built into a number of the neuro-GI peptides.

[1] R. F. Nutt, F. W. Holly, C. Homnick, R. Hirschman, D. F. Veber, and B. H. Arison, *J. Med. Chem.*, 1981, **24**, 692.

[2] O. Trygstad, L. Foss, P. D. Edminson, J. H. Johanson, and K. L. Reichelt, *Acta Endocrinol. (Copenhagen)*, 1978, **89**, 196.

[3] D. H. Coy, J. Jaworek, S. J. Konturek, N. Kwiecien, T. Radecki, A. V. Schally, and J. Tasler, *J. Physiol.*, 1981, **314**, 196.

In one more conventional analogue study with TRH, attempts were made to prepare affinity labels for TRH receptors. Six peptides were synthesized[4] with various groups containing the chloroacetyl moiety in place of <Glu, Phe in place of His, and a pyrrolidone ring in place of Pro-NH_2 of TRH. None of these analogues was found to have significant biological activity *in vitro*.

Luteinizing Hormone-releasing Hormone (LHRH).—Rapid improvements have again been made in the design of competitive antagonists of LHRH. New peptides can block ovulation in the rat, the standard assay method, at single doses of around 1 μg, which represents well over a 1000-fold improvement during the past four years. Two key discoveries during the past twelve months were responsible for the most recent progress.

When D-Ala was incorporated into one of the hitherto most active sequences to give [Ac-D-Phe[1], D-*p*-Cl-Phe[2], D-Trp[3,6], D-Ala[10]]LHRH (Table 1), a doubling of

Table 1 *Anti-ovulatory properties of recent analogues of LHRH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) in rats*

LHRH analogue	*Dose/μg*	*Percentage blockade of ovulation*
Ac-D-Phe[1], D-*p*-Cl-Phe[2], D-Trp[3,6]	31	64
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3,6]	15	70
Ac-D-Trp[1,3], D-*p*-Cl-Phe[2], D-Phe[6]	15	90
Ac-D-Phe[1], D-*p*-Cl-Phe[2], D-Trp[3,6], D-Ala[10]	15	70
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Phe[6], D-Ala[10]	15	90
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Phe[6], D-Ser[10]	15	75
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Phe[6], D-Leu[10]	15	22
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Lys[6], D-Ala[10]	3	50
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Arg[6], D-Ala[10]	3	78
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-His[6], D-Ala[10]	3	22
Ac-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Arg[6]	15	0
Ac-dehydro-Pro[1], D-*p*-Cl-Phe[2], D-Trp[3], D-Arg[6], D-Ala[10]	3	0

activity over corresponding Gly[10] analogues was observed.[5] A similar trend occurred with Ac-D-*p*-Cl-Phe[1] and Ac-D-Trp[1] peptides containing D-Ala[10], and these two peptides gave some anti-ovulatory activity at 5 μg doses. The structural requirements of the D-amino-acid in position 10 appear to be quite strict. Severe losses of activity have been observed with D-Leu[10], D-Phe[10], D-Pro[10], and *N*-Me-D-Ala[10] analogues. Only D-Ser in position 10 preserved high levels of activity (Table 1).

Until quite recently, nearly all of the most active LHRH agonists and antagonists have utilized the presence of D-amino-acids with large aromatic side chains in position 6. The inclusion of so many hydrophobic amino-acids in the LHRH antagonist analogues not surprisingly results in poor solubility in aqueous and most organic media. Coy *et al.*[6] reported a method for improving solubility characteristics by inserting more hydrophilic, basic D-amino-acids into the 6

[4] R. J. Goebel, B. L. Currie, and C. Y. Bowers, *J. Med. Chem.*, 1981, **24**, 366.

[5] J. Erchegyi, D. H. Coy, M. V. Nekola, E. J. Coy, A. V. Schally, I. Mezo, and I. Teplan, *Biochem. Biophys. Res. Commun.*, 1981, **100**, 915.

[6] D. H. Coy, A. Horvath, M. V. Nekola, E. J. Coy, J. Erchegyi, and A. V. Schally, *Endocrinology*, 1982, **110**, 1445.

position. Not only did this improve physical properties, but with D-Lys[6] and D-Arg[6] antagonists (Table 1) significant increases in anti-ovulatory activities unexpectedly resulted. The importance of a highly basic side chain in these compounds was further illustrated by the lower activities of D-His[6] and D-*p*-NH_2-Phe[6] analogues containing aromatic but considerably less basic groups. Increased hydrophilicity was also clearly not the only factor involved in improving biological activity since a D-Glu[6] analogue was also less active. Analogues with neutral alkyl amino-acids, such as D-Ala and D-Leu, were more than five times less active than the D-Arg[6] analogue. Also apparent in this generation of analogues was the increased importance of a hydrophobic aromatic D-amino-acid in position 1 and the D-Ala replacement in position 10.

A recent study [7] on the *in vitro* LHRH antagonist activity of several analogues confirmed earlier conclusions concerning some, but not complete, parallelism between *in vitro* and *in vivo* activities.

The great value of certain LHRH analogues in a general biochemistry sense has also been apparent of late. Hazum [8] allowed [D-Lys[6]]LHRH to react with the photoaffinity label (4-azidobenzoyl)-*N*-hydroxysuccinimide, with retention of high binding affinity to LHRH pituitary receptors. The role of recently discovered peptide hormone cellular internalization in the mechanism of action of LHRH was also studied [9] by use of a similar strategy in which the D-Lys residue was used for both immobilization to agarose beads and fluorescent labelling with rhodamine isothiocyanate. Full biological activity of the immobilized form indicated that internalization, observed directly with the fluorescent peptide, was not essential for gonadotropin-releasing activity.

A new synthesis of ^{3}H-labelled LHRH was reported; [10] in it ^{3}H was introduced by reduction of a dehydro-Pro[10] residue, with a Pd–alumina catalyst. Interestingly, analysis of the labelled product revealed a large percentage of ^{3}H incorporated into the His[2] residue also. Direct tritiation of LHRH itself gave good incorporation of non-exchangeable ^{3}H into the His residue, and it was suggested that this procedure could be of general use for His-containing peptides.

Somatostatin.—The numerous synthetic studies carried out since the isolation and characterization of somatostatin, H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, have resulted in analogues that have some selectivity in four major activity categories: inhibition of growth hormone (GH), insulin, glucagon, and gastric acid secretion.

Extensions of an earlier examination of the effects of different amino-acid replacements for Lys in position 4 of somatostatin have revealed [11] even more GH-selective compounds (Table 2) than the Phe[4] analogue originally described. Although some selectivity was lost with [Phe[4], D-Trp[8]]somatostatin, it still inhibited GH release four times more effectively than somatostatin itself whilst being only twice as active for inhibition of insulin and glucagon release. [*p*-NH_2-Phe[4]]somatostatin contains not only the aromatic side chain necessary for GH

[7] K. Nikolics, C. Bieglmayer, J. Spona, J. Seprodi, and I. Teplan, *Peptides*, 1981, **2**, 65.
[8] E. Hazum, *Endocrinology*, 1981, **109**, 1281.
[9] P. M. Conn and E. Hazum, *Endocrinology*, 1981, **109**, 2040.
[10] E. Klauschenz, M. Bienert, H. Egler, U. Pleiss, H. Niedrich, and K. Nikolics, *Peptides*, 1981, **2**, 445.
[11] W. A. Murphy, C. A. Meyers, and D. H. Coy, *Endocrinology*, 1981, **109**, 491.

selectivity but also a free amino group analogous to the ε-amino group of the original Lys residue. This analogue exhibited a stronger selectivity of about 10 : 1. [*p*-NH_2-Phe^4, D-Trp^8]somatostatin maintained the same ratio of selectivity together with a 15-fold increase in inhibition of GH release. In contrast, replacement of Phe^4 by pentafluoro-Phe^4 completely destroyed selectivity, but at the same time increased overall activity was maintained. It seems possible that the introduction of another benzene ring into somatostatin is producing additional or altered aromatic ring interactions that could lead to important conformational and, hence, bioactivity changes observed.

Table 2 *Effects of some position-4 somatostatin analogues on nembutal-stimulated GH and portal insulin and glucagon release in rats*

Peptide	*Percentage release-inhibiting activity in rats*		
	GH	*Insulin*	*Glucagon*
Somatostatin	100	100	100
Phe^4	374 (96—1465)	2.5 (0.1—55)	16 (4—66)
Phe^4, D-Trp^8	360 (239—542)	156 (85—287)	193 (61—559)
F_5Phe^4	299 (150—598)	285 (162—504)	263 (110—625)
p-NH_2-Phe^4	405 (199—825)	38 (15—97)	43 (12—151)
p-NH_2-Phe^4, D-Trp^8	1524 (491—4735)	139 (84—230)	142 (57—353)

Further analogue investigations have been reported on the short, cyclic, conformationally restricted forms of somatostatin. Veber *et al.*[12] carried out additional computer simulations on *cyclo*(-7-aminoheptanoyl-Cys-Phe-D-Trp-Lys-Thr-Cys-) in an effort to simplify further the molecule by removing the disulphide and aminoheptanoic acid bridges whilst preserving an active conformation. The general structure, *cyclo*(-A-B-Phe-D-Trp-Lys-Thr-), was examined; in it A and B were occupied by Pro and a variety of D and L residues chosen by the computer to mimic the preferred conformation. By far the best activity was obtained with *cyclo*(-Pro-Phe-D-Trp-Lys-Thr-Phe-) in which B = Phe was introduced to create an essential additional hydrophobic binding site missing with other combinations of non-aromatic amino-acids. Thus, in effect 9 of the 14 original residues of somatostatin were replaced by Pro and the resulting hexapeptide was more than twice as active as the parent peptide.

N.m.r. studies on the rigid Pro peptide were quite consistent with the suggested conformation.[13] The author points out that important conclusions about the functions of individual amino-acids in somatostatin itself can now be inferred. Residues 7—10 appear to form the main receptor recognition area of the chain. They constitute an hydrophobic area into which the ε-amino group of Lys protrudes. A complimentary hydrophobic surface on the receptor together with a suitable carboxyl group could be sufficient to account for high-affinity binding and

[12] D. F. Veber, R. M. Freidinger, D. S. Perlow, W. J. Palaveda, F. W. Holly, R. G. Strachan, R. F. Nutt, B. H. Arison, C. Homnick, W. C. Randall, M. S. Glitzer, R. Saperstein, and R. Hirschman, *Nature (London)*, 1981, **292**, 55.

[13] D. F. Veber, 'Peptides: Proceedings of the 7th American Peptide Symposium', ed. D. H. Rich and E. Gross, Pierce, Rockford, Illinois, 1982, p. 685.

hormonal activity. N.m.r. fluorescence, and free-energy calculation studies tend to support most of these conclusions.[14]

A large number of analogues of another of the short, cyclic somatostatin analogues, *cyclo*(-7-aminoheptanoyl-Phe-Phe-D-Trp-Lys-Thr-Phe-), were also recently reported.[15] Alterations were limited to the Phe-D-Trp-Lys-Thr residues, which are equivalent to residues 7—10 of somatostatin. Most changes to and substitutions for the Phe^7 ring, including its replacement by non-aromatic amino-acids, largely retained biological activity, therefore indicating a minimal role for this residue. As in past studies with the full somatostatin sequence, Lys appeared to be the most critical residue since all modifications to basicity and size of side chain destroyed activity. Surprisingly, Thr could also not be replaced, even with very similar residues, without great losses of activity.

Much research continues to be reported on the biological properties of somatostatin-28, a possible precursor of the 14 peptide. It appears to be equipotent, but longer acting, for inhibition of a number of gastrointestinal secretions in dogs.[16] Reports also continue to appear,[17] claiming enormously high (4000% relative to somatostatin) activities for somatostatin-28 in certain other assay systems, particularly inhibition of arginine-induced insulin and glucagon release in rats. These are contrary to most other studies and may be due to bioassay design problems.

Several membrane receptor assays[18] have now been developed for somatostatin, and generally analogues seem to exhibit binding affinities in the most part parallel with their biological activities. This type of assay system has, however, now been used to compare peptide affinities for membranes isolated from different tissue types, for instance from brain and pituitary, and important differences in binding are emerging.[19] This is not too unexpected given the biological selectivity that can be built into somatostatin analogues. In another interesting study[20] ^{125}I-labelled [Leu^8, D-Trp^{22}, Tyr^{25}]somatostatin-28 was used as the radioactive ligand in a comparison of analogue-binding affinities for brain somatostatin receptors and receptors in an insulin-secreting pancreatic tumour composed mainly of B cells. Profound differences in the affinities of various analogues were observed between the two tissue types. In the most part, insulin-specific analogues of the des-Asn variety were noticeably more effectively bound by tumour rather than brain receptors. Glucagon-specific D-Cys^{14} analogues had noticeably reduced B-cell affinities relative to brain-receptor affinities. Again, [D-5-fluoro-

[14] B. H. Arison, R. Hirschman, W. J. Palaveda, S. F. Brady, and D. F. Veber, *Biochem. Biophys. Res. Commun.*, 1981, **100**, 1148; K. Itallenga, P. Deschrijner, G. van Binst, V. U. B. Orgc, M. Knappenberg, C. Dremier, A. Michel, A. Scarso, and J. Brison, 'Peptides: Proceedings of the 7th American Peptide Symposium', ed. D. H. Rich and E. Gross, Pierce, Rockford, Illinois, 1982, p. 695; P. W. Schiller, N. Abraham, F. Bellini, and H. Immer, *ibid.*, p. 367.

[15] S. F. Brady, R. F. Nutt, F. W. Holly, W. J. Palaveda, R. G. Strachan, S. J. Bergstrand, D. F. Veber, and R. Saperstein, 'Peptides: Proceedings of the 7th American Peptide Symposium', ed. D. H. Rich and E. Gross, Pierce, Rockford, Illinois, 1982, p. 653.

[16] S. J. Konturek, J. Tasler, J. Jaworek, W. Pawlik, K. M. Walus, V. Schusdziarra, C. A. Meyers, D. H. Coy, and A. V. Schally, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 1967; N. Vaysse, I. Pradayrol, J. A. Chayvialle, F. Pignal, J. P. Esteve, C. Susini, F. Desco, and A. Ribet, *Endocrinology*, 1981, **108**, 1843.

[17] M. Brown, J. Rivier, and W. Vale, *Endocrinology*, 1981, **108**, 2391.

[18] C. B. Srikant and Y. C. Patel, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 3930; J.-C. Reubi, M. H. Perrin, J. E. Rivier, and W. Vale, *Life Sci.*, 1981, **28**, 2191.

[19] C. B. Srikant and Y. C. Patel, *Endocrinology*, 1981, **108**, 341.

Trp[8]]somatostatin, as previously reported by Srikant and Patel,[19] was the only analogue with much greater affinity for brain over pancreatic receptors.

It should be pointed out that some discrepancies with respect to the actual effectiveness of somatostatin-28 peptides in this type of assay also exist. For instance, Srikant and Patel, using ^{125}I-labelled [Tyr[11]]somatostatin as the ligand, found [21] that somatostatin-28 had much lower affinity (*ca.* 0.3) than somatostatin for brain receptors but about 3 times higher for pituitary receptors. In contrast, Reubi *et al.*[20] found much higher apparent affinities for somatostatin-28 in both tissue types. One explanation for this could be variations in enzyme contents of the tissue preparations employed.

A further paper [222] appeared on the biological activity of catfish pancreatic somatostatin I, which contains sequence differences within the ring compared to mammalian somatostatin. It was 3—10 times less active than somatostatin in several *in vitro* assay systems. A second peptide sequence identical to somatostatin was shown [23] to be present in catfish pancreas by mRNA sequencing, and it was then isolated. In another study [24] somatostatin isolated from rat hypothalami was identical to the pig and sheep type.

Reports continue to be published [25] on the beneficial effects of somatostatin and analogues in diabetes and gastric-ulcer management.[25]

Another paper [26] that should probably be mentioned in this section concerns the design of a peptide, Tyr-D-Trp-Ala-Trp-D-Phe-NH_2, which actually stimulates the release of GH from pituitary cells in culture. This peptide resulted from a series of modifications to [D-Trp[2]]methionine-enkephalin that increased the weak GH-releasing activity of the latter 1000-fold. It is not known whether this peptide is behaving like an endogenous GH-releasing factor or as an antagonist of somatostatin operating on somatostatin receptors. Certainly, free-energy calculations show certain conformational similarities between it and somatostatin.

Corticotropin-releasing Factor (CRF).—A fourth and most elusive hypothalamic peptide can now be added to the list with the recent isolation and sequencing of a 41-residue peptide (see Figure) that possesses very powerful ACTH- and β-endorphin-releasing activity both *in vivo* and *in vitro*.[27] This new peptide contains considerable sequence homology with sauvagine, a peptide isolated from frog skin, and to a much lesser extent with angiotensinogen.

[20] J.-C. Reubi, J. Rivier, M. Perrin, M. Brown, and W. Vale, *Endocrinology*, 1982, **110**, 1049.
[21] C. B. Srikant and Y. C. Patel, *Nature (London)*, 1981, **294**, 259.
[22] H. Oyama, J. Martin, K. Sussman, G. C. Weir, and A. Permutt, *Regul. Peptides*, 1981, **1**, 387.
[23] P. C. Andrews and J. E. Dixon, *J. Biol. Chem.*, 1981, **256**, 8267.
[24] P. Bohlen, R. Benoit, N. Ling, R. Guillemin, and P. Brazeau, *Endocrinology*, 1981, **108**, 2008.
[25] J. L. Selam, D. Chenon, T. C. Pham, J. P. Gagnol, N. Thomas, G. Gravagne, A. Orsetti, and J. Mirouze, *Diabetologia*, 1981, **21**, 126; A. V. Greco, G. Ghirlanda, L. Altomonte, R. Manna, A. G. Rebuzzi, and A. Bertoli, *Horm. Metab. Res.*, 1981, **13**, 310; W. Hofmann, P. O. Schwille, W. Engelhardt, J. Hemmer, and D. Scholtz, *Heptagastroenterology*, 1981, **28**, 38.
[26] F. A. Momany, C. Y. Bowers, G. A. Reynolds, D. Chang, A. Hong, and K. Newlander, *Endocrinology*, 1981, **108**, 31.
[27] W. Vale, J. Spiess, C. Rivier, and J. Rivier, *Science*, 1981, **213**, 1394; J. Spiess, J. Rivier, C. Rivier, and W. Vale, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 6517; C. M. Turkelson, A. Arimura, M. D. Culler, J. B. Fishback, K. Groot, M. Kanda, M. Luciano, C. R. Thomas, D. Chang, J. K. Chang, and M. Schimizu, *Peptides*, 1981, **2**, 425; C. Rivier, M. Brownstein, J. Spiess, J. Rivier, and W. Vale, *Endocrinology*, 1982, **110**, 272.

H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2

Figure *Amino-acid sequence of ovine CRF*

The full 41-residue sequence has been synthesized by solid-phase methods.[27,28] Both methods utilized sophisticated h.p.l.c. purification of HF-cleaved material. No structure–activity studies have thus far been reported for CRF; however, the original paper by Vale *et al.*[27] indicates that the *C*-terminal amide region is critical to biological activity but that acetylation or removal of several *N*-terminal amino-acids has little effect. [Met(O)[21]]CRF was about 10 times less active than the unoxidized peptide.

2 Anterior Pituitary Hormones

By J. Ramachandran

Introduction.—This review will be concerned with the structure–function relationships of the protein and polypeptide hormones of the anterior pituitary gland. Since this subject was covered previously[1] in Volume 13 of this Report, the literature for 1981 and early 1982 will be covered in this review. The nomenclature is according to the suggestions of the IUPAC–IUB Commission of Biochemical Nomenclature.[2] The format will be similar to that used in Volumes 12 and 13 of this Report, and the anterior pituitary hormones will be considered under three structurally and functionally related groups.[1]

Hormones of Group I.—All the hormones of this group are flexible polypeptides derived from a single high-molecular-weight precursor called pro-opiomelanocortin (POMC).[3] POMC is composed of a glycopeptide with an apparent molecular weight of 16000 daltons (16K fragment) at the amino terminal, corticotropin (ACTH) in the middle, and lipotropin (LPH) at the carboxy terminal. Each of the three segments contains a melanotropin (MSH) sequence. γ-MSH is present in the amino-terminal sequence of POMC,[4] α-MSH corresponds to the first 13 residues of ACTH, and β-MSH corresponds to the middle segment of LPH. The tetrapeptide sequence His-Phe-Arg-Trp is common to all the melanotropins and, therefore, is also present in ACTH, LPH, and the amino-terminal fragment of POMC. Processing of POMC in the anterior pituitary gland occurs at selected paired basic residues to generate predominantly ACTH, LPH, and the amino-terminal 16K fragment. In the intermediate lobe these

[28] J. Suieraz-Diaz, D. H. Coy, S. Vigh, T. W. Redding, W.-Y. Huang, I. Torres-Aleman, and A. V. Schally, *Life Sci.*, 1982, **31**, 429.

[1] J. Ramachandran, 'Amino-acids, Peptides, and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), 1982, Vol. 13, p. 330.

[2] 'Amino-acids, Peptides, and Proteins', ed. R. C. Sheppard (Specialist Periodical Reports), The Chemical Society, London, 1976, Vol. 8, p. 442.

[3] M. Chrétien, S. Benjannet, P. Grossard, C. Gianoulakis, P. Crine, M. Lis, and N. G. Seidah, *Can. J. Biochem.*, 1979, **57**, 1111.

[4] S. Nakanishi, A. Inouyi, T. Kita, M. Nakamura, A. C. Y. Chang, S. N. Cohen, and S. Numa, *Nature (London)*, 1979, **278**, 423.

peptides are further processed to yield α-MSH and corticotropin-like intermediate-lobe peptide (CLIP) from ACTH, β-MSH and β-endorphin (β-EP) from LPH, and γ-MSH or related peptides from the amino-terminal 16K fragment of POMC.

γ-MSH and the Amino-terminal Fragment of POMC. The amino-terminal fragment of POMC was isolated from human pituitary glands and its amino-acid sequence was determined.[5] This glycopeptide represents the amino-terminal 76-amino-acid residues of human POMC. It is *O*-glycosylated at Thr-45 and *N*-glycosylated at Asn-65. The amino-acid sequence differed from that predicted by the human genomic DNA sequence [6] in only one residue, namely the presence of Arg-22 instead of the predicted Gly-22. This amino-terminal peptide was found to stimulate aldosterone secretion in a human adrenal aldosteronoma. The complete amino-acid sequence of the glycosylated amino-terminal segment of porcine POMC has also been reported.[7] The sequence represents the first 80 amino-acid residues of porcine POMC and exhibits considerable sequence conservation with its predicted homologues from bovine, murine, and human species. The amino-terminal fragment of salmon POMC has been isolated and characterized.[8] The salmon peptide is composed of 76 residues, which is 27 residues shorter than the bovine [4] and human [6] peptides predicted from the DNA sequences. The first 44 residues of the teleost peptide showed significant sequence homology with the sequences of the mammalian peptides. The salmon peptide, however, does not contain the counterpart of the γ-MSH sequence located between residues 51 and 64 in the mammalian peptides.

A peptide with the amino-acid sequence H-Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2 corresponding to residues 51—63 of bovine POMC has been isolated from the neurointermediate lobes of bovine pituitaries.[9] This suggests that the amino-terminal fragment of POMC may be processed by proteolysis at paired basic residues to γ-MSH peptides in the intermediate lobes of mammalian pituitaries. A novel type of processing at a site lacking the paired basic residues has been reported.[10] A peptide corresponding to the first 30 residues of porcine POMC has been isolated from porcine pituitaries and sequenced.[10] Whether this peptide represents a maturation product of normal processing of POMC *in vivo* remains to be established.

The amino-terminal fragment of human POMC corresponding to residues 1—76 has been detected [11] in circulation at the same time as ACTH. This naturally occurring glycopeptide has been found to potentiate the steroidogenic action of ACTH on isolated perfused rat and human adrenocortical cells.[12] This potentiating action of the human amino-terminal POMC peptide was completely inhibited by actinomycin D, suggesting that RNA synthesis is required for the potentiating

[5] N. G. Seidah and M. Chrétien, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4236.
[6] A. C. Y. Chang, M. Cochet, and S. N. Cohen, *Proc. Natl. Acad. Sci. U.S.A.*, 1980, **77**, 4890.
[7] N. Lariviere, N. G. Seidah, and M. Chrétien, *Int. J. Pept. Protein Res.*, 1981, **18**, 487.
[8] H. Kawauchi, A. Takahashi, and K. I. Abe, *Int. J. Pept. Protein Res.*, 1981, **18**, 223.
[9] P. Böhlen, F. Esch, T. Shibasaki, A. Baird, N. Ling, and R. Guillemin, *FEBS Lett.*, 1981, **128**, 67.
[10] R. Ekman, R. Hakanson, and H. Jornvall, *FEBS Lett.*, 1981, **132**, 265.
[11] J. Hope, S. Ratter, F. E. Estivariz, L. McLoughlin, and P. J. Lowry, *Clin. Endocrinol.*, 1981, **15**, 221.
[12] E. A. S. Al- Dujaili, J. Hope, F. E. Estivariz, P. J. Lowry, and C. R. W. Edwards, *Nature (London)*, 1981, **291**, 156.

action.[13] A possible physiological role for the amino-terminal POMC peptides is suggested by the recent report[14] that the non-γ-MSH portion of the amino-terminal fragment stimulates adrenal DNA synthesis *in vitro* and mitosis *in vivo*.

ACTH. ACTH has been isolated from equine pituitaries and found[15] to have the same amino-acid composition, amino-terminal residue, and biological activity as human ACTH. Two major forms of rat ACTH were purified to homogeneity by reversed-phase high-performance liquid chromatography (r.p.-h.p.l.c.).[16] The two forms of rat ACTH were found in approximately equimolar amounts with very similar amino-acid compositions, which indicated a close similarity to other mammalian corticotropins. Both forms were found to have biological activities and molecular weights comparable to those of standard synthetic human ACTH. Trypsin digestion indicated that the two peptides were identical except for a modification of one form in the carboxy-terminal tryptic peptide. Radiolabelling experiments[17] using cultured rat anterior pituitary cells have shown that the more polar form is *O*-phosphorylated on the serine residue at position 31. A role for the phosphorylation of Ser-31 in rat ACTH is not apparent. It may not be of general significance since porcine ACTH contains a leucine residue in position 31 instead of a serine residue.

Recently, it has been suggested that certain preparations of human leukocyte interferons (IFN-α) are structurally and functionally related to ACTH and β-EP.[18–20] This conclusion was based on the apparent ability of ACTH and EP antisera to neutralize the anti-viral activity of human IFN-α and on the generation of ACTH activity by pepsin treatment of IFN-α. Two reports[21, 22] of inability to confirm these findings have now appeared. The anti-viral activities of purified IFN-αA and IFN-αD prepared by recombinant DNA technology were not affected by a large molar excess of ACTH antiserum, nor did ACTH interfere in interferon immunoassays.[21] Neither IFN-αA, IFN-αD, nor pepsin digests of these proteins were able to stimulate steroidogenesis in adrenocortical cells.[21] Similar results were obtained with natural IFN-α.[22] In view of the lack of significant structural homology between IFN-α and ACTH or EP, the results of Blalock and Smith[18–20] must be viewed with caution.

Two analogues of human ACTH containing cysteine residues have been synthesized by the solid-phase method.[23] [Cys(carbamidomethyl)[25]]ACTH-(1—26) (peptide I) was 6—7 times more potent in stimulating aldosterone production in isolated rat adrenal capsular cells in comparison with the stimulation of

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[14] F. E. Estivariz, F. Iturriza, C. McLean, J. Hope, and P. J. Lowry, *Nature (London)*, 1982, **297**, 419.
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corticosterone production in decapsulated rat adrenal cells. ACTH is equipotent in both assays. The dimer of peptide I, bis{[Cys^{25}]ACTH-(1—26)}, was 3—4 times as potent as ACTH in both assays.

Photoreactive derivatives of ACTH have been prepared by selective modification of the sulphydryl group of [Trp(SH)^9]ACTH with 2-nitro-4-azidophenylsulphenyl chloride (2,4-NAPS-Cl) and 2-nitro-5-azidophenylsulphenyl chloride (2,5-NAPS-Cl).[24] These photoreactive derivatives were stable to neutral and acidic conditions but were readily cleaved above pH 9 and by β-mercaptoethanol. Photolysis of the NAPS derivatives of [Trp(SH)^9]ACTH at neutral pH resulted in the formation of covalently linked polymers and dimers that were converted to monomer by treatment with β-mercaptoethanol. Analysis of the amino-acid composition of acid hydrolysates of photolysed monomeric and dimeric products indicated a decrease in proline, valine, tyrosine, and phenylalanine. The interaction of ACTH with bovine serum albumin was investigated by photoaffinity labelling.[25] Nearly 30 mol% of tritiated [2,4-NAPS-Trp^9]ACTH was covalently bound to BSA at a molar ratio of peptide:BSA of 1.33. The [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex was isolated and the cyanogen bromide fragments of the complex were analysed by sodium dodecylsulphate–polyacrylamide gel electrophoresis. The radioactivity of the ACTH derivative was predominantly associated with the amino-terminal fragment corresponding to residues 1—183 in BSA. Covalent attachment of ACTH to functional receptors on isolated rat adrenocortical cells by photoaffinity labelling has been reported.[26] Photolysis of rat adrenocortical cells in the presence of [2,5-NAPS-Trp^9]ACTH at 24 °C resulted in persistent activation of corticosterone production. The basal rate of steroidogenesis became maximal when photolysis was performed at 24 °C, but it remained the same as that of control cells when irradiation was conducted at 0 °C. No increase in basal rate was observed with dark controls or cells photolysed with [2,4-dinitrophenylsulphenyl-Trp^9]ACTH, a photoresistant analogue of the hormone. Both ACTH and [2,4-dinitrophenylsulphenyl-Trp^9]ACTH blocked the photo-induced activation of steroidogenesis elicited by [2,5-NAPS-Trp^9]ACTH.[26]

Although ACTH was among the first polypeptide hormones to be utilized in direct receptor-binding studies,[27] the detection and characterization of the physiologically relevant receptors for the hormone proved to be a formidable task. One of the major difficulties encountered in binding studies with ACTH was the low biological potency of radiolabelled ACTH preparations generally used in binding studies. Structure–function studies with ACTH analogues showed that the loss of activity following iodination of the hormone results from the introduction of a bulky iodine atom into the tyrosine at position 2 and the oxidation of the methionine residue in position 4. These difficulties encountered in the iodination of native ACTH have been circumvented by the synthesis of an analogue of the hormone in which the tyrosine residue in position 2 is replaced by phenylalanine and the methionine in position 4 is replaced by the isosteric norleucine.[28] This

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analogue, [Phe^2, Nle^4]ACTH-(1—38), was found to be indistinguishable from synthetic human ACTH in its ability to stimulate corticosterone production in isolated rat adrenocortical cells. Iodination of [Phe^2, Nle^4]ACTH-(1—38) resulted in the formation of [monoiodo-Tyr^{23}, Phe^2, Nle^4]ACTH-(1—38) and the corresponding di-iodo derivative, which were completely separated from each other and from unmodified [Phe^2, Nle^4]ACTH-(1—38) by r.p.-h.p.l.c. [Monoiodo-Tyr^{23}, Phe^2, Nle^4]ACTH-(1—38) was also found to be as potent as ACTH in stimulating steroidogenesis. [^{125}I-Tyr^{23}, Phe^2, Nle^4]ACTH-(1—38) (referred to as ^{125}I-ACTH analogue) was prepared [29] and purified by r.p.-h.p.l.c. to give a homogeneous radioligand with full biological potency and a specific radioactivity of 1800 $\pm$ 75 Ci mmol^{-1}. By the use of this ^{125}I-ACTH analogue, a highly sensitive radioimmunoassay capable of detecting 1 pg ACTH was developed.[29]

ACTH receptors on rat adrenocortical cells have been characterized with the aid of ^{125}I-ACTH analogue.[30] Binding was highly specific to adrenocortical cells, and the radioactive peptide was displaced by low concentrations of ACTH but not by other basic peptides. Binding was rapid, reversible, and linearly related to the number of cells. ^{125}I-ACTH analogue was not significantly degraded by incubation with the cells at 23 °C for 1 h. Scatchard analysis of the binding was compatible with a single class of binding sites with K_d = 1.41 $\pm$ 0.21 nM, and the number of sites was estimated to be 3840 $\pm$ 1045 per cell. The binding curve was superimposable on the concentration–response curve for cyclic AMP. Small but significant amounts of ^{125}I-ACTH analogue were bound at concentrations sufficient for maximal stimulation of steroidogenesis. For a series of ACTH analogues, the concentrations of the peptides required for half-maximal stimulation of cAMP production were in excellent agreement with the concentration required for half-maximal inhibition of binding. These results suggest that the adrenocortical cells contain only one class of ACTH receptors and that stimulation of a small fraction of these receptors (<3%) is sufficient for maximal steroidogenesis.

Melanotropins and CLIP. Low-energy structures for α-MSH have been calculated by semi-empirical conformational analysis.[31] A close spacing of the side chains of Glu-5 and Arg-8 as well as an outward orientation of Lys-11 away from the fragment 6—9 were the characteristic features of the low-energy conformations. Considerable conformational rigidity of the active centre, namely the sequence His-Phe-Arg-Trp (residues 6—9), was also indicated by the calculations. A cyclic, conformationally restricted, isosteric analogue of α-MSH was synthesized [32] by substituting cysteine residues in positions 4 and 10 of the α-MSH sequence. This analogue had a potency >10 000 times that of α-MSH in stimulating frog-skin melanophores, 30 times the potency of α-MSH in stimulating lizard-skin melanophores *in vitro*, and 3 times the potency of α-MSH in stimulating adenylate cyclase activity in Cloudman S-91 mouse melanoma plasma membranes. The

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extent of racemization of amino-acid residues in heat–alkali-treated MSH has been reinvestigated[33] by means of a high-resolution gas-chromatographic procedure. Serine was completely racemized and both methionine and phenylalanine were partially racemized to a greater degree than expected. Whereas heat–alkali treatment of β-MSH causes enhancement and prolongation of the response of frog skins to the treated peptide, similar treatment of α-MSH results in a retardation of the response of the frog skins to the treated peptide.[34] Heat–alkali treatment also prolonged and potentiated the activity of synthetic desacetyl-α-MSH (in contrast to the retardation effect on the acetylated peptide, α-MSH).

[13-*p*-Azidophenylalanine]-α-MSH was synthesized in solution by the fragment condensation method and evaluated for its potential as a photoaffinity label for MSH receptors.[35] The photoreactive α-MSH had 65% of the pigment-dispersing activity of α-MSH and 50% of the activity of α-MSH in stimulating tyrosinase activity in cultured mouse melanoma cells. U.v. irradiation of tadpole tail-fin melanophores in the presence of photoreactive α-MSH induced irreversible pigment dispersion.[36] Photoaffinity labelling of MSH receptors in tadpole tail-fin melanophores was useful in distinguishing between receptor-associated and post-receptor requirement of calcium ions.[37] Persistent stimulation of melanophores was not observed if photolysis with photoreactive α-MSH was conducted in calcium-free medium.

In contrast to a recent report,[38] both α-MSH and β-MSH have been found to stimulate corticosterone production in adrenocortical cells, although high concentrations were needed to elicit the response.[39,40] α-MSH stimulated corticosterone production in decapsulated rat and rabbit adrenocortical cells to the same maximal rate as ACTH but the relative potency of α-MSH was found to be 0.04% and 0.01% of ACTH in rat and rabbit cells, respectively.[39] β-MSH was even less potent (<0.01% of ACTH) in both assays.[39,40] Both melanotropins also stimulated aldosterone production in rat adrenal capsular cells and the potencies were again quite low compared to ACTH.[40] The actions of ACTH, α-MSH, and β-MSH were antagonized by corticotropin-inhibiting peptide [ACTH-(7—38)], suggesting that the weak steroidogenic actions of the melanotropins result from interaction with the ACTH receptor in adrenocortical cells. Since both α- and β-MSH contain the sequence His-Phe-Arg-Trp in common with ACTH, it is understandable that pharmacological concentrations of the melanotropins can elicit steroidogenesis.

The biological activities of the melanotropins have been compared by use of the *Anolis*-rate method of assay.[41] In this assay α- and β-MSH were found to be

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[41] A. M. McCormack, R. J. Carter, A. J. Thody, and S. Shuster, *Peptides*, 1982, **3**, 13.

equipotent, but γ-MSH peptides had very low potency (<3% of α-MSH). Desacetyl-α-MSH had a potency of 28.6% of α-MSH. Both γ-MSH and desacetyl-α-MSH appear to act as partial agonists of α-MSH in this assay.

Two major forms of α-MSH and two major forms of CLIP have been isolated from the neurointermediate lobe of rat pituitary by r.p.-h.p.l.c.[42] The main form of α-MSH was found to be [*NO*-diacetyl-Ser[1]]-α-MSH, with the more familiar [mono-*N*-acetyl-Ser[1]]-α-MSH present to a much smaller extent. The major form of CLIP is a peptide that has been modified by phosphorylation of the serine residue at position 31 in the ACTH sequence. The non-phosphorylated form of CLIP was also present at lower concentrations. A new form of CLIP (CLIP-II) has also been isolated from salmon pituitaries.[43] The newly isolated peptide is a tetracosapeptide, two residues longer than the predominant form, CLIP-I. The isolation of this peptide, CLIP-II, represents the fourth line of evidence that the salmon pituitary gland secretes two different forms of POMC, which are coded on two separate genes.

Lipotropin. The complete amino-acid sequence of ostrich β-LPH has been deduced.[44] In contrast to the human, ovine, bovine, and porcine hormones, which contain 89—93 residues, the ostrich β-LPH is composed of only 79 residues. The ostrich hormone appears to be missing residues corresponding to positions 25—34 of human β-LPH. The sequence corresponding to β-EP exhibited the highest degree of homology with the mammalian hormones. The segment His-Phe-Arg-Trp is present in the β-MSH sequence of ostrich β-LPH. The amino-terminal sequence of human β-LPH has been reinvestigated[45] since the amino-acid sequence reported[46] for this hormone differs in 11 positions within the first 26 residues from the sequence predicted[6] from the DNA sequence of human POMC. The revised sequence[45] still differs in 7 positions compared to the structure derived from the DNA sequence. Since much of the sequence variation among LPH molecules of different species are found in the amino-terminal segment devoid of any recognizable biological function, the discrepancies in the structure of human β-LPH may be due to polymorphism. The DNA sequence reported[6] is from a single foetal tissue whereas the amino-acid sequence was determined using β-LPH derived from pooled adult pituitaries.

Two forms of β-LPH have been detected in extracts of rat pituitary.[47] The two forms appear to differ from each other by charge and possibly by size. γ-LPH has been isolated from equine pituitaries.[15] The efficacy of r.p.-h.p.l.c. and partition chromatography in separating closely related analogues of β-LPH and β-EP have been compared.[48] Ovine β-LPH could be effectively separated from the naturally occurring contaminant containing a pyroglutamyl residue at the amino-terminal instead of a glutamyl residue, by partition chromatography but not by r.p.-h.p.l.c.

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Hormones of Group II.—*Growth Hormone (GH).* Growth hormone was isolated from four marsupial species and characterized.[49] The purified marsupial hormones were found to be structurally similar to the eutherian growth hormones but differed in biological and immunological properties. The amino-acid composition of secreted mouse GH was found to be similar to that of rat GH.[50] Human GH (hGH) produced in bacteria by recombinant DNA technology has been characterized by r.p.-h.p.l.c. of intact molecule and tryptic digests of the biosynthetic hormone.[51] The biosynthetic hGH appears to have the same disulphide bridges and amino-acid sequence as the hormone isolated from human pituitaries except for the presence of an extra methionine residue at the amino-terminal, which is due to the initiation of protein synthesis in *E. coli* by formyl methionine and subsequent removal of the formyl group by an enzyme present in the bacteria. When administered to rats, the bacterially derived hGH was found to be equipotent with pituitary-derived preparations.[52]

Proteolytic cleavage of hGH was found to be different in the native and deamidated forms of the hormone.[53] The major desamido-hGH resulting from deamidation of Asn-152 was cleaved by subtilisin at residues 139 and 146 only, whereas subtilisin converted hGH into two cleaved forms, only with cleavages at 139 and 146, and another with cleavages at 139 and 149. Another deamidated form of hGH resulting from deamidation of Gln-137 was cleaved between residues 95 and 127 in addition to the sites cleaved in hGH. Since some of the proteolytically processed forms have high biological activity, deamidation may have some physiological significance in directing specific cleavages.

Bovine GH (bGH) was carbamylated[54] to yield a product that was fully modified at the amino terminal and partially modified at lysines-143 (7%) and -179 (28%). The modified hormone retained full growth-promoting activity. The carbamylated bGH appears to have a different conformation compared to bGH since lysine-69, which is resistant to trinitrophenylation in the native hormone, reacted easily and under mild conditions in the carbamylated protein. The carbamylated, trinitrophenylated bGH was devoid of biological activity. Acetylation of bGH with *N*-acetylimidazole resulted in the modification of several tyrosine residues and lysine residues with concomitant loss of growth-promoting activity.[55] The loss in activity induced by acetylation was prevented by the prior amidination of lysine residues, suggesting that the tyrosine residues (which are modified) are not essential for the manifestation of the growth-promoting activity of bGH.

Iodination of bGH with limited amounts of oxidizing agent yielded a derivative with no more than 1 gram atom of iodine per mole of hormone.[56] Analysis of the tryptic fragments of iodinated bGH revealed that half of the molecules were

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labelled in Tyr-174, followed by Tyr-158 (16%) and Tyr-42 (14%). Frontal gel chromatography indicated that the preparation did not contain significant amounts of unreacted bGH. The circular dichroism spectra of native and iodinated bGH were similar. The small decrease in the self-association of iodinated bGH compared to the unmodified hormone at pH 8.5 could be accounted for by the ionization of the iodotyrosyl residue (average $pK_{app} = 9.3$), indicating that the presence of the iodine atom by itself does not interfere with dimer formation. Iodinated bGH was almost indistinguishable from native bGH in body-growth-promoting activity, rat-liver uptake *in vivo*, binding to rabbit-liver microsomes, and primary antigen–antibody interactions.[57] Microcomplement fixation experiments suggested that iodination introduces minor alterations in the affinity of some antigenic determinants of bGH.

Non-covalent interaction of two fragments of reduced-carbamidomethylated gGH generated by proteolysis with thrombin resulted in the formation of a recombinant with the same receptor-binding activity as reduced-carbamidomethylated hGH when tested on rabbit-liver and mammary-gland membranes.[58] When compared to intact native hGH, the non-covalent recombinant was only 20% as active in receptor binding and 35% as active in the weight-gain test in hypophysectomized rats.[59] Thrombin was able to resynthesize the peptide bond between Arg-134 and Thr-135 when the two thrombin-generated fragments of reduced-carbamylated hGH composed of residues 1—134 and 135—191 were incubated at pH 6 in 90% (v/v) glycerol at room temperature.[60] The maximal amount of synthesis was about 20% as estimated by sodium dodecylsulphate gel electrophoresis and radioreceptor assay. The resynthesized polypeptide was isolated and found to be indistinguishable from reduced-carbamidomethylated hGH in two receptor-binding assays, radioimmunoassay and the rat-tibia test.[60]

In contrast to the human hormone, the homologous fragments of reduced-carbamidomethylated ovine GH (fragments 1—133 and 134—191) do not undergo recombination.[61] Furthermore, neither the reduced-alkylated nor the reduced-non-alkylated carboxy-terminal fragment of the ovine hormone (peptide 134—191) is able to interact with the reduced-carbamidomethylated hGH-(1—134), under conditions that favour the recombination of the two hGH fragments.

Limited digestion of hGH with trypsin at pH 6 in 90% (v/v) glycerol resulted[62] in the selective cleavage of an 11-residue segment corresponding to sequence positions 135—145. Although circular dichroism spectra revealed significant differences between the tertiary structures of the native and trypsin-modified hGH, the biological properties were very similar. The growth-promoting activity of the trypsin-modified hGH was the same as that of intact hGH.

The structural features of the carboxy-terminal fragment necessary for generation of non-covalent recombinants of hGH were examined[63] by the synthesis

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of analogues of the carboxy-terminal fragment, hGH-(140—191). The synthetic peptide [Nle^{170}, Ala^{165,182}]hGH-(140—187) gave a recombinant with 40% of the receptor-binding activity of intact hGH when combined with the natural fragment, [carbamidomethyl-Cys^{53}]hGH-(1—134). The receptor-binding activities of the semisynthetic recombinant and the natural recombinant were the same. Attempts to obtain a semi-synthetic recombinant with the peptide [Nle^{170}, Ala^{165,182}]hGH-(140—182) were unsuccessful. Peptides corresponding to hGH-(73—128) and hGH-(75—120) were synthesized by the solid-phase method.[64] Neither peptide had any growth-promoting activity.

Bovine GH fragments bGH-(6—124), bGH-(125—149), and bGH-(150—179) generated by cyanogen bromide cleavage did not have any somatomedin activity.[65] However, bGH-(87—124) obtained by cleavage of the tryptophanyl and methionyl bonds of bGH displayed weak somatomedin-like activity. The 20 000 dalton variant of hGH, which has diminished insulin-like properties associated with hGH, was able to stimulate production of somatomedin in the hypophysectomized rat.[66]

Prolactin (PRL). Highly purified ovine PRL was resolved into four components by preparative isoelectric focusing.[67] The more acidic of the four enriched fractions of these components appeared to be less active in both the radioreceptor assay using mammary-gland membranes and the pigeon crop-sac assay. The more acidic forms, which are probably due to deamidation of PRL, had a higher immunoreactivity than the unpurified PRL preparation in radioimmunoassay. The lactogenic activities of highly purified tilapia PRL and GH estimated by the rabbit-casein synthesis assay were 10 and 100 times lower, respectively, than the activity of standard ovine PRL.[68] Rat PRL was purified[69] by gel filtration and isoelectric focusing to yield a product showing a single 23 000 dalton band in SDS-gel electrophoresis. The purified rat PRL preparation had full biological activity in a mammary-gland fatty-acid synthesis assay and a receptor-binding assay but was devoid of immunoreactive vasopressin, which was present in the starting material.

Ovine PRL was preferentially cleaved[70] at the Arg-125–Leu-126 bond with trypsin immobilized on polyacrylamide. Although this cleavage of ovine PRL by insolubilized trypsin is much less selective than the cleavages of hGH by plasmin or thrombin, it is of interest that the insoluble trypsin attacked a paired basic-residue site (Lys-124–Arg-125).

The non-covalent recombination of two fragments of ovine PRL produced by proteolysis with fibrinolysin has been studied[71] by the use of fluorescence polarization. Ovine PRL-(1—53) was labelled with fluorescein isothiocyanate, and complementation of this flourescent derivative with ovine PRL-(54—199) was monitored by following the changes in flourescence polarization. The dissociation

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constant of the recombinant PRL was 0.144 μM at 30 °C, with a free energy of dissociation of 9.5 kcal mol^{-1}. The kinetics of association of the two fragments followed second-order kinetics, suggesting that a conformational change in one or both of the fragments is either not essential for the recombination or is not rate-limiting in the reaction. The very low immunoreactivity of the recombinant PRL in a PRL radioimmunoassay could be increased by the addition of an excess of fragment PRL-(1—53), which does not cross-react in the assay.[72]

Thermal denaturation of hGH, ovine PRL, and human choriosomatomammotropin (hCS) has been investigated.[73] The thermal denaturation of ovine PRL and hCS at pH 8.5 is a simple endothermic process that is at least partially reversible and does not produce coagulation of the proteins. The transition temperatures suggest fairly stable conformations for these two proteins, but their enthalpies of denaturation are unusually low. The thermal denaturation of hGH begins with an endothermic event with a considerably greater enthalpy of denaturation and a transition temperature higher than that of the other two hormones. However, before the endothermic process can be completed, hGH coagulates, producing a strong exothermic heat flow.[73]

Hormones of Group III.—Gonadotropins were isolated from pike-eel pituitaries and resolved into four electrophoretically homogeneous forms.[74] The amino-acid compositions of the four isohormones were similar to other known piscine gonadotropins (carp and salmon). The pike-eel hormones were also composed of two non-identical subunits, 15 000 and 10 500 daltons, with Tyr and Ser as amino-terminal amino-acid residues, respectively. The first successful purification of thyrotropin (TSH) from a non-mammalian tetrapod has been reported.[75] TSH was isolated from the pituitary glands of the snapping turtle and the sea turtle.[75] Both species of TSH resembled mammalian TSH in fractionation behaviour, and the amino-acid composition of sea-turtle TSH was strikingly similar to that of bovine TSH. The loss of biological activity during the iodination of bovine TSH has been investigated and ascribed to the oxidation process rather than to the incorporation of iodine.[76]

It is known that dissociation of the glycoprotein hormones into the constituent subunits is accompanied by almost complete loss of biological activity. The low biological activities associated with the α- and β-subunits of ovine lutropin (oLH)[77] and human chorionic gonadotropin (hCG)[78] have been ascribed to contamination of the subunit with the intact hormone. The subunits of oLH and hCG were purified by r.p.-h.p.l.c. and immunoaffinity chromatography and tested[79] for activity in the rat Leydig cell assay.[77] The β-subunits of both hCG and oLH exhibited partial but detectable activity at high concentrations. Furthermore, oLH-β inhibited the action of hCG competitively and hCG-β appeared to act as a

[72] M. D. Jibson, C. H. Li, and C. B. Glaser, *Proc. Natl. Acad. Sci. U.S.A.*, 1982, **78**, 2830.
[73] T. A. Bewley, *Int. J. Pept. Protein Res.*, 1982, **19**, 63.
[74] F.-L. Huang, C. J. Huang, S. H. Lin, T. B. Lo, and H. Papkoff, *Int. J. Pept. Protein Res.*, 1981, **18**, 69.
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[76] N. A. Takai, S. Filetti, and B. Rapoport, *Endocrinology*, 1981, **109**, 1144.
[77] J. Ramachandran and M. R. Sairam, *Arch. Biochem. Biophys.*, 1975, **167**, 294.
[78] F. J. Morgan, R. E. Canfield, J. L. Vaitukaitis, and G. T. Ross, *Endocrinology*, 1974, **94**, 1601.
[79] N. R. Moudgal and C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.*, 1982, **79**, 2500.

non-competitive inhibitor of the action of hCG on rat Leydig cells.[79] The α-subunits were inactive at the concentrations tested. The follicle-stimulating hormone (FSH) activity associated with purified equine lutropin (eLH) has been found to be intrinsic to the eLH molecule.[80] Removal of sialic acid residues from eLH and equine chorionic gonadotropin (eCG) increased the potencies of the hormones in the *in vitro* rat Leydig cell steroidogenesis assay but drastically reduced the activities of eCG and eFSH in the *in vitro* rat Sertoli cell cyclic AMP assay.[81]

Although eLH has intrinsic FSH activity in the female gonads, it does not act like FSH in the *in vitro* rat Sertoli cell assay. In this assay, eLH inhibits the action of FSH. Equine LH-β was found to be ineffective but eLH-α was as effective as eLH in blocking the actions of eFSH in the rat Sertoli cell assay.[82] α-Subunits of other species of LH were without any effect on FSH action. Modification of the histidine residues of eLH and eLH-α abolished their inhibitory actions against FSH.

The kinetics of subunit association and hormone dissociation were determined[83] for hCG, oLH, and porcine LH at pH 6.8 and 37 °C at concentrations ranging from 0.1 μmol dm^{-3} to 8 μmol dm^{-3}. The average second-order association rate constants for the subunits of hCG, oLH, and pLH were 70, 170, and 296 $mol^{-1}\,dm^3\,min^{-1}$, respectively. The dissociation of these three hormones was also extremely slow and the measured half-times were 37, 11, and 46 days, respectively. Each hormone exhibited biphasic dissociation, and the slowly dissociating component comprised the largest relative population (87 ± 6%). The first-order dissociation rate constants of this component were 1.0, 3.7, and 0.94×10^{-5} min^{-1} for hCG, oLH, and pLH, respectively. The rate constants for the rapidly dissociating components were nearly an order of magnitude higher. The intersubunit equilibrium dissociation constants were determined[83] to be 0.60 μmol dm^{-3} for hCG, 0.42 μmol dm^{-3} for oLH, and 0.06 μmol dm^{-3} for pLH. These K_d values are considerably higher than the values expected from the low physiological concentrations of these hormones in circulation. These results suggest that gonadotropin integrity *in vivo* is a kinetically regulated process.[83]

The dissociation of salmon gonadotropin into two subunits has also been reported.[84] The conformations of native and acid-dissociated pLH have been studied by u.v. absorption spectroscopy and far-u.v. circular dichroism.[85]

The disulphide bonds of bLH and bTSH have been investigated by selective-reduction procedures.[86] With LH-β the disulphide bonds between half-cystines-93—100 and -26—110 were found to be reduced sequentially; a third bond was deduced to be between half-cystine-72 and either half-cystine-23 or -88, indicating a specific disulphide interchange between the two last positions. With further reduction, extensive interchange occurs, which prevents further assignment of disulphide bonds. With TSH-β the disulphide bridge between half-cystines-88 and

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[81] B. B. Aggarwal and H. Papkoff, *Biol. Reprod.*, 1981, **24**, 1087.
[82] B. B. Aggarwal, H. Papkoff, and P. Licht, *Endocrinology*, 1981, **108**, 2406.
[83] T. W. Strickland and D. Puett, *J. Biol. Chem.*, 1982, **257**, 2954.
[84] B. Breton, *Gen. Comp. Endocrinol.*, 1981, **45**, 147.
[85] R. Salesse, Y. Combarnous, S. Brahms, and J. Garnier, *Arch. Biochem. Biophys.*, 1981, **209**, 480.
[86] J. R. Reeve and J. G. Pierce, *Int. J. Pept. Protein Res.*, 1981, **18**, 79.

-95 opened first. A similar approach was more successful in the case of hCG-β, for which all six disulphide bonds were assigned.[87]

The structures of the three asparagine-linked carbohydrate units of oLH subunits have been determined.[88] The carbohydrate moieties of hCG subunits have been labelled with fluorescent groups.[89] Physicochemical and immunochemical properties of several synthetic peptides corresponding to the carboxy terminal of hCG-β have been reported.[90]

The role of the α-subunit in the function of LH and hCG has been investigated by chemical modification [91] and by studies of recombinants of the subunits.[92] The α-subunit is common to all the glycoprotein hormones and has an identical amino-acid sequence within a species. Bovine α-subunit selectively alkylated at methionines-8 and -33 recombined with TSH-β to generate an α-modified TSH fully active in radioligand receptor and *in vivo* assays.[91] The α-modified LH produced by recombination of modified α with LH-β lost 70% of its activity in the receptor assay owing to lowered affinity for the receptor. Since no conformational changes could be detected by circular dichroism spectra of α-modified LH, the results suggest that LH-α may be involved in a direct interaction with the receptor. These studies also show that modification of the common subunit leads to a differential loss of activity in one glycoprotein hormone compared to another.[91] Assessment of the steroidogenic activity and ability to inhibit [^{125}I]-hCG binding for all nine homologous and heterologous recombinants of the α- and β-subunits of hCG, oLH, and pLH also indicated that both subunits contribute to biological activity through conformational changes in the complementary subunit as well as by direct interaction with the receptor.[92] Methionine residue-32 in TSH-β could be selectively alkylated with retention of full biological activity.[91]

The galactose residues of asialo-hGH have been modified and the effects of modification on the biological properties have been studied.[93] The effect of chemical deglycosylation by anhydrous HF on the properties of oLH [94] and hCG [95] have been reported.

3 Posterior Pituitary Peptides

By M. Manning, A. Olma, and W. H. Sawyer

This Report covers the literature from early 1981 to early 1982. Two reviews [1, 2] on the design and synthesis of analogues of oxytocin and arginine vasopressin (AVP), reports on over 50 new analogues and new syntheses of oxytocin, AVP, and earlier analogues, together with an editorial on the potential for clinical use of antagonists

[87] T. Mise and O. P. Bahl, *J. Biol. Chem.*, 1981, **256**, 6587.
[88] G. S. Bedi, W. C. French, and O. P. Bahl, *J. Biol. Chem.*, 1982, **257**, 4345.
[89] K. C. Ingham and S. A. Brew, *Biochim. Biophys. Acta*, 1981, **670**, 181.
[90] D. Puett, R. J. Ryan, and V. C. Stevens, *Int. J. Pept. Protein Res.*, 1982, **19**, 506.
[91] J. M. Goverman and J. G. Pierce, *J. Biol. Chem.*, 1981, **256**, 9431.
[92] T. W. Strickland and D. Puett, *Endocrinology*, 1981, **109**, 1933.
[93] H. M. Madnick, N. K. Kalyan, H. L. Segal, and O. P. Bahl, *Arch. Biochem. Biophys.*, 1981, **212**, 432.
[94] M. R. Sairam and P. Manjunath, *Int. J. Pept. Protein Res.*, 1982, **19**, 315.
[95] P. Manjunath and M. R. Sairam, *J. Biol. Chem.*, 1982, **257**, 7109.
[1] W. H. Sawyer, Z. Grzonka, and M. Manning, *Mol. Cell. Endocrinol.*, 1981, **22**, 117.
[2] M. Manning, Z. Grzonka, and M. Manning in 'The Pituitary Butterworths International Medical Reviews', ed. C. Beardwell and G. L. Robertson, Butterworths, London, 1981, p. 265.

of AVP[3] were published during this period. The new analogues fall into the following categories:

1. Antagonists of antidiuretic and vasopressor responses to AVP (Table 1).
2. More selective antagonists of antidiuretic responses to AVP (Table 2).
3. Selective antagonists of vasopressor responses to AVP (Table 3).
4. Antagonists of *in vitro* and *in vivo* responses to oxytocin (Table 4).
5. Agonistic analogues of oxytocin (Table 5).
6. Agonistic analogues of AVP (Table 6).

As will become apparent from the data for these analogues, this division is rather arbitrary especially with regard to the antagonists. Thus the known antidiuretic antagonists also antagonize vasopressor responses to AVP and oxytocic responses to oxytocin. Similarly, all the reported vasopressor and oxytocic antagonists antagonize both vascular and uterine responses to both AVP and oxytocin. However, many of these antagonists exhibit considerably enhanced selectivities with respect to their antagonistic potencies at uterine, vascular, and renal tubular receptors.

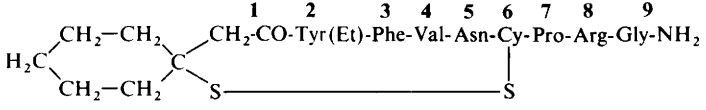
Antagonists of Antidiuretic and Vasopressor Responses to AVP (Table 1).—Since the original report[4] on the first known effective antagonists of both exogenous and endogenous antidiuretic responses to AVP (see Vol. 13, p. 341), many additional antagonists have been reported (Tables 1 and 2). The antagonists in Table 1 are not specific for renal tubular receptors. They all possess more antivasopressor than anti-antidiuretic potency. They also exhibit weak but transient antidiuretic agonistic activity. The antagonists in Table 2 are much more selective for renal tubular than for vascular receptors and the most potent ones are also devoid of any antidiuretic agonistic activity.

In regard to the anti-antidiuretic potencies of the peptides in Table 1, the following structure–activity patterns are discernible. (i) Substitution of D-Tyr(alk) for L-Tyr(alk) consistently enhances potency. (ii) Likewise an L-Arg/D-Arg interchange brings about a significant increase in potency in all but two analogue pairs [*i.e.* $d(CH_2)_5$-D-Tyr(Pr^i)VDAVP/$d(CH_2)_5$-D-Tyr(Pr^i)VAVP (pA_2s 7.51/7.66) and $d(CH_2)_5$-D-Tyr(Pr^n)VDAVP/$d(CH_2)_5$-D-Tyr(Pr^n)VAVP (pA_2s 7.60/7.61)]. (iii) Peptides containing *O*-ethyl-D- and *O*-ethyl-L-tyrosines are the most potent. In the L-Tyr(alk) series, anti-antidiuretic potencies are substantially reduced by increasing the size of the alkyl substituent to n-propyl or isopropyl. This effect is not nearly so pronounced in the D-Tyr(alk) series. (iv) The data in Tables 1 and 2 and data for earlier vasopressor antagonists (See Vol. 10, p. 423, Vol. 11, p. 395, Vol. 12, p. 487, and Vol. 13, p. 343) show that alkylation of the Tyr in $d(CH_2)_5X^2$VAVP or $d(CH_2)_5X^2$VDAVP (X = L- or Tyr) is a requirement for antidiuretic antagonism when the Tyr is in the L configuration but that there is no such requirement when the Tyr is in the D configuration.

More Selective Antagonists of Antidiuretic Responses to AVP (Table 2).—The first generation of antidiuretic antagonists (Table 1) all contained *O*-alkyl substituents

[3] M. Manning and W. H. Sawyer, *Ann. Intern. Med.*, 1982, **96**, 520.
[4] W. H. Sawyer, P. K. T. Pang, J. Seto, M. McEnroe, B. Lammek, and M. Manning, *Science*, 1981, **212**, 49.

Table 1 *Antagonists of antidiuretic and vasopressor responses to AVP*



$d(CH_2)_5Tyr(Et)VAVP$

		Anti-antidiuretic		*Antivasopressor*		
No.	*Peptide*	*Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Ref.*
1.	$d(CH_2)_5Tyr(Me)VDAVP$	15 ± 3	6.68 ± 0.11(4)[c]	0.28 ± 0.05(8)	8.44 ± 0.07(8)	4, 1*a*
2.	$d(CH_2)_5Tyr(Et)VDAVP$	5.7 ± 0.5	7.10 ± 0.08(4)	0.34 ± 0.04(8)	8.31 ± 0.05(8)	4, 1*a*
3.	$d(CH_2)_5Tyr(Pr^i)VDAVP$	8.5 ± 1.7	6.88 ± 0.07(4)	0.28 ± 0.07(4)	8.41 ± 0.08(4)	1*a*
4.	$d(CH_2)_5Tyr(Pr^n)VDAVP$	14 ± 2	6.67 ± 0.05(4)	1.1 ± 0.2(8)	7.86 ± 0.09(8)	1*a*
5.	$d(CH_2)_5Tyr(Me)VAVP$	3.1 ± 0.4	7.35 ± 0.06(4)	0.29 ± 0.06(4)	8.32 ± 0.08(4)	4, 1*a*
6.	$d(CH_2)_5Tyr(Et)VAVP$	1.9 ± 0.2	7.57 ± 0.06(4)	0.49 ± 0.11(4)	8.16 ± 0.09(4)	4, 1*a*
7.	$d(CH_2)_5Tyr(Pr^i)VAVP$	3.6 ± 0.9	7.32 ± 0.10(6)	0.31 ± 0.06(4)	8.36 ± 0.09(4)	1*a*
8.	$d(CH_2)_5Tyr(Pr^n)VAVP$	3.5 ± 0.6	7.29 ± 0.07(4)	0.40 ± 0.04(4)	8.22 ± 0.04(4)	1*a*
9.	$d(CH_2)_5$-D-Tyr(Me)VDAVP	4.9 ± 1.3	7.19 ± 0.12(4)	0.54 ± 0.07	8.10 ± 0.04(4)	1*b*, 1*c*
10.	$d(CH_2)_5$-D-Tyr(Et)VDAVP	1.8 ± 0.2	7.59 ± 0.04(4)	0.88 ± 0.12	7.88 ± 0·05(4)	1*b*, 1*c*
11.	$d(CH_2)_5$-D-Tyr(Pr^i)VDAVP	2.2 ± 0.3	7.51 ± 0.06(4)	0.46 ± 0.08	8.19 ± 0.08(5)	1*b*, 1*c*

12.	d(CH_2)$_5$-D-Tyr(Pr^n)VDAVP	1.7 ± 0.2	7.60 ± 0.05(4)	0.82 ± 0.10	7.92 ± 0.05(4)	1*b*, 1*c*
13.	d(CH_2)$_5$-D-Tyr(Me)VAVP	1.2 ± 0.3	7.77 ± 0.07(6)	0.23 ± 0.04	8.48 ± 0.08(4)	1*b*, 1*c*
14.	d(CH_2)$_5$-D-Tyr(Et)VAVP	1.1 ± 0.2	7.81 ± 0.07(5)	0.45 ± 0.11	8.22 ± 0.12(4)	1*b*, 1*c*
15.	d(CH_2)$_5$-D-Tyr(Pr^i)VAVP	1.6 ± 0.4	7.66 ± 0.11(4)	0.28 ± 0.04	8.40 ± 0.06(4)	1*b*, 1*c*
16.	d(CH_2)$_5$-D-Tyr(Pr^n)VAVP	1.7 ± 0.2	7.61 ± 0.06(4)	0.51 ± 0.10	8.14 ± 0.08(4)	1*b*, 1*c*

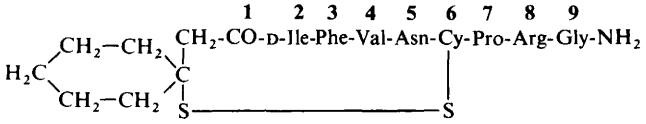
[a] The effective dose is defined as the dose (in nmol kg^{-1}) that reduces the response seen with 2*x* units of agonist to equal the response seen with *x* units of agonist administered before antagonist. [b] Estimated *in vivo* pA_2 values represent the negative logarithms of the 'effective dose' divided by the estimated volume of distribution (67 ml kg^{-1}). [c] Mean ± S.E.; number of assay groups in parentheses. [d] The abbreviations and their full names are as follows: d(CH_2)$_5$Tyr(Me)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$Tyr(Et)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$Tyr(Pr^i)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-isopropyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$Tyr(Pr^n)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-propyltyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$Tyr(Me)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-methyltyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$Tyr(Et)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$Tyr(Pr^i)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-isopropyltyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$Tyr(Pr^n)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-propyltyrosine,4-valine]-arginine-vasopressin; d(CH_2)$_5$-D-Tyr(Me)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)-D-tyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$-D-Tyr(Et)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid,2-(*O*-ethyl)-D-tyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$-D-Tyr(Pr^i)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)-D-tyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$-D-Tyr(Pr^n)VDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-propyl)-D-tyrosine,4-valine,8-D-arginine]vasopressin; d(CH_2)$_5$-D-Tyr(Me)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-methyl)-D-tyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$-D-Tyr(Et)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)-D-tyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$-D-Tyr(Pr^i)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl-D-tyrosine,4-valine]arginine-vasopressin; d(CH_2)$_5$-D-Tyr(Pr^n)VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-propyl)-D-tyrosine,4-valine]-arginine-vasopressin.

1*a* M. Manning, B. Lammek, A. M. Kolodziejczyk, J. Seto, and W. H. Sawyer, *J. Med. Chem.*, 1981, **24**, 701.

1*b* M. Manning, A. Olma, W. A. Klis, A. M. Kolodziejczyk, J. Seto, and W. H. Sawyer, *J. Med. Chem.*, 1982, **25**, 45.

1*c* M. Manning, A. Olma, W. A. Klis, A. M. Kolodziejczyk, J. Seto, and W. H. Sawyer, 'Proceedings of the Seventh American Peptide Symposium', ed. D. Rich and E. Gross, Pierce Chemical Co., Rockford, Illinois, U.S.A., 1981, p. 257.

Table 2 *More selective antagonists of antidiuretic responses to AVP and related peptides*



$d(CH_2)_5$-D-IleVAVP

		Anti-antidiuretic		*Antivasopressor*		
No.	*Peptide*[e]	*Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Ref.*
1.	$d(CH_2)_5$VAVP	Agonist	—	0.76 ± 0.10	7.97 ± 0.06(8)[c]	2*a*
2.	$d(CH_2)_5$-D-TyrVAVP	2.2 ± 0.2	7.51 ± 0.08(4)[c]	0.29 ± 0.09	8.41 ± 0.11(4)	1*b*, 1*c*
3.	$d(CH_2)_5$-D-TyrVDAVP	6.3 ± 0.8	7.03 ± 0.05(4)	0.60 ± 0.04	8.05 ± 0.03(4)	1*b*, 1*c*
4.	$d(CH_2)_5$-D-PheVAVP	0.67 ± 0.13[d]	8.06 ± 0.09(8)	0.58 ± 0.04	8.06 ± 0.03(4)	2*b*
5.	$d(CH_2)_5$-D-PheVDAVP	6.9 ± 1.3	7.07 ± 0.10(9)	0.73	7.98 ± 0.07(4)	2*b*
6.	$d(CH_2)_5$-D-IleVAVP	0.70 ± 0.08[d]	7.98 ± 0.05(4)	8.2 ± 1.4	6.94 ± 0.08(5)	2*b*
7.	$d(CH_2)_5$-D-LeuVAVP	1.2 ± 0.3[d]	7.79 ± 0.12(4)	26 ± 5	6.45 ± 0.09(4)	2*b*
8.	$d(CH_2)_5$-D-ValVAVP	2.3 ± 0.3[d]	7.48 ± 0.06(4)	27 ± 3	6.41 ± 0.05(4)	2*b*
9.	$d(CH_2)_5$-D-AlaVAVP	Agonist	—	172 ± 31	5.79 ± 0.08(4)	2*b*
10.	$d(CH_2)_5$GlyVAVP	Agonist	—	Agonist	—	2*b*
11.	$d(CH_2)_5$-D-ArgVAVP	>90	<5.9	~260	~5.4	2*b*

[a, b, c] See Table 1. [d] No evident agonistic activity. [e] The abbreviations and their full names are as follows: $d(CH_2)_5$VAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-valine]arginine-vasopressin; $d(CH_2)_5$-D-TyrVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-tyrosine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-PheVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-phenylalanine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-PheVDAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-phenylalanine,4-valine,8-D-arginine]vasopressin; $d(CH_2)_5$-D-IleVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-isoleucine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-LeuVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-leucine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-ValVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-valine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-AlaVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-alanine,4-valine]arginine-vasopressin; $d(CH_2)_5$GlyVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-glycine,4-valine]arginine-vasopressin; $d(CH_2)_5$-D-ArgVAVP = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-arginine,4-valine]arginine-vasopressin.

2*a* M. Manning, B. Lammek, M. Kruszynski, J. Seto, and W. H. Sawyer, *J. Med. Chem.*, 1982, **25**, 408.
2*b* M. Manning, W. A. Klis, A. Olma, J. Seto, and W. H. Sawyer, *J. Med. Chem.*, 1982, **25**, 414.

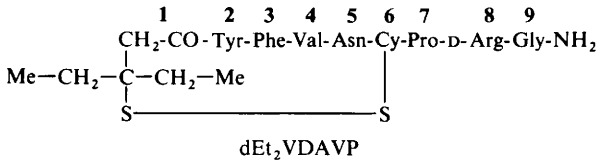
on the L- or D-tyrosine residues at position two. The discovery that a mere D-Tyr/L-Tyr interchange at position two in $d(CH_2)_5$VAVP and $d(CH_2)_5$VDAVP converted these weak antidiuretic agonists into the potent antidiuretic antagonists $d(CH_2)_5$-D-TyrVAVP and $d(CH_2)_5$-D-TyrVDAVP led to the development of a second generation of antidiuretic antagonists possessing a number of advantages (Table 2). Thus the substitution of the D-Tyr in $d(CH_2)_5$-D-TyrVAVP by D-Phe, D-Ile, D-Leu, or D-Val resulted in analogues that (i) are devoid of detectable antidiuretic agonistic activity, (ii) possess equal or increased anti-antidiuretic potency, and (iii) exhibit increased anti-antidiuretic/antivasopressor and anti-antidiuretic/antioxytocic selectivities.

The incorporation of D-Ala, Gly, and D-Arg in addition to D-Phe, D-Ile, D-Leu, and D-Val at position two in $d(CH_2)_5$-D-TyrVAVP allowed the delineation of the structural features of the residue at position two required for antagonism (Table 2). The analogues containing D-Ala and Gly are weak antidiuretic agonists and the analogue with D-Arg at position two exhibits a drastic loss in anti-antidiuretic potency relative to $d(CH_2)_5$-D-TyrVAVP. The data for these analogues indicate clearly that for anti-antidiuretic activity (*a*) an aromatic D-amino-acid is not a requirement, (*b*) there is a lower limit to the size of the aliphatic D-amino-acid required, and (*c*) a positively charged D-amino-acid is deleterious. The data on these analogues provide useful clues to the design of antagonists possessing more enhanced potencies and selectivities for possible clinical use in the treatment of AVP-induced water-retention states.[3]

Selective Antagonists of Vasopressor Responses to AVP (Table 3).—The design and synthesis of antagonists of the vasopressor responses to AVP continue to be a fruitful line of inquiry. For earlier studies see Vol. 10, p. 423, Vol. 11, p. 395, Vol. 12, p. 487, and Vol. 13, p. 343. A number of previously reported vasopressor antagonists are proving to be valuable pharmacological tools for investigations of (*a*) the role(s) of AVP in the regulation of blood pressure under normal and pathophysiological states, (*b*) the characterization of AVP vascular type (V_1) receptors in other tissues, and (*c*) the effects of AVP on memory processes (see ref. 2*a* and references cited therein).

In addition to the antidiuretic/vasopressor antagonists in Tables 1 and 2, eight new selective vasopressor antagonists have been reported (Table 3). Using the β,β-dialkyl substituents at position 1 originally developed in du Vigneaud's laboratory for oxytocin and lysine vasopressin, these analogues were designed by (*a*) incorporating a β,β-diethyl group (Et_2) on the β-carbons of 1-deaminoarginine-vasopressin (dAVP) and 1-deamino-[4-valine, 8-D-arginine]vasopressin (dVDAVP) to give, respectively, dEt_2AVP and dEt_2VDAVP and (*b*) incorporating β,β-dimethyl (P), Et_2, and β,β-cyclopentamethylene [$(CH_2)_5$] groups on the β-carbons of 1-deamino-[4-valine]arginine-vasopressin (dVAVP) and 1-deamino-[8-D-arginine]vasopressin (dDAVP). No consistent pattern of increasing or decreasing antivasopressor potencies with increasing size of the β,β-dialkyl groups has emerged. Three of these analogues, namely dEt_2AVP (pA_2 = 8.36), dEt_2VAVP (pA_2 = 8.29), and $d(CH_2)_5$DAVP (pA_2 = 8.52), are among the most potent vasopressor antagonists reported to date. These three antagonists also exhibit low antidiuretic activities. They are thus highly selective vasopressor antagonists. The

Table 3 *Selective antagonists of vasopressor responses to AVP*



dEt_2VDAVP

No.	*Peptide*[d]	*Antivasopressor* *Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Antidiuretic activity*/ units mg^{-1}	*Ref.*
1.	dEt_2AVP	0.32 ± 0.10(10)[c]	8.36 ± 0.07	0.38 ± 0.07(3)	2*a*
2.	dEt_2VDAVP	0.35 ± 0.10(7)	8.18 ± 0.06	0.71 ± 0.07(4)	2*a*
3.	dPVAVP	0.84 ± 0.13(4)	7.92 ± 0.07	312 ± 23(4)	2*a*
4.	dEt_2VAVP	0.38 ± 0.09(6)	8.29 ± 0.08	1.52 ± 0.11(4)	2*a*
5.	$d(CH_2)_5VAVP$	0.76 ± 0.11(8)	7.97 ± 0.06	0.32 ± 0.02(4)	2*a*
6.	dPDAVP	2.5 ± 0.5(4)	7.45 ± 0.08	5.8 ± 0.7(6)	2*a*
7.	dEt_2DAVP	0.81 ± 0.15(7)	7.96 ± 0.08	0.067 ± 0.006(4)	2*a*
8.	$d(CH_2)_5DAVP$	0.20 ± 0.02(4)	8.52 ± 0.03	0.31 ± 0.01(4)	2*a*

[a, b, c] See Table 1. [d] The abbreviations and their full names are as follows: dEt_2AVP = [1-(β-mercapto-β,β-diethylpropionic acid)]arginine-vasopressin; dEt_2VDAVP = [1-(β-mercapto-β,β-diethylpropionic acid),4-valine,8-D-arginine]vasopressin; dPVAVP = [1-deaminopenicillamine,4-valine]arginine-vasopressin; dEt_2VAVP = [1-(β-mercapto-β,β-diethylpropionic acid),4-valine]arginine-vasopressin; $d(CH_2)_5VAVP$ = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-valine]arginine-vasopressin; dPDAVP = [1-deaminopenicillamine,8-D-arginine]vasopressin; dEt_2DAVP = [1-(β-mercapto-β,β-diethylpropionic acid),8-D-arginine]vasopressin; $d(CH_2)_5DAVP$ = [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),8-D-arginine]vasopressin.

availability of these antagonists should broaden the scope of potential studies on the putative physiological roles of AVP.[2a, 3]

Antagonists of *in vitro* and *in vivo* Responses to Oxytocin (Table 4).—Analogues from Table 2 and 3 are listed in Table 4 because, in addition to their anti-antidiuretic/antivasopressor properties (analogues 1—10) and their antivasopressor/antidiuretic properties (analogues 11—18). all of these analogues exhibit varying degrees of *in vitro* antioxytocic activities. A number have also been shown to antagonize oxytocic responses to oxytocin *in vivo*. They are for the most part much less potent as *in vivo* antagonists of oxytocin than as antagonists of AVP responses. In addition, those analogues that contain D-Ile, D-Leu, or D-Val at position 2 exhibit significant increases in anti-antidiuretic/antioxytocic selectivity relative to $d(CH_2)_5$-D-TyrVAVP.

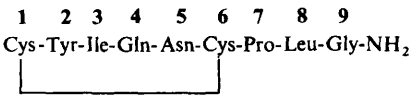
Agonistic Analogues of Oxytocin (Table 5).—A number of oxytocin analogues possessing interesting properties were reported during the past 12 months. The interchange of Pro/Leu at positions 7 and 8 brought about substantial reductions in rat-uterine and rat-milk-ejection activities. While the resynthesis of [D-Gln[4]]oxytocin has shown that it possesses greater oxytocic potency than that originally reported,[5j] it none the less confirms that a D-Gln/LGln interchange in oxytocin leads to a drastic reduction in the characteristic activities of oxytocin. One of the most interesting analogues reported to date is that in which the *C*-terminal $CONH_2$ group of oxytocin was replaced with the CN group. This

Table 4 *Antagonists of* in vitro *and* in vivo *responses to oxytocin*

		Antioxytocic activities (rat uterus)				
		in vitro		in vivo		
No.	*Peptide*[d]	*No* Mg^{2+} pA_2[b]	0.5 mmol dm^{-3} Mg^{2+} pA_2[b]	*Effective dose*[a]/ nmol kg^{-1}	pA_2[b]	*Ref.*
1.	$d(CH_2)_5$-D-TyrVAVP	8.11 ± 0.12(12)[c]	7.78 ± 0.12(8)	6.9 ± 3	7.01 ± 0.08(5)	2*b*
2.	$d(CH_2)_5$-D-TyrVDAVP	7.99 ± 0.12(12)	7.78 ± 0.08(6)	8.1 ± 1.2	6.93 ± 0.06(4)	2*b*
3.	$d(CH_2)_5$-D-PheVAVP	7.74 ± 0.06(6)	8.29 ± 0.05(7)	8.9 ± 1.9	6.92 ± 0.10(5)	2*b*
4.	$d(CH_2)_5$-D-PheVDAVP	7.70 ± 0.05(4)	8.21 ± 0.06(9)	—	—	2*b*
5.	$d(CH_2)_5$-D-IleVAVP	7.98 ± 0.10(10)	7.53 ± 0.07(8)	44 ± 10	6.21 ± 0.09(4)	2*b*
6.	$d(CH_2)_5$-D-LeuVAVP	7.32 ± 0.11(8)	7.38 ± 0.08(8)	61 ± 9	6.05 ± 0.09(4)	2*b*
7.	$d(CH_2)_5$-D-ValVAVP	7.90 ± 0.08(8)	6.89 ± 0.04(9)	220 ± 30	5.49 ± 0.05(4)	2*b*
8.	$d(CH_2)_5$-D-AlaVAVP	6.16 ± 0.05(7)	5.01 ± 0.09(6)	—	—	2*b*
9.	$d(CH_2)_5$GlyVAVP	5.54 ± 0.15(9)	<4.7	—	—	2*b*
10.	$d(CH_2)_5$-D-ArgVAVP	<5.0	—	—	—	2*b*
11.	dEt_2AVP	7.30 ± 0.07(4)[c]	7.16 ± 0.09(8)	—	—	2*a*
12.	dEt_2VDAVP	7.29 ± 0.11(6)	7.21 ± 0.04(9)	—	—	2*a*
13.	dPVAVP	8.09 ± 0.08(4)	7.72 ± 0.10(6)	—	—	2*a*
14.	dEt_2VAVP	7.32 ± 0.11(6)	7.66 ± 0.11(10)	—	—	2*a*
15.	$d(CH_2)_5$VAVP	7.34 ± 0.07(4)	7.31 ± 0.03(10)	26 ± 7	6.47 ± 0.11(5)	2*a*, 2*b*
16.	dPDAVP	7.87 ± 0.15(6)	6.71 ± 0.18(17)	—	—	2*a*
17.	dEt_2DAVP	6.95 ± 0.05(4)	6.70 ± 0.05(8)	—	—	2*a*
18.	$d(CH_2)_5$DAVP	6.97 ± 0.10(4)	6.59 ± 0.10(9)	—	—	2*a*

[a, b, c] See Table 1. [d] Abbreviations as in Tables 2 and 3.

Table 5 *Agonistic analogues of oxytocin*



Oxytocin

No.	*Peptides*	*Rat uterus*		*Rat-milk ejection*	*Rat anti-diuretic*	*Rat pressor*	*Ref.*
		No Mg^{2+}	0.5 mmol dm^{-3} Mg^{2+}				
	Oxytocin	520 ± 12	486 ± 15	—	4.0 ± 0.8	4.3 ± 0.1	5*a*
1.	[7-Leucine, 8-proline]oxytocin	27	—	~45	—	—	5*b*
2.	Deamino-1-carba-[4-glutamic acid]oxytocin	1 ± 0.25	22.7 ± 5.9	—	—	—	5*c*, 5*d*
3.	Deamino-1-carba-[4-(γ-methylamide)glutamic acid]oxytocin	40.0 ± 4.5	80.1 ± 6.7	—	—	—	5*c*, 5*e*
4.	Deamino-1-carba-[4-(γ-dimethylamide)glutamic acid]oxytocin	4.3 ± 0.3	13.5 ± 2.4	8.1	—	—	5*c*, 5*e*
5.	Deamino-1-carba-[4-(γ-*p*-tolylamide)glutamic acid]oxytocin	21.2 ± 7.6	58·6 ± 20.2	4.7	—	—	5*c*, 5*e*
6.	Deamino-1-carba-[4-(γ-hydrazide)glutamic acid]oxytocin	19.4 ± 5.1	43.7 ± 3.8	0.71	—	—	5*c*, 5*e*
7.	Deamino-1-carba-[2-*p*-fluorophenylalanine]oxytocin	400.0	—	319.0	—	—	5*f*
8.	Deamino-1-carba-[2-*p*-fluorophenylalanine]oxytocin sulphoxide (two diastereoisomers)	0.11 6.9	— —	0.15 34.7	— —	— —	5*f*
9.	[9-α-Aminoacetonitrile]oxytocin	662 ± 45	658 ± 40	389 ± 24	—	5.2 ± 0.2	5*g*
10.	[9-β-Alanine]oxytocin	0.187 ± 0.027	0.088 ± 0.016*	—	0.2	—	5*h*
11.	[4-D-Glutamine]oxytocin	0.1	—	0.09 ± 0.02	0.05	<0.1	5*i*, 5*j*

* *in vivo* 0.2.

5*a* M. Manning, E. J. Coy, and W. H. Sawyer, *Experientia*, 1971, **27**, 1372.
5*b* I. Blaha, V. Krchnak, and M. Zaoral, *Collect. Czech. Chem. Commun.*, 1981, **46**, 2136.
5*c* J. Slaninowa, M. Lebel, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 1689.
5*d* M. Lebel and K. Jost, *Collect. Czech. Chem. Commun.*, 1978, **43**, 523.
5*e* M. Lebel, A. Dimeli, V. Bohanovska, J. Slaninova, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1979, **44**, 2556.
5*f* Z. Prochazka, M. Lebel, L. Seritova, and T. Barth, *Collect. Czech. Chem. Commun.*, 1981, **46**, 947.
5*g* J. Roy, D. Gazis, and I. L. Schwartz, 'Proceedings of the Seventh American Peptide Symposium', ed. D. Rich and E. Gross, Pierce Chemical Co., Rockford, Illinois, U.S.A., 1981, p. 237.
5*h* P. Anagnostaras, P. Cordopatis, and D. Theodoropoulos, *Eur. J. Med. Chem.*, 1981, **16**, 171.
5*i* V. J. Hruby, H. I. Mosberg, and V. Viswanatha, *J. Am. Chem. Soc.*, 1982, **104**, 837.
5*j* A. S. Dutta and N. Anand, *J. Med. Chem.*, 1966, **9**, 497.

substitution led to a substantial increase in rat-uterine activity while retaining milk-ejection and vasopressor activities equal to those of oxytocin. These findings (*a*) are strong evidence that the C=O group of the terminal $CONH_2$ is much more important than the NH_2 group for interactions with oxytocin receptors and (*b*) provide a very useful modification for incorporation in other biologically active peptides that terminate in a $CONH_2$ group.

Agonistic Analogues of AVP (Table 6).—Although none of the analogues in Table 6 exhibits enhanced antidiuretic or vasopressor potencies relative to AVP, they provide useful S/A insights. Thus, the Pro/Arg interchange at positions 7 and 8 in AVP led to a drastic reduction in the AVP-like characteristics of the molecule, whereas the oxytocin-like properties were left unaffected. The low vasopressor and antidiuretic activities of [D-Gln^4]AVP provide further evidence for a critical requirement of the L configuration for Gln at position 4 in AVP for binding to vascular and renal tubular receptors. These findings have been interpreted in terms of a proposed bio-active conformation for AVP.

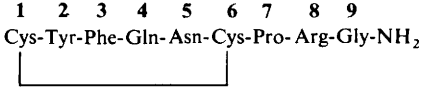
New Syntheses of Oxytocin, AVP, and Analogues.—New syntheses of oxytocin, AVP, and dAVP that utilize the 2,4,6-trimethylbenzyl group for protection of the sulphydryl group of cysteine (removable by HF or trifluoromethanesulphonic acid) have been reported.[5] [8-Tryptophan]oxytocin has been resynthesized by the use of Fmoc-amino-acids and active ester couplings.[6] New syntheses of [1-deamino]lysine-vasopressin (phenacyl protection of the ξ-NH_2 group of Lys removed by penicillin amidohydrolase[7]) and [1-deamino]oxytocin using pentafluorophenyl active esters[8] have also been reported.

Other Studies.—The availability of effective antagonists of the vasopressor action of AVP has revived interest in the contribution of this hormone to cardiovascular regulation.[2a, 3] After modest dehydration in rats[9] or non-hypotensive haemorrhage in dogs[10] the administration of an antagonist produces a transient fall in blood pressure. Even more impressive is the effect of an antagonist on dehydrated rats in which the renin–angiotensin system has been rendered inoperative by prior nephrectomy.[11] In such rats the mean arterial pressure drops from about 100 mm Hg to less than 60 and does not show signs of recovery in the ensuing 45 minutes. This clearly demonstrates the importance of AVP and the renin–angiotensin system for the maintenance of blood pressure during mild volume depletion and, surprisingly, the inability of the adrenergic vasomotor system alone to prevent hypotension.

An AVP antagonist has also proved useful to demonstrate that endogenous AVP contributes to the ability of rats to remember, as tested by a conditioned

[5] F. Brtnik, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 1983.
[6] M. Bodanszky, J. C. Tolle, M. S. Bednarek, and P. W. Schiller, *Int. J. Pept. Protein Res.*, 1981, **17**, 444.
[7] F. Brtnik, M. Krojidlo, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 286.
[8] K. L. Polevaya, R. Vegners, G. Ars, J. Grinsteine, and G. Cipens, *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.*, 1981, 469 (*Chem. Abstr.*, 1981, **96**, 7059).
[9] G. A. Aisenbrey, W. A. Handelman, P. Arnold, M. Manning, and R. W. Schrier, *J. Clin. Invest.*, 1981, **67**, 961.
[10] J. Schwartz and I. A. Reid, *Endocrinology*, 1981, **109(5)**, 1778.
[11] C. E. Andrews, jun. and B. M. Brenner, *Circ. Res.*, 1981, **48**, 254.

Table 6 *Agonistic analogues of AVP*



AVP

No.	*Peptides*	*Rat-uterus oxytocic*	*Rat-milk ejection*	*Rat anti-diuretic*	*Rat pressor*	*Ref.*
	AVP	13.9 ± 0.5	—	323 ± 16	369 ± 6	6*a*
1.	[7-Arginine, 8-proline]vasopressin	20	1.6	3.2	< 1	5*b*
2.	Deamino-1-carba-[7-glycine]arginine-vasopressin	30	50	47.5	< 1	6*b*
3.	Deamino-1-carba-des-Pro^7-Arg^8-Gly-NH$_2$^{9}-vasopressin (deamino-1-carbapressinoic acid)	9.0	—	—	2	6*b*
4.	[4-D-Glutamine]arginine-vasopressin	0.11	—	0.45 ± 0.01	0.26 ± 0.004	5*i*
5.	Valyl-aspartyl-arginine-vasopressin	—	—	—	—	6*c*

6*a* M. Manning, E. J. Coy, W. H. Sawyer, and M. Acosta, *J. Med. Chem.*, 1973, **16**, 463.
6*b* F. Brtnic, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, 1981, **46**, 278.
6*c* Y. L. Chan, C. A. Peterson, Y. F. Ting, and C. W. Smith, *Life Sci.*, 1981, **28**, 415.

avoidance paradigm.[12] The antagonist actually hastens extinction of the learned response. Unlike AVP, which crosses the blood-brain barrier with great difficulty, the antagonist is almost as active when given systemically as it is when injected into the cerebral ventricles.[13] This suggests that these more lipophilic peptides can penetrate into the brain. This may pose a problem if these peptides come into clinical use unless specific antagonists that interact with peripheral and not central receptors can be designed.

4 Pancreatic Hormones

By D. Brandenburg, D. Saunders, and A. Schüttler

A book 'Structural Studies on Molecules of Biological Interest',[1] written in honour of Professor Dorothy Hodgkin, reflects the influence of her work on insulin research and covers many aspects relevant to this chapter. Natural insulin precursors[2] and evolution[3] are discussed, as are the insulin synthesis,[4] the semisynthesis of insulin and precursors,[5-7] structure in crystals[8-11] and in solution,[12] structure–function relationships,[4, 6, 7] and pharmacokinetic studies.[13] Much information on the recombinant DNA technology of insulin is found in a recent publication,[14] which also refers to radioimmunoassay and immunological properties of insulin, proinsulin, glucagon, and pancreatic polypeptide. The properties of biosynthetic human insulin[15] as well as the results of its biochemical characterization and of first clinical tests have been reported.[16] Further reviews discuss the chemistry of insulin,[17] its total synthesis,[18] semisynthesis,[19-21] and structure–activity relationships.[21]

[12] G. F. Koob, M. Le Moal, O. Gaffori, M. Manning, W. H. Sawyer, J. Rivier, and F. E. Bloom, *Regul. Pept.*, 1981, **2**, 153.
[13] M. Le Moal, G. F. Koob, L. Y. Koda, F. E. Bloom, M. Manning, W. H. Sawyer, and J. Rivier, *Nature (London)*, 1981, **291**, 491.
[1] 'Structural Studies on Molecules of Biological Interest', ed. G. Dodson, J. P. Glusker, and D. Sayre, Clarendon Press, Oxford, 1981.
[2] D. F. Steiner in ref. 1, p. 407.
[3] S. Falkmer and S. O. Emdin in ref. 1, p. 420.
[4] P. G. Katsoyannis in ref. 1, p. 454.
[5] V. K. Naithani, H.-G. Gattner, E. E. Büllesbach, and H. Zahn in ref. 1, p. 441.
[6] Y. Zhang in ref. 1, p. 492.
[7] D. Brandenburg in ref. 1, p. 583.
[8] The Beijing Insulin Research Group in ref. 1, p. 501.
[9] N. Sakabe, K. Sakabe, and K. Sasaki in ref. 1, p. 509.
[10] J. F. Cutfield, S. M. Cutfield, E. J. Dodson, C. D. Reynolds, and D. Vallely in ref. 1, p. 527.
[11] R. A. G. de Graaff, A. Lewit-Bentley, and S. P. Tolley in ref. 1, p. 547.
[12] D. Mercola and A. Wollmer in ref. 1, p. 583.
[13] M. Berger, H. J. Cüppers, J. G. Davies, P. A. Halban, S. M. Hoare, and R. E. Offord in ref. 1, p. 487.
[14] 'Insulins, Growth Hormone and Recombinant DNA Technology', ed. J. L. Gueriguian, Raven Press, New York, 1981.
[15] R. E. Chance, E. P. Kroeff, and J. A. Hoffmann in ref. 14, p. 71.
[16] 'Symposium on Biosynthetic Human Insulin', ed. J. S. Skyler and S. Raptis, *Diabetes Care*, 1981, **4**, 139.
[17] R. Geiger in 'Structure and Activity of Natural Peptides', ed. W. Voelter and G. Weitzel, Walter de Gruyter, Berlin and New York, 1981, p. 41.
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Various aspects of insulin action are covered by several authors.[22-25] The present state of research on insulin receptors is well documented in the proceedings of the First International Receptor Symposium[26] and in other reviews.[27-30] Basic and clinical aspects of the immunology of insulin have been discussed,[31] and recent developments in diabetes research have been summarized.[32]

Insulin.—Evidence has been presented that material very similar in biological and immunological properties to mammalian insulin is present also in insects[33] (fruit-fly *Drosophila melanogaster*), in annelids[33] (earth-worm *Annelida oligocheta*), and in *Escherichia coli*.[34]

Previous reports of extrapancreatic insulin synthesis have been questioned[35] since the measured insulin levels were markedly below plasma concentrations.

New analogues and derivatives of insulin that have been described in 1981 are listed in the Table. Compounds of known structure are included if new data and/or further details have been published.

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Table *Analogues and derivatives of insulin*[a]

Compound number	*Structure,*[b] *species*[c]	*Receptor binding*[d]	*Biological activity*[d] in vitro	in vivo	*Other studies*	*Ref.*
1a	Insulin, b				n.m.r.	36
					c.d.	38, 39
					semisynth.	40
1b	Insulin, h (synth.)				c.d.	41
	(biosynth.)					42, 43, 44
	(semisynth.)				c.d.	45
						46, 47, 81
1c	Insulin, bonito		42 D		160% E	49
1d	Insulin, casiragua		2 D		43% E	49
					modelling	50
1e	Insulin, coypu		1 D		190% E	49
1f	Insulin, guinea pig		D		E	49
1g	Insulin, hagfish		7 D		8% E	49
1h	Insulin, porcupine		3 D		110% E	49

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Table (*cont.*)

Compound number	*Structure,*[b] *species*[c]	*Receptor binding*[d]	*Biological activity*[d] in vitro	in vivo	*Other studies*	*Ref.*
1i	Insulin, pig (recomb.)	+			imm.	44
1j	Insulin, turkey		240 D		350% E	49
1k	[^{3}H-Phe^{B1}], b	+ *in vivo*				51
1l*	[^{3}H-Ile], rat					52
1m*	[^{3}H-Phe], rat					52
A-Chain Modification						
2	A1-Boc		50 D		80% E	49
3	A1-(3-Nitro-4-azidophenyl)	+ covalent				53
4*	[D-A_2Bu(Apa)^{A1}], [^{125}I], p		66 C			54
5*	[(Me)$_3$Gly^{A1}], b		49 C	70 A		55
6*	[Nle^{A2}], ovine	0.6 Lm	D			56
7	[Tyr(3-^{125}I)^{A14}], p					57
8*	[Tyr(3-^{125}I)^{A19}], p	65 Ad 88 Lc				58
9*	Pro^{A22}-Leu-Lys-Pro-Ala-Lys-Ser-Ala, h		20 D			59
B-Chain Modification						
10*	B1-Biotinyl,streptavidin (3 : 1)	6% Lm			avidin	60, 61
11*	B1-Biotinyl,streptavidin (1 : 1)	6% Lm			complex	61
12*	B1-Iminobiotinyl-					61
13*	B1-6-Iminobiotinylamidohexyl-					61
14*	B1-Desthiobiotinyl-					61
15*	B1-Desthiobiotinylamidohexyl-					61
16*	B1-Msc-des(B26—30)					62
17	B1,B29-(Suc)$_2$		50 C			63
18*	[Tyr(3-^{125}I)^{B1}], p	100 Lc			degrad.	64
19	Des-Phe^{B1}					65
20*	Des-Phe^{B1}[Gly^2, Pro^3, Glu^4,Thr^5], p			12 B		66
21	B2-Napa-des-Phe^{B1}, b	+ covalent	+ F			67
22*	[Asn^{B12}]	*ca.* 0.05 Lm	*ca.* 0.03 D			68

23*	[Tyr(3-^{125}I)^{B16}], p	100 Ad 100 Lc				58
24*	[Arg(Ad)^{B22}]	90 P		100 B		69
25*	[Arg(DHCH)^{B22}]	80 P		100 B		69
26	[Glu^{B22}]des(B26—30)			25 A		70
27	Des(B23—30), b				c.d.	38, 39
			D		E	49
	(highly purified)		D, F			71
		+ P				72

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Table (*cont.*)

Compound number	*Structure,[b] species[c]*	*Receptor binding[d]*	*Biological activity[d]* in vitro	in vivo	*Other studies*	*Ref.*
28	[Leu^{B24}], h	20—50 H	30 D, F	100 B	c.d., imm.	45, 73
		8—12 H	11 C		neg. co-op.	74
					c.d.	41
29*	[Leu^{B24}, Leu^{B25}], h	0.5 H	—	5 B	c.d.	45, 73
		0.4 H	0.6 C			75
30*	[Ala^{B24}, Thr^{B30}], b	5 H	4—10 D, F	10—30 B	c.d., imm.	45
31	Des(B24—30)				c.d.	38
32	[Leu^{B25}], h	1—2 H	2 D, F	10—20 B	c.d.	45
		1 H	3 C		neg. co-op.	74
					c.d.	41
33*	[Ala^{B25}, Thr^{B30}], b	10—15 H	15—25 D, F	50 B	c.d., imm.	45
34	Des(B25—30)			30 A	cryst.	76
35*	[Tyr(3-^{125}I)^{B26}], p	200 Ad				58
		115 Lc				58
36*	[Ala^{B26}, Thr^{B30}], b	100 H	100 D, F	90—120 B	c.d.	45
37	Des(B26—30), b				c.d.	38, 77, 78
38	B29-(4-Azidobenzoyl) [^{125}I]	+ covalent				79
39	B29-Napa		+		coval. bind.	53
39a	B29-Napa [^{125}I]	+ covalent				80
40*	[Leu^{B30}]	100 P	100 F	100 B	imm.	81
41*	[D-Leu^{B30}]	100 P	100 F			81
42*	[Leu-NH$_2$^{30}]	100 P	100 F			81
43*	[Phe^{B30}]	100 P				81
44	[Thr^{B30}], b	100 H	100 D, F	100 B		45
45	Des-Ala^{B30}	100 P	100 F			81
				100 B		82
46*	Ala^{B31}-Ala^{B32}, p	small decr. P	small decr. F			81
A- + B-Chain Modifications						
47*	Lys^{AO}-des(B26—30), b		7.8 C			78
48	A1,B29-(Ac)$_2$			+	gluc. met.	83
49	A1,B29-(Msc)$_2$				degrad.	84

50*	[Ala^{A1}]-des(B26—30), b		2 C			78
51*	[D-Ala^{A1}]-des(B26—30), b		6.5 C			78
52	Des-Gly^{A1}, des(B26—30), b		0.7 C			78
53	Des-Gly^{A1}, des-Phe^{B1}, b		0.8 D		14% E	49
					imm.	109
54	A1,B1-(Boc)$_2$-des(B23—30)	0.01		0.5 B		85
55*	A1,B1-(Boc)$_2$-des(B23—30)-NHNHPh, b				semisyn.	40
56	A1,B1,B29-(Boc)$_3$	0.01		5.6 B		85
57	Des-Asn^{A21}, des-Ala^{B30}		5 D		10% E	49
58	Des(B22—30) (OMe)$_5$	0.01		0.01		85
59*	Hexadesamido		69			86
60	Hexamethyl ester	0.01		0.01		85
Crosslinked Derivatives						
61	A1—B29-Adipoyl		6 D		98% E	49
62	A1—B29-A$_2$Sb, b				*X*-ray str.	88
63	A1—B29-Azoisobutyryl			+ B		87
64	A1—B29-(Boc)$_2$-A$_2$Sb				semisyn.	77

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Table (*cont.*)

Compound number	*Structure,[b] species[c]*	*Receptor binding[d]*	*Biological activity[d]* in vitro	in vivo	*Other studies*	*Ref.*
65	A1—B29-CBM				semisyn.	64, 77
					degrad.	84
66*	A1—B29-CBM, B22/23 split				semisyn.	77
			1 C		c.d.	89
67*	A1—B29-CBM, B25/26 split		1.6 C		c.d.	89
68	A1—B29-Dodecoyl				gluc. met.	83
					degrad.	84
69	A1—B29-Oxaloyl				gluc. met.	83
					degrad.	84
70	A1—B29-Sebacoyl				degrad.	84
71	B1—B1′-Suberoyl(insulin)$_2$	+			degrad.	90, 91
					imm.	109
72	B1—B29′-Suberoyl(insulin)$_2$	+			degrad.	90, 91
Heterocrosslinked Derivatives						
73*	B1-(Tetramethylrhodamine)$_{11}$-lactalbumin-^{14}C-adipoyl (A1,B29-bisguanylinsulin)$_{0.24}$		17 C			92
74*	B29-(Ricin-B-chain)-3-thiopropionyl	+	+		Aib transp.	93
Structure Not Determined						
75	4-Azidobenzoyl [^{125}I]	+ coval.				94
76	Ferritin/glutaraldehyde	+				95, 96

[a] New compounds are marked with an asterisk. [b] Abbreviations: A_2Bu = α,γ-diaminobutyric acid, A_2Sb = 2,7-diaminosuberoyl, Apa = 4-azidophenylacetyl, CBM = carbonylbismethionyl, Napa = 2-nitro-4-azidophenylacetyl, Nle = L-norleucine, Suc = succinoyl. [c] Species: b = bovine, h = human, p = porcine. [d] Potency or affinity based on insulin (b,p) = 100%. The following test systems are indicated: A = mouse convulsion assay, Ad = fat cells or membranes, B = blood glucose assay (*in vivo*), C = lipogenesis in isolated adipocytes, D = glucose oxidation in isolated adipocytes, E = [^{3}H]thymidine incorporation into cultured fibroblasts, F = 2-deoxyglucose or 3-*O*-methylglucose transport in adipocytes, H = binding to IM-9 lymphocytes, Lm/c = binding to liver cells or membranes, P = binding to human placenta membranes.

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Synthesis, Semisynthesis, and Chemical Modification. Human insulin can now be produced by recombinant DNA technology. Current synthesis is based on production of A- and B-chains in separate *E. coli* fermentations (see Goeddel *et al.*, *cf.* Vol. 12).[14, 16] The problem of chain combination has been overcome, and yields as high as 60% (based on B-chain, molar ratio A : B = 2.6 : 1) have been reported.[42] Synthesis of insulin by processing the products from an inserted proinsulin gene has also been reported.[43] As a further development of the single-chain procedure, products from a gene of a proinsulin analogue with a shortened connecting peptide have been investigated.[97]

Gattner *et al.*[63] have published their combination method in detail. As found previously in experiments with recombination of modified A- and B-chains, maximal yields (15%, ratio 1 : 1) are obtained with insulin, while analogous chains give often considerably reduced yields. [D-Phe[19]]A-chain and [Tyr[25]]B-chain did not recombine with natural partner chains to give insulin analogues at all. Chemical synthesis has yielded two new insulin analogues (6, 22) and a hybrid of insulin and IGF I (9) containing the A-chain extension equivalent to residues 63—70 of the growth factor. Des(26—30)[Tyr[24], Ala[25]]B-chain and five B-chains in which one to five amino-acids were omitted have been synthesized by Losse and co-workers.[98, 99] The combination products with natural A-chain have not yet been characterized. The specific removal and replacement of A1-glycine was accomplished either after direct reaction of insulin with phenylisothiocyanate and subsequent protection of the B-chain [55] (5) or *via* temporary protection of A1 [54, 78] (compounds 4, 50, 51).

Another insulin/IGF I hybrid (20) was prepared by subjecting A1,B29$(Msc)_2$-insulin to five cycles of Edman degradation and coupling the sequence Gly-Pro-Glu-Thr.

Chu has now described the chemical semisynthesis of compound 26 and several other analogues of des(B25—30)- and des(B26—30)-insulin in detail. They were prepared by coupling synthetic D-Ala-X-Y-OMe or D-Ala-X-Y-Z-OMe to A1,B1$(Boc)_2$-des(B22—30)insulin-pentamethylester or to related derivatives with substitutions in position B22. Biopotencies (*in vivo only*) have already been reported (*cf.* Vols. 8 and 12). Enzymatic semisynthesis is clearly the method of choice for introducing modifications in the region B23—30, but conditions have to be optimized in each case.[45, 100] For trypsin-catalysed coupling to Arg^{B22} the *N*-terminal residues of des(B23—30)insulin are blocked by Boc groups,[40, 45, 73, 75, 100] but in one case by Msc groups.[76] In addition to [Leu^{B24}]- and [Leu^{B25}]-insulin (see Vol. 13, also refs. 45 and 73 of this volume), compound 29 [73, 75] and Ala-substituted analogues (30, 33, 36) have been synthesized in organic aqueous media at pH 6.5. A rather high pH of 8 was reported as optimal for coupling Gly-Phe.[76] Phenylhydrazine was condensed with Boc_2-des(B23—

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[100] H.-G. Gattner, W. Danho, R. Knorr, V. K. Naithani, and H. Zahn in 'Peptides 1980', Proc. 16th European Peptide Symposium, Helsingor, Denmark, ed. K. Brunfeldt, The Danish Institute of Protein Chemistry, Copenhagen, 1981, p. 372.

30)insulin, and the B23—30 octapeptide was coupled after activation of the phenylhydrazone to a phenyldi-imide.[40]

A first example of intramolecular enzymatic synthesis with insulin has been reported.[77] The split Arg-Gly bond in compounds 66 and 64 is resealed by trypsin in 50—60% yield. Trypsin, carboxypeptidase Y,[101] and *Achromobacter lyticus* protease I[81, 102] have been used to couple threonine and other amino-acid esters (compounds 40—44 and 46) to des-Ala^{B30}-insulin. The replacement of Ala with threonine-t-butyl ester can now be performed in a single-step trypsin-catalysed transpeptidation.[46, 47] Analogous processes are already used to produce semisynthetic human insulin commercially.

Radioactive insulins have been obtained by incubation of rat islets with [^{3}H]Ile or [^{3}H]Phe.[52] The B1-labelled derivative 18 (spec. act. 1100 Ci mmol^{-1}) was prepared by semisynthetic replacement[64] of Phe in compound 65, the iodo-insulins 23 and 35 by careful fractionation[58] of iodination mixtures. Periodate oxidation of a compound with modified Arg, 25, gave the N^8N^9-adipoyl derivative 24. The crosslinked insulin 65 was split with trypsin to give 66; compound 67 was synthesized from B1-des(B26—30)insulin and a synthetic pentapeptide.[89]

Heterocrosslinking aimed at linking receptor groups (fluorescent, or heavy atoms) to insulin is still problematic. New experiments in this direction have been started and gave, among others, compound 73.[92] The reactivity of insulin groups towards 1-fluoro-2,4-dinitrobenzene has been studied.[103]

Structure, Receptor Binding, and Biological Activity. Further c.d. studies on the structure of insulin under a variety of solution conditions have been reported,[12, 37, 38, 39] and the quaternary structure of insulin has been discussed.[104] C.d. spectra of chemically synthesized[41] and semisynthetic[45] human insulin have been obtained, and c.d. spectra of the analogues 6,[56] 27,[38, 39] the series of B24/26 modified insulins (28—33, 36),[38, 41, 45] and the crosslinked derivatives 66 and 67[89] have been measured to deduce structure–function relationships. Information on the His residues[36] and on calcium binding[105] has been obtained by means of proton[36] and ^{113}Cd n.m.r. spectroscopy,[105] respectively. The *X*-ray structure of a crosslinked insulin 62 has been refined.[88] Computer graphics helped to explain the structural differences between the Leu-insulins 28 and 32[41] and were applied to construct a three-dimensional model of casiragua insulin.[50] The model provides an explanation for the low potency and inability to aggregate, for which the exchange of Tyr^{B26} by Arg is assumed to be important.

A detailed comparison between the properties of porcine and human insulin is of great practical bearing. The availability of bio- and semi-synthetic human insulin is now making large-scale and long-term clinical studies possible. So far, a large variety of *in vitro* and *in vivo* studies have shown biological equivalence of human and porcine insulins. The potential advantages in terms of immunogenicity and resorption are being investigated further.[16]

[101] K. Breddam, F. Widmer, and J. T. Johansen, *Carlsberg Res. Commun.*, 1981, **46**, 361.
[102] T. Oka, K. Morihara, S. Oghaku, M. Kobayashi, M. Iwasaki, and Y. Shigeta in 'Peptide Chemistry', ed. K. Okawa, Protein Research Foundation, Osaka, 1981.
[103] Y.-K. Chan, G. Oda, and H. Kaplan, *Biochem. J.*, 1981, **193**, 419.
[104] T. G. Vickers, *Trends Biochem. Sci.*, 1981, **6**, No. 5, v.
[105] J. L. Sudmeier, S. J. Bell, M. C. Storm, and M. F. Dunn, *Science*, 1981, **212**, 560.

A-Chain. Replacement of the branched Ile side chain in position A2 by the linear C_4 chain leads to an almost complete loss of receptor binding (6). This is presumably due to a distortion of the A2—8 α-helix.[56] Photoaffinity labelling has shown a specific binding of (3) to receptors, but the hormone–receptor complex appears to be metabolically inactive.[53] Increasing the bulk at the A1-amino group (5) or that of the side chain (4) has little effect on potency. Elongation at the *C* terminus by 8 amino-acids, although markedly reducing potency, indicates that the presence of a free A21-carboxy group is functionally not stringent.

B-Chain. Des-Phe^{B1}-insulin (19) is the first insulin analogue to be commercially available and is used mostly in combination with normal insulin. It may be advantageous in certain cases owing to its lower immunogenicity and antigenicity and better solubility. Covalent binding of the photo-insulin 21 has now been shown to stimulate glucose transport in adipocytes.[67] This seems to suggest a proximity of the *N* terminus to the receptor. However, avidin complexes of B1-biotinyl-insulin are still biologically active so that it seems unlikely that the *N* terminus of the B-chain is essentially involved in receptor interaction.[60, 61]

Replacement of the B1—5 sequence by the analogous IGF 1 tetrapeptide sequence (20) gave a derivative that is no more active in lowering blood sugar than des(B1—5)insulin. In the absence of c.d. data this may suggest a critical role for B5-histidine or a template role of the *N*-terminal sequence for the remaining structure.

The properties of compounds 24—26 are further evidence against a crucial role of Arg^{B22} for binding and potency. B16-Monoiodo-insulin (23) is a valid new tracer; iodotyrosine in position B26 (35) seems to enhance binding to adipocytes but not to hepatocytes.[58]

The occurrence of a Phe → Leu replacement in the insulin of a diabetic patient (*cf.* Vol. 13) has stimulated further studies. Human [Leu^{B24}]- and [Leu^{B25}]-insulins have now also been synthesized by Inouye *et al.*[45] Data from several groups show that the activity of the former (28) is moderate and that of the latter (32) is low; the activity of the [Leu^{B24}-Leu^{B25}] analogue (29) is exceptionally low. A similar trend was observed with respect to the ability to induce negative co-operativity.[74] Neither 28 nor 32 was confirmed to have an antagonistic effect on the lipogenic action of insulin.[74] C.d. spectra, supplemented by gel-filtration experiments, showed that conformational changes had occurred in the B24 analogue, while the B25 analogue was structurally very similar to insulin and was still able to dimerize. Computer graphics indicated that the Leu side chain could be accommodated when in position B25 but not in position B24. C.d. changes were similar with the [Ala^{B24}] (30) and [Ala^{B25}] (33) analogues, but a reverse order was found with bioactivity (*i.e.* 33 > 30). At present, it is not possible to explain these findings on the basis of the insulin model. As in previous cases, there are considerable differences between *in vitro* and *in vivo* data, indicating a different handling of the analogues in the organism. This makes conclusions based on such data alone questionable. The c.d. data of Lu[38] with des-octapeptide- (27) and des-pentapeptide-insulin (37) confirm earlier studies and show that the structure of des-heptapeptide-insulin (31) is intermediate. The c.d. spectrum of des-octapeptide-insulin (27) is not further disturbed by denaturing agents.[39] Changes in position B30 are without effect on binding and potency (40—45), although elongation causes a slight decrease (46).[81]

A-Chains, B-chains, and crosslinking. Alterations in A1 (compounds 47 and 50—52) have similar effects on the potency of des-pentapeptide-insulin (37) and insulin, but Gly^{A1} may be more important for stabilizing the native insulin structure than that of the already partially destabilized analogue 37.[78] Cleavage of the B25/26 bond or of the B22/23 bond in crosslinked insulins (62, 65) causes structural alterations comparable to those seen with des-pentapeptide- and des-octapeptide-insulins (37, 27). This proves that the crosslink is unable to maintain the penta- or the octa-peptide in its original positions.[89]

The refinement of the crystal structure of diaminosuberoyl-insulin (62) to 3 Å (*cf.* Vol. 13) shows that it has a strong structural similarity to porcine insulin, especially for the A-chain invariant residues and the dimer-forming residues of the B-chain. These findings have contributed to new ideas about the receptor-binding region of insulin (see below).

Structure–function relationships. Saunders[48] has considered the evidence brought forward to support the model of Blundell *et al.* in which the *C* terminus of the B-chain plays a dominant role in receptor binding. In the light of parallel changes in binding affinity and biological activity in des-octapeptide-insulin (27) it seems that little biologically relevant information has been lost. Using supplementary arguments from data on negative co-operativity, it was postulated that the exposed surfaces of the A-chain helices were equally attractive as binding surfaces for the receptor.

In discussing the strong similarities of the structures of native insulin to crosslinked A1,B29-diaminosuberoyl-insulin, Cutfield *et al.*[10] were drawn to the conclusions that A-chain structures, including backbone carbonyl and amide functions, should be more emphasized in considering the interaction of insulin with its receptor.

Receptor studies. Qualitative and quantitative differences with respect to the dependency of receptor binding on NaCl concentration and pH were observed between two dimers (71 and 72) and insulin.[90,91] Complex formation between biotinyl-insulin and several derivatives (10—15) has been studied in more detail[60,61] for receptor isolation. Photoaffinity labelling of receptors in H4 hepatoma cells with 75 defined a major subunit at 130 K.[94] The covalent Triton X100 solubility complex of the B29-photo-insulin (39a) receptor from rat-liver membranes was found in two forms with Stokes radii of 7.08 and 3.62 Å. These forms were interconvertible without the use of reducing conditions, a result that stands in contrast to the hypothesis of Czech.[106] The same photo-insulin (39a) was also shown to effect a 'permanent' stimulation of lipogenesis in viable adipocytes.[53] Another B29-photo-insulin (38) was used to demonstrate specific intracellular binding sites in several Golgi fractions of hepatocytes.[79] Internalization studies were carried out with the conjugates 76 and 74.

Biological studies. In contrast to insulin and derivative (48), which exert a hypoglycaemic effect (in dogs) entirely by stimulating peripheral glucose uptake, two crosslinked compounds (68 and 69) acted almost exclusively by decreasing hepatic glucose production. This may be an interesting lead in the development of new insulins for therapeutic purposes.

B1-Iodotyrosyl-insulin (18) was used to follow insulin degradation in isolated

[106] M. P. Czech, J. Massague, and P. F. Pilch, *Trends Biochem. Sci.*, 1981, **6**, 222.

hepatocytes.[64] The mechanism appeared to be qualitatively similar to degradation of A-chain-labelled insulin. A cleavage product of the B-chain could be detected, which was considered to be indicative of an early event in insulin breakdown. *In vitro* cleavage of insulin by pepsin was found to be strongly inhibited by crosslinking (62, 65, 68—70), but the effect was less pronounced towards chymotryptic cleavage.[84]

Derivatives of the pentapeptide Arg-Gly-Phe-Phe-Tyr (B22—26) at concentrations of at least 10^{-4} mol dm^{-3} have been reported to increase binding of ^{125}I-labelled insulin to adipocytes and to stimulate several actions of submaximal doses of insulin and des-octapeptide-insulin (27) in these cells.[107, 108]

A large number of natural insulins (beef, pork, horse, mouse, rat, sheep) and several derivatives (Dnp-, Ftc-, TNBS-insulins, insulin ester) have been used in immunization and antigenicity studies.[31] Hexa-desamido-insulin (59) did not give IgG antibodies but rather IgE (reagin-type) antibodies in rabbits and rats, and it may, if present at all, contribute to allergic reactions during treatment. [Leu^{B30}]-insulin (40) is very poorly immunoreactive but fully active and may thus have therapeutic merits.[81]

Anti-insulin antibodies bind avidly to insulin affinity columns and are often difficult or even impossible to elute. Several column-bound modified insulins {des(B1—3)[Pyr-Glu^{B4}]insulin, 53, the dimer 71, and a B29—29′ isomer} retained the antibodies less well and allowed their elution.[109]

In the light of strong sequence homologies between insulins and IGFs and a limited degree of cross-interaction with the respective receptors[110] the relative activities of several natural and semisynthetic insulin sequences in promoting glucose oxidation and thyridine incorporation have been examined.[49] Several examples of insulin displaying much higher relative growth-promoting activity than conventional glucose oxidation activity were found; no example of inverse relative activity was observed. Although it seems obvious that the growth-promoting activity is therefore less readily modified by natural or synthetic means, no clear picture emerges as to how this difference can be explained.

In an insulin IGF hybrid (20) containing the extended IGF A-chain sequence[66] the growth-promoting activity was double that of insulin whereas the metabolic activity was reduced to *ca.* 20%. In another hybrid (9) containing the *N*-terminal B-chain IGF tetrapeptide sequence[59] no improvement of the growth-promoting activity was observed, and the metabolic potency was reduced.

Glucagon.—Two proteins (14.5 K and 12.5 K) immunologically related to glucagon have been isolated from the products of synthesis of a wheatgerm cell-free system by the use of poly(A) RNA extracted from anglerfish islets.[111] They are produced from different mRNA sequences. One partial RNA sequence suggests that the glucagon sequence is flanked by Lys-Arg sequences, as so often in prohormones. Three products have been detected by another group (16 K and two of 14 K) and were shown to be processed differently by microsomal mem-

[107] K. Kikuchi and J. Larner, *Biochem. Biophys. Acta*, 1981, **670**, 117.
[108] K. Kikuchi and J. Larner, *Biochem. Biophys. Acta*, 1981, **670**, 190.
[109] K.-G. Petersen, K. Schlüter, A. Schüttler, and D. Brandenburg in ref. 31, p. 361.
[110] P. K. Lund, R. H. Goodman, and J. F. Habener, *J. Biol. Chem.*, 1981, **256**, 6515.
[111] D. Shields, T. G. Warren, S. E. Roth, and M. J. Brenner, *Nature (London)*, 1981, **289**, 511.

branes.[112] It is likely that the slight differences reported by different groups will be resolved by the use of standardized methods of analysis, and when the complete gene sequences become known. A preliminary report of the sequence of a 14.5 K precursor, deduced from a cloned gene sequence, has appeared.[113] After the 24-residue signal peptide, four sequence segments separated by Lys-Arg dibasic spacers are observed (Figure 1). The *C*-terminal 34-residue segment is homologous with other members of the glucagon family, particularly GIP; maybe the glucagon precursor is a polyprohormone.

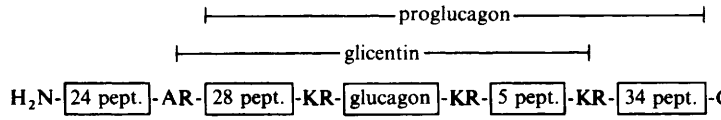


Figure 1 *Diagram of the primary structure of anglerfish islet preproglucagon*

The purification by ion-exchange chromatography of cyanogen bromide-cleaved glucagon has been described.[114] The analytical criteria have to be stringent as the biological activities of the derivatives that have been synthesized by coupling to the *C*-terminal homoserine lactone residue (*cf.* Vols. 10 and 12) are low, so that traces of contaminating uncleaved glucagon would lead to misleading results. *S*-Methyl glucagon is more immunogenic than glucagon and was used to raise high-affinity antibodies.[115] Several semisynthetic glucagon derivatives have been re-examined in an assay based on their abilities to stimulate glucose production by glycogenolysis in isolated hepatocytes.[116] As this system is much more biologically valid than the membrane adenylate cyclase assay, it is reassuring that nearly the same relative activities were observed in the two systems. The antagonistic effect of several semisynthetic partial agonists has been studied again.[117] As this effect is often greater than would be expected from the binding affinity, the authors are drawn to the conclusion that glucagon activates glycogenolysis *via* cAMP-dependent and cAMP-independent mechanisms; antagonists can only act as agonists *via* the latter mechanism (*cf.* Vol. 11).

A reduction of the glucagon-induced hyperglycaemia in streptozotocin-diabetic rats was activated by infusion of N^{α}-TNP-[homoArg^{12}]glucagon,[118] demonstrating a possible clinical use for such analogues.

Six glucagon analogues containing sequence deletions at positions 1—4, 5—9, 10—15, 16—21, 22—26, and 27—29 have been produced by conventional peptide

[112] B. N. Jones and R. S. Gurd, *Arch. Biochem. Biophys.*, 1981, **206**, 227.
[113] W. J. Nooijen and P. W. Koppert, *Ann. Clin. Biochem.*, 1981, **18**, 118.
[114] V. J. Hruby, N. S. Agarwal, A. Griffen, M. D. Bregman, C. A. Nugent, and K. Brendel, *Biochem. Biophys. Acta*, 1981, **674**, 383.
[115] M. D. Bregman, V. J. Hruby, and D. Trivedi in 'Peptides 1980', Proc. 16th European Peptide Symposium, Helsingor, Denmark, ed. K. Brunfeldt, The Danish Institute Protein Chemistry, Copenhagen, 1981, p. 488.
[116] V. J. Hruby, M. D. Bregman, D. Trivedi, D. G. Johnson, and C. Ulichny in 'Peptides: Synthesis–Structure–Function', Proc. 7th American Peptide Symposium, ed. D. H. Rich and E. Gross, 1981, p. 813.
[117] E. K. Frandsen, F. C. Gronvald, L. G. Heding, N. L. Johansen, B. F. Lundt, A. J. Moody, J. Markussen, and A. Volund, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 665.
[118] G. L. Johnson, V. I. MacAndrew, jun., and P. F. Pilch, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 875.

synthesis.[119] The synthetic strategy used step-wise chain elongations to produce fragments mainly protected by t-butyl-based groups. For selective amino-group liberation the Bpoc group was employed. Histidine-1 was protected with the *N*-Adoc group, and the arginine guanido function was incorporated as the hydrobromide. After deprotection the analogues were purified by ion exchange by the use of a volatile buffer system; yields were not reported. All of the analogues were capable of displacing ^{125}I-glucagon; the most potent was des(1—4)glucagon (5.7%), the least potent was des(22—26)glucagon (0.0006%!). Only des(27—29) glucagon had a measurable stimulatory activity in adenylate cyclase and lipolysis assays (*ca.* 0.2%), which correlated well with results from semisynthetic material. It was concluded that the receptor address and transduction sequence were separate, the former being a function of the *C*-terminal sequence and the latter a function of the *N*-terminal sequence.

The structure of the glucagon receptor has been studied by photoaffinity labelling.[120] Membranes treated with the *N*-hydroxysuccinimide ester of *p*-azidobenzoic acid were incubated with ^{125}I-glucagon and irradiated. After solubilization and reduction a 53 K-labelled component was observed. As this was readily reduced in intensity by competition with glucagon or treatment of the membranes with fluoride ion before irradiation (to reduce the affinity of the receptor), it was concluded to be a receptor component. It is thus uncertain whether the 23 K unit labelled with a biologically inactive photosensitive glucagon derivative[121] is a different receptor component or is unrelated to the receptor.

5 Vasoactive Peptides

By P. D. Roy

Bradykinin.—The relationship between the kallikrein–kinin system and pathophysiological conditions such as inflammation and hypertension awaits further characterization before drug development can be considered. A review article by Margolius points out some of the remaining problem areas and examines the pharmacological prospects in the system.[1] The need for more information on kinin receptors in particular has always been apparent, and accordingly this area has received considerable attention in recent investigations.

The results of extensive pharmacological studies on kinin receptors have been reported by the research group at the University of Sherbrooke, Canada.[2–5] The Table lists some of the bradykinin analogues described with accompanying biological data. In general these findings support the interpretation that kinins act

[119] M. D. Bregman and D. Levy. *Biochem. Biophys. Res. Commun.*, 1977, **78**, 584.
[120] J. F. Habener, P. K. Lund, J. W. Jacobs, P. C. Dee, and R. H. Goodman, in 'Peptides: Synthesis–Structure–Function', Proc. 7th American Peptide Symposium, ed. D. H. Rich and E. Gross, 1981, p. 457.
[121] E. R. Froesch, J. Zapf, E. Rinderknecht, B. Morell, E. Schoenle, and R. E. Humbell, 'Hormones and Cell Culture', Cold Spring Harbour Conference on Cell Proliferation, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, 1979, Vol. 6, p. 61.
[1] H. S. Margolius, *Trends Pharmacol. Sci.*, 1980, **1**, 293.
[2] P. Gaudreau, J. Barabé, S. St.-Pierre, and D. Regoli, *Can. J. Physiol. Pharmacol.*, 1981, **59**, 380.
[3] P. Gaudreau, J. Barabé, S. St.-Pierre, and D. Regoli, *Can. J. Physiol. Pharmacol.*, 1981, **59**, 371.
[4] E. Laczko and E. Escher, *Helv. Chim. Acta*, 1981, **64**, 621.
[5] E. Escher, E. Laczko, G. Guillemette, and D. Regoli, *J. Med. Chem.*, 1981, **24**, 1409.

Table *Analogues of bradykinin*

1 5 9

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Bradykinin

Compound number	*Structure*	*Biological activity*[a] A	B	C	D	E	*Ref.*
(1)	Bradykinin	100	100	100	100	8.5	
(2)	Des-Arg^9-bradykinin	0	0	0	0·16	100	
(3)	[Ala^5]	0·05	0·09	<0·01	0·16		
(4)	[Ala^8]	0	0	0.06	0		
(5)	[Leu^5]	12.0	17.0	0.7	2.0		
(6)	[Leu^8]	1.0	0.2	0.1	0.1		
(7)	[Trp^5]	0.7	0.7	1.4	2.1		
(8)	[Trp^8]	56	24	27	75		
(9)	[Tyr(Me)^5]	2.5	30.0	9.2	30.0		
(10)	[Tyr(Me)^8][b]	86	140	100	105	0.24	2, 3
(11)	[Cha^5]	24	49	18	37		
(12)	[Cha^8]	85	49	15	100		
(13)	Kallidin[c]	150	150	64	89	95	
(14)	Lys-Lys-(1—9)	30	78	58	14		
(15)	Lys-Lys-Lys-(1—9)	12	39	26	7	180	
(16)	(1—9)-Leu	4	0.3	24	1.3		
(17)	(1—9)-Lys	9.3	4.5	1.0	5.8		
(18)	(1—9)-Glu	0.05	0.10	0.43	0.01		
(19)	[Leu^8]-(1—8)	—*	—*	—*	—*	*	
(20)	[Phe(4-NO$_2$)^5]-(1—8)					16 92%[d]	
(21)	[Phe(4-NO$_2$)^8]-(1—8)					0 76%[d]	
(22)	[Phe(4-NH$_2$)^5]-(1—8)					1.2	
(23)	[Phe(4-NH$_2$)^8]-(1—8)					1.5	
(24)	[Phe(4-N$_3$)^5]-(1—8)					15 90%[d]	
(25)	[Phe(4-N$_3$)^8]-(1—8)					0.8 $\alpha^E = 0$ * 37%[d]	4, 5
(26)	[Phe(4-NO$_2$)^5]					7 88%[d]	
(27)	[Phe(4-NO$_2$)^8]					25 91%[d]	
(28)	[Phe(4-NH$_2$)^5]					3	
(29)	[Phe(4-NH$_2$)^8]					9	
(30)	[Phe(4-N$_3$)^5]					17 44%[d]	
(31)	[Phe(4-N$_3$)^8]					23 85%[d]	
(32)	[Phe(4-NH$_2$-3,5-I$_2$)^5]					<0.01	

[a] A = Guinea-pig anterior mesenteric vein, B = rabbit jugular vein, C = dog common carotid artery, D = cat terminal ileum, E = rabbit aorta. Values shown refer to relative affinity expressed as a percentage of bradykinin (100%) for assays A—D (B_2-receptor) or as a percentage of des-Arg^9-bradykinin (100%) for assay E (B_1-receptor) except where otherwise indicated. α^E = intrinsic activity, * = antagonist activity, —* = inactive as an antagonist. [b] Tyr(Me)^8 inhibits chemotaxis of rat polymorphonuclear cells (ref. 7). [c] Kallidin is lysyl-bradykinin. [d] Mean residual activity after treatment with photolabile analogue.

on two different receptor types, so-called B_1- and B_2-receptors. Specific and competitive antagonists, such as [Leu^8]-des-Arg^9-bradykinin (19), are known for B_1-receptors (assay E) whereas such compounds are not available for B_2-receptors (assays A—D). The area of kinin pharmacology as a whole has been extensively reviewed recently.[6]

Analogues (1)—(18) listed in the Table were synthesized by the solid-phase method and activities determined in vascular and intestinal smooth-muscle preparations containing a B_2-receptor type (rabbit jugular vein, guinea-pig anterior mesenteric vein, dog common carotid artery, and cat terminal ileum).[2] Where comparisons have been made, these activities differ significantly from those obtained in a B_1-receptor containing preparation [rabbit-aorta assay E; see compounds (1), (2), (10), and (19)].

One series of analogues was designed to examine the importance of the phenylalanine residues in bradykinin for interaction with the B_2-receptor. Monosubstitution in position 5 with aliphatic residues such as leucine (5) is more consistent with activity than the corresponding substitution in position 8 (6). The reverse situation applies to substitutions involving a large aromatic residue or one containing an aliphatic ring. In this case compounds substituted in position 8 are found to be more active [compare analogues (7) and (8), (9) and (10)]. One of these compounds, [Tyr(Me)^8]bradykinin (10), was 1.4 times more potent than bradykinin in the rabbit jugular-vein assay (B). These results point to the presence of a relatively larger B_2-receptor pocket for the Phe^8 residue of bradykinin.

The effect on activity of *N*- and *C*-terminal extension of bradykinin was also studied.

Kallidin (13) is considerably more potent than bradykinin in the two venous B_2-receptor preparations (assays A and B), suggesting perhaps that this is a target tissue for kallidin *in vivo*. *N*-Terminal addition of one or two further lysyl residues gave progressively less active analogues (14) and (15), respectively, indicating the decapeptide (kallidin) to be of optimum chain length. By contrast in the B_1-receptor preparation the latter *N*-trilysylbradykinin compound (15) is highly active, providing further evidence for differential bradykinin receptors. *C*-Terminal extension of bradykinin with leucine (16), lysine (17), and in particular with glutamic acid (18) leads to significant loss of activity in the B_2-receptor preparations.

Several photoaffinity-labelling analogues derived from either bradykinin or des-Arg^9-bradykinin have been synthesized[4] and evaluated[5] for purposes of kinin-receptor isolation [Table, analogues (20)—(32)]. This technique requires the development of compounds containing sufficient radioactivity capable of specifically and irreversibly binding to kinin receptors upon activation by ultraviolet light (365 nm). Labelled receptors may subsequently be isolated by biochemical techniques following solubilization of the cell membrane. In these studies, analogues were synthesized by the solid-phase method introducing 4-nitrophenylalanine into aromatic position 5 [analogues (20) and (26)] or 8 [analogues (21) and (27)]. Reduction of this series gives the corresponding 4-aminophenylalanine analogues (photostable series), which on diazotization and sodium azide treatment afforded the 4-azidophenylalanine analogues (24), (25), (30), and (31).

[6] D. Regoli and J. Barabé, *Pharmacol. Rev.*, 1980, **32**, 1.

Analogues were initially tested in the absence of u.v. light to obtain information on receptor-binding capacity. The assays used were either rabbit aorta (B_1) sensitive to the octapeptide series or rabbit jugular vein (B_2) highly sensitive to bradykinin nonapeptide analogues. Results from the octapeptide-B_1 system show analogues modified in position 5 to have greater affinity for the receptor than the corresponding position-8 analogues. However, the [Phe(4-N_3)^8] octapeptide (25) is a weak competitive antagonist of des-Arg^9-bradykinin and in subsequent photoaffinity-labelling tests significantly inactivated tissues. In the nonapeptide-B_2 system no antagonists were evident and position-8 analogues are more active when tested in the absence of u.v. light. Photolabelling tests here showed [Phe(4-N_3)^5]bradykinin (30) to be the best analogue in terms of receptor inactivation. On the basis of these findings the synthesis of radioactive photolabile kinin analogues starting with [Phe(4-NH_2-3,5-I_2)^5]bradykinin (32) is planned.

Bradykinin and [Tyr(Me)^8]bradykinin (10), potent stimulants of the B_2-receptor, both exert a direct inhibitory effect on rat polymorphonuclear chemotaxis.[7] In contrast, a B_1-receptor stimulant, des-Arg^9-bradykinin, was inactive in the same test. This indicates the presence of kinin-membrane receptors of the B_2 type in rat polymorphonuclear cells that mediate a chemotactic inhibition. Whether this represents a mechanism of kinin modulation of the inflammatory response is an interesting point raised in this study.

The synthesis of *cyclo*[Lys^1, Gly^6]bradykinin, in which the N^{ε}-amino group of Lys^1 is coupled to the *C*-terminal carboxy group, has been described in detail.[8] The cyclic analogue, like bradykinin, reduces arterial blood pressure in rat but differs in exerting no myotropic action *in vitro*. This appears to be the first reported case of differential activity between depressor and myotropic effects among bradykinin analogues. A further interesting feature in the activity profile is the longer duration of the depressor action (several hours compared to *ca.* 1 minute for bradykinin). The cyclic configuration presumably enhances stability to degradation enzymes (kininase II, carboxypeptidases) that require a free *C*-terminal carboxy group for activity. Collectively these features may provide grounds for the development of a selectively acting hypotensive agent with a prolonged duration of action.

The Met-Lys bond in the model kallikrein substrate Gly-Leu-Met-Lys-bradykinin is hydrolysed selectively by horse urinary and porcine pancreatic kallikreins. The methionyl methylthiol group and the lysyl ε-amino group are not essential for peptide-bond hydrolysis at this position. This is evident from kallikrein cleavage of the Met-Nle and Nle-Lys bonds in the substrates Gly-Leu-Met-Nle-bradykinin and Gly-Leu-Nle-Lys-bradykinin, respectively. On the other hand, introduction of a more polar methionine sulphoxide residue gives a substrate, Gly-Leu-Met-(*O*)-Lys-bradykinin, resistant to both kallikreins. The information in this study is consistent with kallikrein cleavage of the Leu-Lys bond in the natural sequence Ser-Met-Leu-Lys-bradykinin, recently identified in horse kininogen.[9]

[7] M. Roch-Arveiller, D. Regoli, and J.-P. Girond, *Agents Actions*, 1981, **11**, 499.
[8] G. I. Chipens, F. K. Mutulis, B. S. Katayev, V. E. Klusha, I. P. Misina, and N. V. Myshlyiakov, *Int. J. Pept. Protein Res.*, 1981, **18**, 302.
[9] M. S. Araujo-Viel, L. Juliano, and E. S. A. Prado, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 337.

Angiotensin.—Earlier studies of structure–activity relationships in the angiotensin series (>200 analogues) have identified residues involved in affinity for the receptor, such as Tyr^4 > His^6 > Arg^2, and those involved in the receptor-stimulation process, such as Phe^8. [The structure of angiotensin II is shown in (33).] Replacement of Phe^8 by an aliphatic residue such as Ala or Ile leads to

1 5 8

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

(33) Angiotensin II

inhibitory analogues. Further modification at strategic positions in the sequence with residues such as sarcosine (Sar) confers greater enzymatic stability. Hence [Sar^1, Ile^8]angiotensin II is an angiotensin inhibitor resistant to aminopeptidase activity and currently used for the diagnosis of renin–angiotensin-mediated hypertension. As is evident, considerable progress in this field has been made, and the search for new clinically useful angiotensin inhibitors with prolonged *in vivo* half-life devoid of residual pressor activity continues. At the same time it is noteworthy that the recently developed series of non-peptidic inhibitors of angiotensin-converting enzyme by Cushman, Ondetti, and co-workers [10] underlines the limitations of the peptidic series of angiotensin inhibitors.[11] As with peptide drugs in general, the latter lack oral activity (no orally active peptide drugs are available in the clinic today) and are relatively difficult and costly to synthesize. Hence in the design of future analogues the feasibility of developing economical, orally effective, and metabolically stable angiotensin inhibitors requires consideration.

The use of analogues containing alkylating groups has been further studied as an alternative approach to developing longer-acting angiotensin inhibitors.[12] Replacement of Phe^8 in [Ac-Asn^1, Val^5]angiotensin II by the potential alkylating moiety melphalan (Mel) or its methyl or benzyl esters gives analogues 34a, b, or c, respectively, which show antagonistic activity *in vitro*.

[Ac-Asn^1, Val^5, Mel-R^8]angiotensin II

(34) a; R = OH
b; R = OMe
c; R = OBzl

The best inhibitor of [Asn^1]angiotensin II-induced rat uterine contraction was [Ac-Asn^1, Val^5, Mel^8]angiotensin II (34a) with a pA_2 of 8.50. Antagonism was prolonged (2—3 hours) but reversible, presumably since the analogue did not alkylate the receptor. Moreover the inhibitory effect of (34a) was only transient *in vivo* (rat pressor), indicating rapid degradation of the inhibitor. Simultaneous modification to produce greater resistance to enzymatic degradation is required. An additional restriction in this approach is the requirement of a suitable receptor nucleophile in proper alignment with the alkylating group for reaction to occur.

Methylation of the α-carbon in Tyr^4 affords stability to α-chymotrypsin with retention of significant agonist activity (93% relative to angiotensin II in rat

[10] D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry*, 1977, **16**, 5484.
[11] P. S. Farmer in 'Drug Design', ed. E. J. Ariëns, Academic Press, New York, 1980, Vol. 10, p. 119.
[12] K. Hsieh and G. R. Marshall, *J. Med. Chem.*, 1981, **24**, 1304.

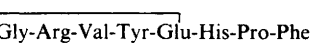
pressor assay). Conformational analysis (c.d. and n.m.r.) confirms that α-methylation does not lead to major changes in the overall solution conformation, so that receptor recognition and activity are retained. In addition to α-methylation in position 4 suitable modifications at the termini are probably necessary for resistance to attack by endopeptidases and exopeptidases.[13]

Further results from c.d. and n.m.r. studies of angiotensin II and selected analogues with well defined agonist and antagonist properties have established a clear correlation between conformation and biological activity. For example, *N*-methylation at either position 4 or 5 (in contrast to α-methylation) leads to profound conformational changes of Tyr^4 and His^5 side chains, with corresponding loss of activity. For maximum activity the conformation of the analogue should resemble at each pH that of the parent compound.[14]

Substitution of valine by γ,γ,γ,γ′,γ′,γ′-hexafluorovaline (Hfv) in position 5 of [Val^5]angiotensin II does not significantly alter agonist activity. In the rat-uterus assay [Hfv^5]angiotensin II has 133% activity whereas the corresponding [D-Hfv^5]compound was inactive. On the other hand, the position-8 analogue [Ac-Asn^1, Val^5, Hfv^8]angiotensin II is an effective inhibitor in the same assay (pA_2 value of 7.88). These analogues containing highly fluorinated residues in strategic positions within the angiotensin II sequence with respect to receptor interaction are potentially useful ^{19}F n.m.r. probes for studying conformational differences between angiotensin agonists and antagonists.[15]

The aza-α′-homovaline (aza-Hva) analogues [Asn^1, aza-Hva^3, Val^5]- and [Asn^1, aza-Hva^3, Val^5, Ile^8]-angiotensin II are potent antagonists in the rat colon ascendens *in vitro* (pA_2 9.2 and 9.7, respectively). The former compound is a partial agonist but the latter is reported to be essentially devoid of intrinsic activity.[16]

Attempts have been made to identify a receptor-active conformation of angiotensin II. Synthesis of the cyclic analogue (35) in which the side-chain carboxy group of Glu^5 is coupled to the *N*-terminal α-amino group is reported.

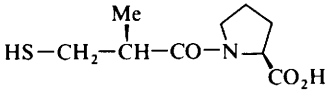


(35)

The pentafluorophenyl ester method was employed in the cyclization step. This was prepared to test the model postulating a biologically active conformation of angiotensin with closely located side chains in positions 1 and 5. The cyclic analogue, however, was found to exhibit considerably lower myotropic and pressor activity than angiotensin.[17] The numerous models proposed for the solution conformation of angiotensin II have recently been critically examined in the light of a specific hydrogen-bonded conformation.[18]

[13] M. C. Khosla, K. Stachowiak, R. R. Smeky, F. M. Bumpus, F. Piriou, K. Lintner, and S. Fermandjian, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 757.
[14] S. Fermandjian, F. Piriou, C. Sakarellos, K. Lintner, M. C. Khosla, R. S. Smeby, and F. M. Bumpus, *Biopolymers*, 1981, **20**, 1971.
[15] W. H. Vine, K. Hsieh, and G. R. Marshall, *J. Med. Chem.*, 1981, **24**, 1043.
[16] J. Ancans, N. A. Makarova, and G. Chipens, *Bioorg. Khim.*, 1981, **7**, 336.
[17] R. Vegners, G. Cipens, I. Grinsteine, N. V. Myshlyakova, G. A. Afanaséva, and I. A. Vosekalna, *Bioorg. Khim.*, 1981, **7**, 325.
[18] R. E. Lenkinski, R. L. Stephens, and N. R. Krishna, *Biochemistry*, 1981, **20**, 3122.

Potentiating Peptides and Enzyme Inhibitors.—Much of the current attention in this area focuses on angiotensin-converting enzyme inhibitors with either proven or potential clinical antihypertensive properties. In the recently published proceedings of the 1980 A. N. Richards Symposium on angiotensin-converting enzyme inhibitors [19] there is coverage by leading authorities on the mechanism of action and clinical implications of the antihypertensive drugs captopril (36) and teprotide (37).

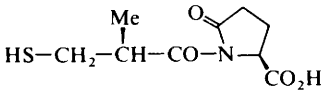


(36) Captopril

Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

(37) Teprotide

Teprotide (SQ 20881, also known as BPP9a) is a nonapeptide suitable for i.v. administration only and may be used for the diagnosis and initial treatment of selected hypertensive emergencies. Captopril (SQ 14225, 2-D-methyl-3-mercaptopropanoylproline) is orally active and has been used successfully for long-term therapy of hypertension and congestive cardiac failure. Gavras and co-workers have reviewed the status and clinical applications of these drugs.[20] In addition, updated reviews on the design and successful development of active-site-directed inhibitors of angiotensin-converting enzyme, with special reference to captopril, have been presented by Ondetti and Cushman.[21,22] The latter successful approach to antihypertensive drugs has somewhat expectedly prompted the synthesis of numerous possible alternative 'captopril-type' derivatives.[23-27] For example, the pyroglutamic acid analogue (38), incorporating the captopril side

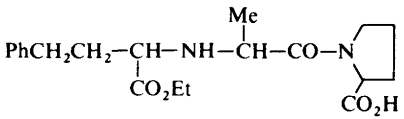


(38)

chain, is reported to be the most potent orally active inhibitor of angiotensin-converting enzyme from a large structure–activity series studied. Its *in vitro* activity was six-fold greater than that of captopril.[23]

[19] 'Angiotensin Converting Enzyme Inhibitors. Mechanisms of Action and Clinical Implications', ed. Z. P. Horovitz, Urban and Schwarzenberg, Baltimore, 1981.
[20] H. Gavras, F. Charocopos, H. Brunner, and I. Gavras, *Bull. N.Y. Acad. Med.*, 1981, **57**, 304.
[21] M. A. Ondetti and D. W. Cushman, *J. Med. Chem.*, 1981, **24**, 355.
[22] D. W. Cushman and M. A. Ondetti, *Trends Pharmacol. Sci.*, 1980, **1**, 260.
[23] K. Imaki, S. Sakuyama, T. Okada, M. Toda, M. Hayashi, T. Miyamoto, A. Kawasaki, and T. Okegawa, *Chem. Pharm. Bull.*, 1981, **29**, 2210.
[24] M. Oya, J. Matsumoto, H. Takashina, J.-I. Iwao, and Y. Funae, *Chem. Pharm. Bull.*, 1981, **29**, 63.
[25] M. Oya, I. Matsumoto, H. Takashina, T. Watanabe, and J.-I. Iwao, *Chem. Pharm. Bull.*, 1981, **29**, 940.
[26] R. D. Smith, A. D. Essenburg, R. B. Parker, V. L. Nemeth, M. J. Ryan, D. H. Dugan, and H. R. Kaplan, *J. Med. Chem.*, 1981, **24**, 104.
[27] R. F. Meyer, E. D. Nicolaides, F. J. Tinney, E. A. Lunney, A. Holmes, M. L. Hoefle, R. D. Smith, A. D. Essenburg, and H. R. Kaplan, *J. Med. Chem.*, 1981, **24**, 964.

Several types of side effects have been observed during therapy with captopril (rashes, transient loss or alteration of taste). The spectrum of side effects is reminiscent of the drug penicillamine; perhaps significantly both drugs contain a free mercapto function (—SH), which may account for these adverse effects. In this connection a powerful new class of angiotensin-converting enzyme inhibitors has been developed by Patchett and collaborators;[28] these inhibitors are structurally distinct from captopril in the absence of a mercapto function. From a series of over 200 variants clinical trials are being undertaken with the orally active compound MK 421 (39) to determine whether such inhibitors have advantages over captopril with regard to the incidence of side effects.[29]



(39) MK 421 (*S,S,S* configuration)

Another prominent non-sulphydryl-containing angiotensin-converting enzyme inhibitor appears to be an analogue of benzyl-Phe-Gly-Pro, in which the Phe-Gly amide bond is replaced by the enzyme-resistant ketomethylene group (—CO—CH_2—). It is evident from kinetic studies that this tripeptide analogue differs from captopril in its mode of inhibition of human converting enzyme. It is proposed that the tripeptide class of compound binds to hydrophobic sites of converting enzyme and does not require metal binding *via* a sulphydryl group.[30]

The inhibitory activity of two analogues of teprotide (**BPP9a**) in which one or more proline residues have been replaced by L-3,4-dehydroproline (△-Pro) has been compared with that of the parent compound. Whilst the analogues [△-Pro^9]-**BPP9a** and [△-$Pro^{3,9}$]**BPP9a** are at least as potent and effective antihypertensive agents as teprotide in rat, they represent fewer economic alternatives owing to the presence of the uncommon amino-acid △-Pro in the sequence.[31]

Tachykinins.—Research in this area relates mainly to naturally occurring kinins present in the amphibian skin and to the smooth-muscle action of substance P (40) and analogues.

1 5 10

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

(40) Substance P

The recently sequenced polypeptide sauvagine has been proposed as the prototype of a new family of amphibian-skin peptides, in addition to the

[28] A. A. Patchett, E. Harris, E. W. Tristram, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, J. ten Broeke, L. G. Payne, D. L. Ondeyka, E. D. Thorsett, W. J. Greenlee, N. S. Lohr, R. D. Hoffsommer, H. Joshua, W. V. Ruyle, J. W. Rothrock, S. D. Aster, A. L. Maycock, F. M. Robinson, and R. Hirschamm, *Nature (London)*, 1980, **288**, 280.

[29] G. A. MacGregor, N. D. Markandu, J. Bayliss, J. E. Roulston, M. Squires, and J. J. Morton, *Br. Med. J.*, 1981, **283**, 401.

[30] J. A. Weare, T. A. Stewart, J. T. Gafford, and E. G. Erdös, *Hypertension (Dallas)*, 1981, **3** (3, Pt. 2), 50.

[31] P. Hatzini Rolaou, H. Gavras, B. Waeber, G. H. Fisher, and J. W. Ryan, *Biochem. Pharmacol.*, 1981, **30**, 1869.

tachykinins (including substance P), bradykinin-like, caerulein-like, bombesin-like, and opiate-like peptides previously identified in the skin of the South American frog *Phyllomedusa*. The sequence comprises 40 amino-acid residues none of which is aromatic, though the occurrence of adjoining basic residues (-Arg^{15}-Lys^{16}-) in the central part of the molecule is consistent with the idea of enzymatic degradation of the parent precursor and generation of smaller active fragments. Synthetic studies are currently in progress to establish the proposed sequence and to determine the minimum chain length required for activity. This includes a prolonged hypotensive action in man and a number of animal species.[32]

As is often discovered in this area of study, hormonal peptides in man have counterparts in amphibian skin. The latest example in this category is an angiotensin-like peptide from the skin of the Australian frog *Crinia georgiana* identified as the undecapeptide Ala-Pro-Gly-[Ile^3, Val^5]angiotensin II. The latter differs from human angiotensin II in the presence of an extra *N*-terminal tripeptide, and in addition it is the first example of species variation in which the residues in positions 3 and 5 (Ile, Val) have been interchanged. Activity in the rat pressor assay was 90.6% of that of human angiotensin II.[33]

The smooth-muscle pharmacology of substance P has been reviewed, and a comparative study of the effects of the undecapeptide and its *C*-terminal fragments in vascular and intestinal preparations has been carried out. Results indicate that substance P inhibits arterial and stimulates venous smooth muscles by direct interaction with specific receptors in the vascular system. The hypotensive effect of substance P is thus probably mediated by an inhibitory action on arteriolar smooth muscle. The observed differences in the order of potencies of substance P and its fragments in the various pharmacological preparations used (arterial, venous, and intestinal smooth muscles) indicate the existence of different receptor types for substance P. To establish this point requires the development of specific and competitive inhibitors of substance P for the various assays employed.[34] Folkers and co-workers have examined a new series of substance P analogues for possible antagonist activity. They report the trisubstituted analogue [D-Pro^2, D-Phe^7, D-Trp^9]substance P to be devoid of agonist activity and to be an inhibitor of substance P both *in vitro* (guinea-pig ileum) and *in vivo* (rat).[35]

Substitution of Gly^9 in the *C*-terminal hexapeptide fragment of substance P by D- or L-Ala gives analogues [Glp^6, D-Ala^9]SP_{6-11} and [Glp^6, Ala^9]SP_{6-11}, respectively, with drastically reduced activities in guinea-pig ileum (0.2% and 0.4% compared to [Glp^6]SP_{6-11}).[36] However, the results of a separate structure–activity study on [Boc-Gln^1]substance P_{6-11} are somewhat contradictory and show that Gly^9 can be substituted without significant effect on activity by L-Ala or sarcosine but not by D-Ala or β-Ala.[37]

Replacement of the *C*-terminal amide group of substance P with a methyl ester gives an analogue with 110% of the activity of the parent compound in guinea-pig

[32] P. C. Montecucchi and A. Henschen, *Int. J. Pept. Protein Res.*, 1981, **18**, 113.
[33] M. C. Khosla, F. M. Bumpus, T. Yasuhara, and T. Nakajima, *J. Med. Chem.*, 1981, **24**, 885.
[34] R. Couture and D. Regoli, *Pharmacology*, 1982, **24**, 1.
[35] K. Folkers, J. Hörig, S. Rosell, and U. Björkroth, *Acta Physiol. Scand.*, 1981, **111**, 505.
[36] A. W. Lipkowski, S. Drabarek, T. Majewski, A. M. Konecka, and B. Sadowski, *Experientia*, 1981, **37**, 499.
[37] S. Blumberg and V. I. Teichberg, *Biochem. Biophys. Res. Commun.*, 1981, **99**, 752.

ileum. In contrast, the corresponding free-acid form of substance P only retains 0.9% activity.[38] The study of further substance P esters is required before the importance of a hydrophobic group at the *C*-terminus can be evaluated.

The degradation of substance P and *C*-terminal fragments by rat plasma enzymes has been examined. Both substance P and substance P_{4-11} octapeptide are inactivated rapidly by an endopeptidase, but interestingly this degradation was completely blocked by the angiotensin-converting enzyme inhibitor captopril. The action of the latter agent thus appears not to be as specific as was thought.[39]

Two other analogues of substance P that incorporate *N*-methylated amino-acid residues [Me-Phe^8, Me-Gly^9]substance P and [Glp^5, Me-Phe^8, Me-Gly^9]substance P_{5-11} are reported to be stable when incubated with substance P-degrading enzyme for prolonged periods.[40] These peptides were synthesized using the base-labile fluorenylmethoxycarbonyl (Fmoc) group in place of the Boc group for α-amino protection and the more polar polydimethylacrylamide-based polymer developed by the Sheppard group in Cambridge. Use of the latter Fmoc-polyamide procedure for the synthesis of substance P gives an overall yield of 47% undecapeptide amide.[41]

6 Enkephalins, Endorphins, and Related Peptides

By G. W. Hardy

Introduction.—1981 proved to be an exceptional year for the publication of structure–activity data on analogues of the enkephalins. Over 300 novel peptides were described, together with aspects of their biological activities; these data have been tabulated here as far as possible. Such a wealth of information has severely limited the space available for discussion of other areas of interest. Consequently, only the more significant events are highlighted, but a comprehensive list of references is provided. The sources of references were 'Current Contents, Life Sciences' (Institute for Scientific Information, Philadelphia, U.S.A.) and a 'Medlars II' profile (National Library of Medicine, Bethesda, U.S.A.).

The role of the endorphins in neuropsychiatry,[1] the pathology of headaches,[2] memory and learning,[3] neurotransmission,[4] and their interaction with opiate receptors[5] have been reviewed. Morley[6] has discussed the endocrinology of the opioid peptides. A volume[7] devoted to β-endorphin includes a chapter on structure–activity relationships. A general review[8] of the enkephalins has ap-

[38] M. A. Cascieri, M. M. Goldenberg, and T. Liang, *Mol. Pharmacol.*, 1981, **20**, 457.
[39] R. Couture and D. Regoli, *Can. J. Physiol. Pharmacol.*, 1981, **59**, 621.
[40] B. E. B. Sandberg, C.-M. Lee, M. R. Hanley, and L. L. Iverson, *Eur. J. Biochem.*, 1981, **114**, 329.
[41] E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
[1] 'Modern Problems of Pharmacopsychiatry', ed. H. M. Emrich, S. Karger A. G., Basel, 1981, Vol. 17.
[2] 'Advances in Neurology', ed. M. Critchley, S. Gorini, A. P. Friedman, and F. Sicuteri, Raven Press, New York, 1981, Vol. 33, p. 59.
[3] 'Endogenous Peptides and Learning and Memory, Behavioural Biology', ed. J. L. Martinez, R. A. Jensen, R. B. Messing, H. Rigter, and J. L. McGough, Academic Press, New York, 1981.
[4] R. J. Miller, *Pharmacol. Ther.*, 1981, **12**, 73.
[5] J. R. Smith and E. J. Simon in 'Pathobiology Annual, 1981', ed. H. L. Ioachim, Raven Press, New York, 1982, p. 87.
[6] J. E. Morley, *Metabolism*, 1981, **30**, 195.
[7] 'Hormonal Peptides and Proteins', ed. C. H. Li, Academic Press, New York, 1981, Vol. 10 (β-Endorphin).
[8] A. M. Konecka, *Postepy Biochem.*, 1980, **26(3)**, 443.

peared; others deal with their biosynthesis [9] and degradation.[10] Structure–activity relationships of enkephalin analogues were a major topic at the 7th American peptide symposium.[11] A review [12] of analgesic agents includes some information on opioid peptides.

New Opioid Peptides and Biosynthesis.—The complete structure of dynorphin (1—17), YGGFLRRIRPKLKWDNQ, was reported independently by two groups.[13, 14] Dynorphin (1—8) was found to be the major form in rat pituitary,[15] whereas dynorphin immunoreactivity in rat adenohypophysis was associated with a 6000 dalton protein.[16] The complete sequence of α-neoendorphin, YGGFLRKYPK, was determined [17] and a related peptide, β-neoendorphin, found to lack only the Lys [10] residue of α-neoendorphin.[18] Horse pituitary β-endorphin has been isolated and sequenced.[19] Further work with high-molecular-weight precursors revealed high potency,[20, 21] and molecular cloning provided structural information confirming the presence of multiple enkephalin sequences flanked by pairs of basic residues.[22–24] Several groups have identified inactive, *N*-acetylated opioid peptides from brain.[25–27] Active opioid peptides have been characterized in human pheochromocytomas,[28, 29] placenta,[30] and bovine adrenals.[31, 32] Unsworth *et al.*[33] have isolated the *O*-sulphate of [Leu ^{5}]enkephalin

[9] J. Rossier *Trends Neurochem. Sci.*, 1981, 94.
[10] J.-C. Schwartz, B. Malfroy, and S. De La Baume, *Life Sci.*, 1981, **29**, 1715.
[11] 'Peptides: Synthesis–Structure–Function', Proceedings of the 7th American Peptide Symposium, ed. D. H. Rich and E. Gross, Pierce Chemical Company, Rockford, Il, U.S.A., 1981.
[12] P. D. Gesellchen and D. M. Zimmerman, *Annu. Rep. Med. Chem.*, 1981, **16**, 41.
[13] A. M. Goldstein, W. Fischli, L. I. Lowney, M. Hunkapiller, and L. Hood, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 7219.
[14] S. Tachibana, K. Araki, S. Ohya, and S. Yoshida, *Nature (London)*, 1982, **295**, 339.
[15] B. R. Seizinger, V. Hoellt, and A. Herz, *Biochem. Biophys. Res. Commun.*, 1981, **102(1)**, 197.
[16] B. R. Seizinger, V. Hoellt, and A. Herz, *Biochem. Biophys. Res. Commun.*, 1981, **103(1)**, 256.
[17] K. Kangawa, N. Minamino, N. Chino, S. Sakakibara, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1981, **99(3)**, 871.
[18] N. Minamino, K. Kangawa, N. Chino, S. Sakakibara, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 1981, **99(3)**, 864.
[19] C. H. Li, T. B. Ng, D. Yamashiro, D. Chung, R. G. Hammonds, jun., and L.-F. Tseng, *Int. J. Pept. Protein Res.*, 1981, **18**, 242.
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from mouse brain. The mechanism by which kyotorphin releases enkephalins *in vivo* was investigated.[34]

Degradation of Opioid Peptides.—Metal-dependent aminopeptidases that degrade enkephalins have been purified from rat-brain preparations[35,36] and from human plasma.[37,38] A further study of the subcellular distribution of the dipeptidyl carboxypeptidase 'enkephalinase' supports the hypothesis that it inactivates enkephalin neurotransmitters.[39] However, other groups have questioned the specificity of this enzyme, drawing parallels with a similar ubiquitous metallo-endopeptidase isolated from kidney[40-43] and pituitary.[44] A multi-catalytic protease complex that will release enkephalins from precursor molecules was isolated from bovine pituitaries.[45] Enkephalins extended at the *C*-terminus with basic residues are efficiently converted to the native opioids by a novel carboxypeptidase isolated from chromaffin granules.[46]

Fournie-Zaluski *et al.*[47] described the synthesis of tritiated thiorphan and analogues modified to act as photoaffinity labels for enkephalinase. Thiorphan stabilizes [Met^5]enkephalin released from rat striatum by depolarization.[48] Three other groups have reported novel potent inhibitors of enkephalinase.[49-51] Wagner and Dixon[52] have demonstrated inhibition of rat-brain enkephalin aminopeptidase by inhibitors of microbial origin.

Conformational Studies.—The conformations adopted by the enkephalins and analogues have been investigated by c.d. measurements,[53] fluorescence spectroscopy,[54] calculation,[55,56] and n.m.r. spectroscopy using ^{1}H,[57-59] ^{15}N,[60] and

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^2H[61] nuclei, the latter in the solid state. In addition, combinations of n.m.r. and fluorescence spectroscopy with calculation were employed.[62,63] Generally a family of allowed conformations was observed; in some cases attempts were made to relate these observations to biological activity.[53,57,62] Calculation of favourable enkephalin conformations and comparison with morphine led to proposals for the conformational requirements of the μ-receptor.[64] Maroun and Mattice have studied the conformation of dynorphin (1—13) in solution.[65]

Receptors.—The occurrence of opiate receptors in intestinal tissue has been reviewed by Oka,[66] and a mini-symposium surveyed the evidence for multiple opiate receptors.[67-70] Perhaps the most significant development of the year came from investigations with the irreversible receptor antagonist naloxazone. It was suggested that the sites of highest affinity ($Kd < 1$ nm) blocked by the antagonist mediate analgesia induced by opioid peptides as well as by morphine and that lower affinity sites are responsible for other activities.[71-73] Specific receptors have been claimed for truncated sequences of dynorphin[74,75] and for β-endorphin.[76,77] Morphiceptin,[78] a tetrapeptide derived from β-casomorphin, is a highly selective μ-agonist but also shows evidence of δ-antagonism in rat vas deferens.[79] Wüster *et al.*[80] investigated the interactions of various opioid peptides with different receptors.

Structure–Activity Studies.—*Enkephalin.* The majority of the new enkephalin analogues reported during 1981 appear in the Table, together with their biological activities on the guinea-pig ileum (GPI) and mouse vas deferens (MVD), in receptor-binding assays, and in various anti-nociceptive tests. The wide variety of assay techniques applied makes direct comparison of results from different

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Table *Structures and biological activities of enkephalin analogues*

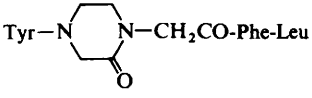
No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
1	Tyr-D-A_2bu-Gly-Phe-Leu (cyclic)	17.5[a]		2[a]		81
2	Tyr-A_2bu-Gly-Phe-Leu (cyclic)	low		low		81
3	Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 (cyclic)	73[b]		1.63[c]		82
4	Tyr-D-Cys-Gly-Phe-Cys-NH_2 (cyclic)	38[b]		0.83[c]		82
5	Tyr-Gly-Gly-Phe-Leu-Lys-Lys (cyclic)	No effect at 2 μM		4.2,[d] 2.7[e] 7.1,[f] 2.1[g]		83
6	Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl	~8.5[a]				84
7	Tyr-Sar-Gly-Phe-Leu	0.05[a]	0.006[e]		0.38[h]	85
8	Tyr-N(CH_2CO_2H)CH_2CO-Gly-Phe-Leu	0.16[a]	0.25[a]		1.18[h]	85
9		<0.003[a]	<0.003[a]		0.55[h]	85
10	Pr^i-Tyr-Gly-Gly-Phe-Met	0.013[b]	0.058[b]	1100,[i] 21[j]		86
11	[$PhCH_2CH_2$-Tyr^1]-10	0.15[b]	0.01[b]	35,[i] 15.6[j]		86
12	[Cyclopropylmethyl-Tyr^1]-10	0.014[b]	0.0025[b]	460,[i] 206[j]		86
13	[$MeTyr^1$]-10	1.45[b]	0.23[b]			86
14	[$EtTyr^1$]-10	0.04[b]	0.0086[b]	50,[i] 22.4[j]		86
15	[Pr^nTyr^1]-10	0.045[b]	0.0081[b]	56,[i] 25[j]		86
16	[Bu^nTyr^1]-10	0.20[b]	0.0078[b]	58,[i] 26[j]		86
17	[n-C_5H_{11}-Tyr^1]-10	0.67[b]	0.011[b]	26,[i] 11.6[j]		86
18	[n-C_6H_{13}-Tyr^1]-10	0.16[b]	0.027[b]	10,[i] 4.5[j]		86

Table (*cont.*)

No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
19	[n-C_7H_{15}-Tyr^1]-10	0.11[b]	0.0064[b]	36,[i] 16.1[j]		86
20	[n-C_8H_{17}-Tyr^1]-10	0.08[b]	0.0078[b]	45,[i] 20[j]		86
21	Tyr-D-Ala-Phe-Met	0.02[b]				87, 88
22	[Gly^2]-21	0.004[b]				87, 88
23	[Gly^2, Met^4-NH_2]-21	0.02[b]				87, 88
24	[Met^4-NH_2]-21	1.2[b]			~0.2[k]	87, 88
25	[Met^4-OMe]-21	0.4[b]				87, 88
26	[Metol^4]-21	0.5[b]				87, 88
27	[Met(O)ol^4]-21	0.2[b]				87, 88
28	[Met^4-NHEt]-21	0.2[b]				87, 88
29	[Met^4-NHCH$_2$CH$_2$OH]-21	0.1[b]				87, 88
30	[Leu^4-NH_2]-21	1.2[b]				87, 88
31	[Ac-Tyr^1, Met^4-NH_2]-21	0.04[b]				87, 88
32	[△-Phe^3, Met^4-NH_2]-21	0.004[b]				87, 88
33	Tyr-D-Ala-Gly-△-Phe-Met-NH_2	7[b]				87, 88
34	Tyr-Gly-Gly-Pheol	0.013[b]				89, 90, 91
35	[D-Ala^2]-34	0.94[b]			~0.1[l]	89, 90, 91
36	[D-Met(O)^2]-34	2.13[b]			1.4,[l] 0.9[m]	89, 90, 91
37	[MeTyr^1, D-Met(O)^2]-34	0.69[b]			1.2,[l] 1.5[m]	89, 90, 91
38	[D-Met(O)^2, MePheol^4]-34	14 560[b]			9.1,[l] 6.6[m]	89, 90, 91
39	[MeTyr^1, D-Met(O)^2, MePheol^4]-34	17 125[b]			7.1,[l] 6.4[m]	89, 90, 91
40	[D-Met(O)^2, Pheol^4-Ac]-34	3.7[b]			0.5,[l] 0.7[m]	89, 90, 91
41	[Tyr(Ac)^1, D-Met(O)^2, Pheol^4-Ac]-34	0.5[b]			0.3,[l] 0.5[m]	89, 90, 91
42	Tyr-D-Met(O)-Gly-NHCH$_2$CH$_2$Ph	8.51[n]			0.09[m]	92, 93
43	Tyr-D-Met(O)-Gly-N(Me)CH$_2$CH$_2$Ph	11.24[n]			1.14,[l] 1.13[m]	92, 93
44	Tyr-△-Ala-Gly-Phe-Leu			0.68,[o] 1.25[p]		94
45	Tyr-D-Ala-△-Ala-Phe-Leu			0.42,[o] 0.29[p]		94
46	Tyr-D-Ala-Gly-△-Phe-Leu			1.00,[o] 1.19[p]		94
47	Tyr-D-Ala-Gly-Phe-△-Leu			0.76,[o] 0.87[p]		94
48	Tyr-△-Ala-Gly-Phe-OMe			0.01,[o] 0.10[p]		94

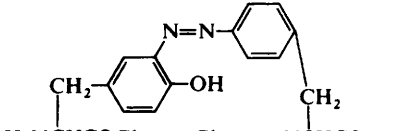
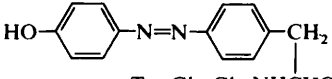
49	Tyr-D-Ala-Gly-Phe-Ser(CH_2Ph)	1.38[n]	15 700[n]			95
50	Tyr-D-Ala-Gly-Phe-Cys(CH_2Ph)	1.55[n]	2 500[n]			95
51	Tyr-D-Ala-Gly-MePhe-$NHCH_2CH_2OH$	6.5[q]	8.15,[q] 0.3[b]	95.9,[r] 0.89[s]	14,[m] 0.57[t]	96, 97
52	Tyr-D-Ala-Gly-MePhe-$NHCH_2CH_2NMe_2$	9.34[q]	5.88,[q] 0.36[b]	113.6,[r] 2.89[s]	2.3,[m] 1.18[t]	96, 97
53	Tyr-D-Ala-Gly-MePhe-$NHCH_2CH_2N(O)Me_2$	12.5[q]	10.6,[q] 0.76[b]	120.0,[r] 1.29[s]	70—87, 500,[m] 5.2[s]	96, 97
54	Tyr-D-Ala-Gly-$NHCH(Me)CH_2CHMe_2$	0.91[b]	94[b]		1—3[u]	98
55	Tyr-D-Met-Gly-$NHCH(Me)CH_2CHMe_2$	0.5[b]	84.6[b]		~2—3[u]	98
56	Tyr-Gly-Gly-Phe-Met-Thr	1.9[b]	0.47[b]		>0.1[u]	98
57	Tyr-D-Ala-Gly-Phe-Met-Thr	1.2[b]	0.084[b]		2—4[u]	98
58	Tyr-D-Ser-Gly-Phe-Leu-Thr	1.8[b]	0.045[b]		2—4[u]	98
59	Tyr-Gly-Gly-Phe(4-NH_2)-Leu				~0.9[u]	99
60					~1[u]	99
61					Inactive[u]	99
62	Tyr-$N(CH_2CO\text{-}Gly\text{-}Phe\text{-}Leu)_2$	<0.003[a]	0.08[a]		0.4[h]	100
63	Tyr-D-Ala-Gly-Phe-MeMet-NH_2		1[w]		1[x]	101
64	[D-Alg^2]-63[v]		2.1[w]		0.3[x]	101
65	[L-Alg^2]-63[v]		0.04[w]		<0.04[x]	101
66	[D-Ser^2]-63		2.4[w]		0.2[x]	101

Table (*cont.*)

No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
67	[D-Thr^2]-63		10.1^w		1.2^x	101
68	[D-Met^2]-63				0.18^x	101
69	[β-Ala^3]-63		$<10^{-3w}$		$<10^{-2x}$	101
70	[γ-Abu^3]-63^v		$<10^{-3w}$		$<10^{-2x}$	101
71	[δ-Ape^3]-63^v		$<10^{-3w}$		$<10^{-2x}$	101
72	[ε-Ahx^3]-63^v		$<10^{-3w}$		$<10^{-2x}$	101
73	[Phe(4-F)^4]-63		15.8^w		16.5^x	101
74	[Phe(4-Cl)^4]-63		5.6^w		0.54^x	101
75	[D-Phe(4-Cl)^4]-63		0.07^w		$<10^{-2x}$	101
76	[Phe(4-Br)^4]-63		5.6^w		0.23^x	101
77	[Phe(4-I)^4]-63		0.94^w		$<10^{-2x}$	101
78	[Phe(4-CF_3)^4]-63		8.1^w		0.84^w	101
79	[Phe(4-NO_2)^4]-63		19.1^w		2.3^x	101
80	[Tyr^4]-63		0.03^w		$<0.04^x$	101
81	[β-MePhe^4]-63		0.39^w		0.26^x	101
82	[D-β-MePhe^4]-63		0.008^w			101
83	[Cha^4]-63		0.03^w		0.007^x	101
84	[HomoPhe^4]-63		$<10^{-2w}$		$<10^{-2x}$	101
85	(Tyr-D-Ala-Gly-Phe-MeMet-$NHCH_2CH_2$)$_2$		17.2^w		0.04^x	101
86	Tyr-D-Ala-Gly-Phe-Phg-NH_2^{v}		10.2^w		0.91^x	101
87	[Phe^5]-86		0.16^w		0.04^x	101
88	[MePhe^4]-86		0.49^w		2.42^x	101
89	[EtPhe^4]-86		2.1^w		5.7^x	101
90	[Pr^{n}Phe^4]-86		1.94^w		2.5^x	101
91	[MeTyr^1, MePhe^4]-86		1.0^w		1.73^x	101
92	[MeTyr^1, EtPhe^4]-86		6.4^w		72.6^x	101
93	[Phe(4-F)^4]-86		64.2^w		5.76^x	101
94	[D-Phe(4-F)^4]-86		0.07^w			101
95	[D-Phe^5]-86				0.02^x	101
96	[D-Phg^5]-86		2.84^w		1.56^x	101
97	[MePhe^4, D-Phg^5]-86		0.58^w		0.095^x	101

98	[MeTyr^1, MePhe^4, D-Phg^5]-86	0.49[w]	0.72[x]	101
99	[Phe(4-F)^4, D-Phg^5]-86	22.6[w]	3.52[x]	101
100	[D-Tyr^5]-86		<0.04[x]	101
101	[D-Phg(4-OH)^5]-86		0.36[x]	101
102	[Phg(4-OMe)^5]-86	4.5[w]	0.12[x]	101
103	[Phe(4-F)^4, Phg(4-OMe)^5]-86	27.1[w]	0.36[x]	101
104	Tyr-D-Ala-Gly-Phe-HyM[v]-NH_2	0.21[w]	0.09[x]	101
105	[HyL^5][v]-104	0.39[w]	0.08[x]	101
106	[HyF^4][v]-104		<0.012[x]	101
107	[HyF^5][v]-104	0.045[w]	0.08[x]	101
108	[HyF^4]-63	0.07[w]	0.009[x]	101
109	[HyG^3]-63	10^{-3} [w]	<0.012[x]	101
110—122	Tyr-D-Ala-Gly-Phe-NH_2 | R			
110	R = H	0.064[w]	0.43,[x] >30[y]	102
111	R = Me	0.10[w]	0.77[x]	102
112	R = Et	5.81[w]	121,[x] 26[y]	102
113	R = Pr^n	1.11[w]	2.4[x]	102
114	R = $C_8H_{17}{}^n$	0.46[w]	0.03[x]	102
115	R = —$CH_2CH{=}CH_2$	1.44[w]	36.3[x]	102
116	R = —CH_2— 	7.53[w]	30.25[x]	102
117	R = —$CH_2C{\equiv}CH$	2.3[w]	4.54[x]	102
118	R = —$CH_2CH{=}CMe_2$	1.89[w]	0.91[x]	102
119	R = —CH_2CH_2SMe	2.87[w]	7.3[x]	102
120	R = —CH_2CO_2Me	0.06[w]	0.26[x]	102
121	R = —CH_2CH_2OH	0.24[w]	1.1[x]	102
122	R = —CH_2CH_2F	3.2[w]	27.9[x]	102
123	MeTyr-D-Ala-Gly-EtPhe-NH_2	0.72[w]	17.3,[x] 4[y]	102
124	Me_2Tyr-D-Ala-Gly-EtPhe-NH_2	0.04[x]	0.21[x]	102
125	EtTyr-D-Ala-Gly-EtPhe-NH_2	0.14[w]	0.25,[x] 145[y]	102
126	Tyr-D-Ala-Gly-HyF[v]-NH_2	0.04[w]	<0.01,[x] ≫30[y]	102
127	MeTyr-D-Ala-Gly-EtPhe(4-F)-NH_2	6.5[w]	30.25,[x] 3.55[y]	102
128	Tyr-D-Ala-Gly-EtPhe(4-F)-NH_2	5.1[w]	20.21,[x] 19[y]	102

Table (*cont.*)

No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
129	Tyr-D-Ala-Gly-Phe-D-Leu				1[z]	51
130	[MeTyr^1]-129				7.55[z]	51
131	[Phe(4-NO_2)^1]-129				0.66[z]	51
132	[Tyr(3-OH)^1]-129				0.64[z]	51
133	[Me_2Tyr^1]-129				0.53[z]	51
134	[D-Tyr^1]-129				<0.013[z]	51
135	[D-Met^2]-129				7.55[z]	51
136	[α-MeAla^2]-129				0.52[z]	51
137	[D-Leu^2]-129				0.18[z]	51
138	[D-Trp^2]-129				<0.013[z]	51
139	[Phe(4-NO_2)^4]-129				27.7[z]	51
140	[Phe(4-Cl)^4]-129				7.55[z]	51
141	[Tyr(Me)^4]-129				1.05[z]	51
142	[MePhe^4]-129				0.97[z]	51
143	[Phe(4-NH_2)]-129				0.10[z]	51
144	[Pro^5]-129				10.4,[z] >66[aa]	51
145	[*cyclo*Leu^5]-129				6.4[z]	51
146	[D-Leuol^5]-129				4.6[z]	51
147	[D-Met^5]-129				1.04[z]	51
148	[Thr^5]-129				1.0[z]	51
149	Tyr-D-Met-Gly-Phe(4-NO_2)-Pro				8.3,[z] >66[aa]	51
150	[Pro^5-NH_2]-149	106[b]	1.6[b]		83,[z] 2.4[aa]	51
151	[*cyclo*Leu^5-NH_2]-149				83,[z] >66[aa]	51
152	[*cyclo*Leu^5-NHEt]-149				21,[z] >66[aa]	51
153	[D-Met^5-NHEt]-149				0.13,[z] >66[aa]	51
154	[D-Met^5-NH_2]-149				0.13,[z] >66[aa]	51
155	[D-Leu^5-NHEt]-149				0.11,[z] >66[aa]	51
156	[Pip^5-NH_2][v]-149				83,[z] 56.5[aa]	51
157	[MeTyr^1, Pro^5-NH_2]-149	70.6[b]	1.77[b]		4.1[aa]	51

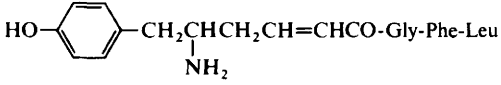
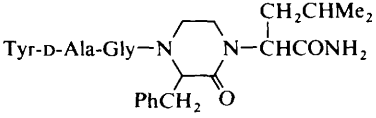
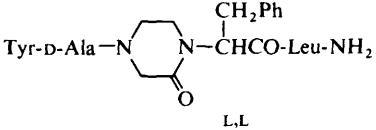
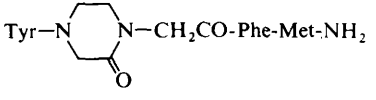
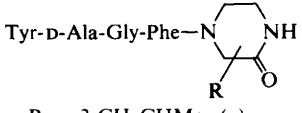
158	[Prool^5]-149	38.9[b]	1.22[b]		4.9[aa]	51
159	[BishomoProol^5]-149	59.6[b]	0.29[b]		14.9[aa]	51
160	Descarboxy-149	477[b]	1.81[b]		49.9[aa]	51
161	Tyr-D-Ala-Gly-Phe(4-NO_2)-Leu	8.69[b]				103
162	[Leu^5-NH_2]-161	18.3[b]				103
163	[Met^5-NH_2]-161	24.8[b]				103
164	[Ada^5-NH_2]-161	15.0[b]				103
165	[Neo^5-NH_2]-161	30.3[b]				103
166	[Car^4, Leu^5-NH_2]-161	1.9[bb]		1.55[bb, cc]		104
167	[Car^4, Met^5-NH_2]-161	3.2[bb]		5.8[bb, cc]		104
168	[Car^4, Leu^5]-161	1.0[bb]		0.7[bb, cc]		104
169	[D-Ada^4, Leu^5]-161	Inactive		Inactive		105
170	[Ada^4, Leu^5-NH_2]-161	Inactive		Inactive		105
171	[Ada^4, Ada^5-NH_2]-161	Inactive		Inactive		105
172		0.094[b]				107
173	TyrNHCH$_2$CHSCH$_2$CO-Phe-Leu					
	Isomer I	Weak antagonist				108
	Isomer II	0.1[a]				108
174–179	[Tyr-D-Ala-Gly-Phe-Leu-NH$(CH_2)_n$]$_2$					
174	n = 1			1.78,[dd] 0.27[ee]		109
175	n = 2			2.28,[dd] 0.33[ee]		109
176	n = 3			2.61,[dd] 0.43[ee]		109
177	n = 4			3.37,[dd] 0.83[ee]		109
178	n = 5			5.82,[dd] 1.92[ee]		109
179	n = 6			8.51,[dd] 9.12[ee]		109
180	Tyr(3-F)-D-Ala-Gly-Phe-MeMet-NH_2		0.98[w]		0.25[x]	110
181	D-Tyr(3-F)-D-Ala-Gly-Phe-MeMet-NH_2		0.004[w]		$<10^{-2}$[x]	110

Table (*cont.*)

No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
182	 L,L/D,D			0.05^{ff}	0.29^{gg}	111
183	L,D/D,L-182			$<0.002^{ff}$	$<0.14^{gg}$	111
184	 L,L			0.006^{ff}	$<0.14^{gg}$	111
185	D,L-184			0.0084^{ff}	$<0.14^{ee}$	111
186				$<0.002^{ff}$	$<0.14^{ee}$	111
187—196						
187	R = 3-CH_2CHMe_2 (L)			1.4^{ff}	4.5^{ee}	111
188	R = 3-$CHCH_2SMe$ (L)			16.2^{ff}	1.5^{ee}	111
189	R = 3-CH_2CH_2SMe (D)			7.2^{ff}	0.18^{ee}	111
190	R = H			0.47^{ff}	$<0.07^{ee}$	111
191	R = 3-Me (L)			0.78^{ff}	0.2^{ee}	111
192	R = 3-Me (D)			0.1^{ff}	$<0.14^{ee}$	111
193	R = 3,3-Me_2			0.3^{ff}	0.23^{ee}	111
194	R = 6,6-Me_2			1.2^{ff}	$<0.14^{ee}$	111

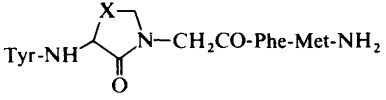
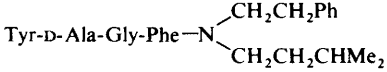
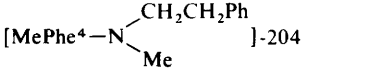
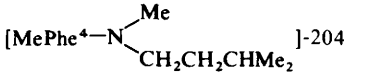
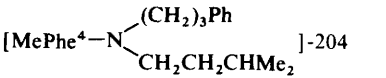
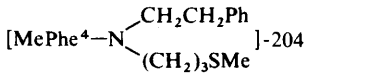
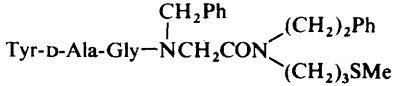
195	R = 3-CH_2CHMe_2-1-Me (L)			0.26[ff]	<0.14[ee]	111
196	R = 3-CH_2CHMe_2-6,6-Me_2 (L)			1.5[ff]	<0.14[ee]	111
197—203						
197	X = CH_2 (D)	$<10^{-2}$[b]		0.002[b, cc]		112
198	X = CH_2 (L)	$<10^{-2}$[b]		$<10^{-2}$[b, cc]		112
199	X = $(CH_2)_2$ (D)	0.09[b]		0.13[b, cc]		112
200	X = $(CH_2)_2$ (L)	$<10^{-2}$[b]		0.002[b, cc]		112
201	X = CH_2S (D)	0.03[b]		0.013[b, cc]		112
202	X = CH_2S (L)	$<10^{-2}$[b]		$<10^{-2}$[b, cc]		112
203	X = $(CH_2)_3$ (D)	$<10^{-2}$[b]		$<10^{-2}$[b, cc]		112
204		0.92[b]	0.005[b]			113
205	[MePhe[4]]-204	$<10^{-3}$[b]	$<2\times10^{-4}$[b]	0.74[hh]		113
206		$<10^{-2}$[b]	$<10^{-3}$[b]	9.34[hh]		113
207		0.65[b]	0.03[b]			113
208		0.04[b]	10^{-4}[b]			113
209	[MePhe[4]—N—$(CH_2CH_2Ph)_2$]-204	$<10^{-3}$[b]	$<5\times10^{-5}$[b]	0.6[hh]		113
210		$<10^{-3}$[b]	$<10^{-4}$[b]	1.9[hh]		113
211	[Sar[4]]-204	$<10^{-3}$[b]	$<10^{-4}$[b]			113
212		0.1[b]	$<10^{-3}$[b]			113

Table *(cont.)*

No.	*Compound*	*Guinea-pig ileum*	*Mouse vas deferens*	*Receptor binding*	*Analgesia*	*Ref.*
213	[MePhe^4-NH(CH$_2$)$_2$CHMe$_2$]-204			33.5[hh]		113
214	Tyr-OGly-Gly-Phe-Leu[v]	<0.008[a]	<10^{-2}[a]			114
215	Tyr-Gly-OGly-Phe-Leu[v]	<0.008[a]	0.01[a]			114
216	Tyr-Gly-Gly-OPhe-Leu[v]	<0.008[a]	<10^{-3}[a]			114
217	Tyr-Gly-Gly-D-OPhe-Leu[v]	<0.008[a]	<10^{-3}[a]			114
218	Tyr-Gly-Gly-Phe-OLeu[v]	0.1[a]	0.14[a]			114
219	Tyr-Gly-Gly-Phe-D-OLeu[v]	0.18[a]	0.06[a]			114
220	Tyr-D-Ala-Gly-Phe-D-OLeu[v]	0.55[a]	1.92[a]			114
221	Tyr-Pro-Phe-NH$_2$	0.19[b]				115
222	Tyr-Aib-Phe-NH$_2$	0.02[b]				115
223	Tyr-D-Phe-Phe	0.001[b]				115
224	Tyr-D-Ala-Phe-NH$_2$	0.25[b]				115
225	Tyr-D-Ala-Pheol	0.24[b]				115
226	Tyr-D-Pro-Phe-NH$_2$	0.001[b]				115
227	Tyr-D-Phe-Met-NH$_2$	0.09[b]				115
228	Tyr-D-Phe-Leu-NH$_2$	0.04[b]				115
229	Tyr-D-Phe-Phe-NH$_2$	0.23[b]				115
230	Tyr-D-Ala-D-Phe	0.001[b]				115
231	Tyr-D-Phe-D-Met-NH$_2$	0.01[b]				115
232	Tyr-D-Phe-D-Leu-NH$_2$	0.002[b]				115
233	Tyr-D-Ala-D-Phe-NH$_2$	0.02[b]				115
234	Tyr-D-Phe-D-Phe-NH$_2$	0.01[b]				115
235	Tyr-D-Phe-NH$_2$	0.001[b]				115
236	Tyr-D-Ala-NHCH$_2$Ph	0.08[b]				115
237	Tyr-D-Ala-NH(CH$_2$)$_2$Ph	0.06[b]				115
238	Tyr-D-Ala-NH(CH$_2$)$_3$Ph	0.77[b]				115
239	Tyr-D-Ala-NH(CH$_2$)$_4$Ph	0.13[b]				115

[a] [Leu^5]enkephalin = 1. [b] [Met^5]enkephalin = 1. [c] *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin, [Met^5]enkephalin = 1. [d] *vs.* [^{3}H]-[D-Ala^2, MePhe^4, Glyol^5]enkephalin at 25 °C, K_I, μmol dm^{-3}. [e] *vs.* [^{3}H]-[D-Ala^2, MePhe^4, Glyol^5]enkephalin at 0 °C, K_I, μmol dm^{-3}. [f] *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin at 25 °C, K_I, μmol dm^{-3}. [g] *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin at 0 °C, K_I, μmol dm^{-3}. [h] Rat-tail flick, i.c.v., [Leu^5]enkephalin = 1. [i] IC_{50}, nmol dm^{-3}, rat-brain homogenates *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin. [j] K_D, nmol dm^{-3}. [k] Mouse-tail flick, i.c.v., [Met^5]enkephalin = 1. [l] Mouse-tail flick, s.c., morphine = 1. [m] Mouse stretch, phenylbenzoquinone, morphine = 1. [n] Morphine = 1. [o] Binding relative to [D-Ala^2, D-Leu^5]enkephalin *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin. [p] Binding relative to [D-Ala^2, D-Leu^5]enkephalin *vs.* [^{3}H]-dihydromorphine. [q] Normorphine = 1. [r] Rat-brain synaptosome fraction *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin, K_I, nmol dm^{-3}. [s] Rat-brain synaptosome fraction *vs.* [^{3}H]-53, K_i, nmol dm^{-3}. [t] Rat-tail flick, i.v., morphine = 1. [u] Rat hot-plate, i.c.v., morphine = 1. [v] A_2bu = α, γ-diaminobutyric acid, Alg = 2-NH_2-4-pentenoic acid, δ-Ape = δ-NH_2-pentanoic acid, ε-Ahx = ε-NH_2-hexanoic acid, γ-Abu = γ-NH_2-butyric acid, Pip = pipecolyl, Phg = C^{α}-phenylglycine. HyX denotes the hydroxy acid equivalent to the amino-acid X. OYyy denotes the aminoxy acid equivalent to the amino-acid Yyy. [w] Potency relative to metkephamide, 63, = 1. [x] Mouse hot-plate, s.c., 63 = 1. [y] Mouse hot-plate, p.o., ED_{50} mg kg^{-1}. [z] Mouse hot-plate, i.c.v., 129 = 1. [aa] Mouse hot-plate, s.c., μmol kg^{-1}. [bb] [D-Ala^2, Met^5]enkephalinamide = 1. [cc] *vs.* [^{3}H]naloxone, rat-brain cell membranes, [D-Ala^2, Met^5]enkephalinamide = 1. [dd] ED_{50} *vs.* [^{3}H]naloxone, rat-brain membranes, nmol dm^{-3}. [ee] ED_{50} *vs.* [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin, rat-brain membranes, nmol dm^{-3}. [ff] *vs.* [^{3}H]etorphine, morphine = 1. [gg] Mouse-tail flick, i.v., morphine = 1. [hh] Sodium index, rat-brain membranes *vs.* [^{3}H]naloxone. Pure agonists ~30, pure antagonists ~1, partial agonists ~10.

laboratories impossible; consequently, the biological activities of each group of compounds have been related to separate standards as indicated in the footnotes. Significant points from the Table are highlighted in the discussion below and analogues displaying activities other than those tabulated are detailed at the end.

The high activities associated with some of the cyclic structures (1—6, 60) are significant as they suggest that the conformation the compounds may adopt are akin to that bound at the receptor.[81-83,99] The piperazinone derivative (9) is unusual in that it has little activity *in vitro* but displays moderate analgesic activity.[85] Studies with a more extensive series of piperazinone analogues (183—196) revealed that high activity is expressed only when this modification is restricted to the *C*-terminus.[111] Another series of conformationally restricted analogues (197—203) had very little activity.[112]

Investigations with a series of deletion analogues (21—32) have shown that, in

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contrast to the prevailing dogma, analogues lacking Gly[3] are active opiates.[87,88] The study was extended more recently and significant activity was found in dipeptide amides (221—239).[115] Exceptionally high activity on GPI was discovered in a series of tetrapeptides[89-92] (34—43, 'syndyphalins'), and the compounds were potent analgesics when given subcutaneously. A Chinese group described two peptides that are very highly specific for the δ-receptors of MVD (49, 50).[95] Systematic modifications in a series of tetrapeptides (110—128)[102] coupled with the observation that improvements in activity were additive led to the development of potent, orally active peptide analgesics (127, 128). Similar studies in different pentapeptide series by two groups (63—109, 180, 181)[101,110] and (129—160)[51] also produced potent compounds, but no oral activity was reported. Further examples of analogues containing 'fat' amino-acid residues were described (165—171),[103-106] and several groups investigated the properties of enkephalin oligomers (*e.g.* 62, 85, 174—179).[100,101,109,116,117] A series of analogues containing aminoxy-acid residues have been prepared (213—220);[114] activity was highest in compounds with *C*-terminal substitutions.

The pharmacological effects of three previously described potent tetrapeptide amides (51—53) were studied in depth.[96,97] One of these (53) had very high but variable activity in the mouse stretch assay. The same group has also discovered the first examples of peptides that act as μ-selective opiate receptor antagonists (204—213).[113]

Shinagawa *et al.*[118] have published accounts of the synthesis and activities of tetrapeptide hydrazide derivatives including 39 new analogues, but the data were not available for tabulation. A detailed study of the biological activities of four of the compounds has also appeared.[119] Further studies of the properties of acetaldehyde–enkephalin condensation products were described.[120,121] Momany *et al.*[122] studied the structure–activity requirements for growth-hormone-releasing activity in a series of aromatic enkephalin-derived peptides. Preliminary investigations of the activities of deletion analogues of the dermorphins have been reported[123,134] and the synthesis of a chromophoric dehydro analogue of

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[Leu^5]enkephalin has been described.[125] Detailed studies of the pharmacology of [D-Met^2, Pro^5]enkephalinamide[126,127] revealed that ovulation is blocked in rats after i.c.v. administration; consequently, opioid peptides may be involved in the central physiological regulation of events leading to ovulation. Investigations of the enhanced agonist potency of tobacco mosaic virus–enkephalin conjugates have been extended;[128] their stability to protease degradation was studied by Do and Schwyzer.[129]

Endorphins. Very little structure–activity work with analogues of the endorphins was reported in 1981. Further studies with [Gln^8]-β-endorphin (human) have reinforced the observation that this substitution enhances both analgesic potency and receptor binding.[130,131] Human β-endorphin dimethylated at all six amino groups (N^{α}- and $5 \times N^{\varepsilon}$-Lys) has greatly increased stability to proteolytic degradation and is expected to exhibit a prolonged half-life *in vivo*.[132] Finally, Taylor *et al.*[133] have synthesized a peptide modelled on β-endorphin in which the *C*-terminal sequence is modified to have a high amphiphilic α-helix-forming potential. The conformation of the peptide was studied and the presence of α-helix confirmed. The analogue was highly resistant to proteolysis and exhibited enhanced receptor-binding potency.

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